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EDITORIAL INTRODUCTION SPECIAL ISSUE "TRENDS IN FOOD SCIENCE AND TECHNOLOGY"

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Progress in the field of food science and technology has been observed in last several years both in developed and developing countries. Carpathian Journal of food Science and Technology an open publication offer space for publication and encourage scientists from all the countries of the world. Special issue "Trends in Food Science and Technology" promote valuable researchers from Nigeria, Iran, India, Taiwan, Thailand, Algeria etc. We welcome Authors to contribute with original research as well review articles, in several connected fields, such as agriculture, food-science, food processing, food chemistry. First 2 manuscripts are reviews studies and refer to the possibility to use blends made it by mixt of polyethylene and starch to increase the mechanical properties of LDPE and possibility to use octacosanol in food technology. A new trend, even in Europe, is the possibility of using amino acids in the regular diet by extracting them from different types of insects. The study shows 5 amino acids profiles of five commonly consumed insects in southwestern Nigeria. Yellow yam tubers and jack bean seed were processed into flour, blended together and D-optimal mixture design. Pulsed electric field can be used with best results in food technology on the one hand to reduce the electric costs and on the other hand to keep the integrity in chemical composition of raw materials processed in food industry. New models of chambers and results obtained are presented in another article. The rapid development antibiotic-resistant food pathogens pose a heightened threat to public health. This study investigated the antibiotic resistance pattern of bacteria associated with three hundred *suya meat* samples, a ready-to-eat beef product, in Nigeria. Problems regarding the particles produced through the micronization process and possibility to introduce in food technology processes are presented in manuscript the application of micronization technology on a fruiting body extract of *Antrodia Cinnamomea*. Pineapple wine production a new food product can be obtained from fermenting pineapple, watermelon and cashew juices using yeast species isolated from fruits as starter cultures. Cooking methods and cooking time affected carotenoid retention in Umudike Sweet Potato Variety. Carotenoid retention was higher in oven-dried samples than in roasted and boiled samples. Oven-drying for 24h decreased total carotenoid retention in UMUSPO potato. Half-dried salted round *scad* fish can be stored for only a short time due to high-moisture content. Study evaluates the quality changes during storage under 3 packaging: air, vacuum and air with oxygen absorber. Colon cancer is an important worldwide cause of death in human which is treated commonly by chemotherapy, radiotherapy and surgery methods with different side effects. Natural treatment such as microbial cell wall extract is suggested to be used as an effective alternative of chemical drugs for treatment of colon cancers without any side effect. Study in vitro investigate the anticancer properties of *Saccharomyces boulardii* supernatant (SBS) on colon cancer cells.

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FACTORS AFFECTING MECHANICAL PROPERTIES OF LOW-DENSITY POLYETHYLENE (LDPE)/STARCH BLENDS: A REVIEW

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ABSTRACT

Due to the environmental concerns associated with non-biodegradable polymers like low-density polyethylene (LDPE), the production of eco-friendly and partially biodegradable films based on starch/LDPE blends is the point of interest. However, due to immiscibility between hydrophilic starch and hydrophobic LDPE, the mechanical properties of the produced films are not good enough. In this context, several investigations have been conducted to improve the mechanical properties of the films, mainly through the improvements in miscibility and compatibility of two layers. The mechanical characteristics of the films fabricated from these blends are affected by different factors including modification of starch via esterification, cross-linking and thermo-plasticization, starch granule characteristics (amylose to amylopectin ratio, size, and shape) and their content, type, and quantity of compatibilizers, presence of nanoparticles (type and content), using of recycled polymers, type of plasticizers and processing technique. The current article presents an overview of the factors that affect the mechanical properties of LDPE/starch blends.

1.Introduction

Every year million tons of plastics are produced and discarded after using into the environment (Shimao, 2001). Due to environmental problems of using synthetic polymers, the use of renewable sources to fabricate packaging systems has attracted the attention of the researchers in recent years (Oromiehie & Rabiee, 2013). Due to unique characteristics of low-density polyethylene (LDPE) such as durability, flexibility, processability, and being lightweight and low price, it is employed in agricultural, pharmaceutical, medical, and food packaging industries. However, non-biodegradability is one of the disadvantages of LDPE in terms of environmental issues(Torabi Angaji & Hagheeghatpadjooh, 2004). The addition of biodegradable ingredients can be considered as one of the possible approaches to overcome this challenge (J. C. Huang, Shetty, & Wang, 1990;

Obasi & Igwe, 2014; Swift, 2000; Torabi Angaji & Hagheeghatpadjooh, 2004).

Starch, as an eco-friendly packaging material, is commonly used to make partially biodegradable plastics. Some properties such as low cost, high availability, renewability, and processability made the starch a promising additive for producing partially biodegradable plastics (Aminabhavi, Balundgi, & Cassidy, 1990; Gage, 1990; Raghavan & Emekalam, 2001). In the mid of 1970s, for the first time, native starch was used as a filler into LDPE-based plastic (G. Griffin, 1994; G. J. L. Griffin, 1977; Mali & Grossmann, 2003). After discarding of packaging containing LDPE/starch into the environment, in the effect of microorganisms' attack to starch, the porosity and surface area/volume ratios are increased, and as a result, the improvement in the rate of oxidation, ultraviolet photodegradation, and biodegradability of the mentioned polymers can be achieved (G. Davis, 2003; Nikazar, Safari, Bonakdarpour, & Milani, 2005).

The effect of starch on the mechanical properties of LDPE/starch blends has been previously investigated by different authors (Arvanitoyannis, Biliaderis, Ogawa, & Kawasaki, 1998; W. Liu, Wang, & Sun, 2003; Lu, Xiao, & Xu, 2009; Psomiadou, Arvanitoyannis, Biliaderis, Ogawa, & Kawasaki, 1997; Thakore, Iyer, Desai, Lele, & Devi, 1999). Nearly all of these researches indicated that the addition of starch has a negative impact on the mechanical properties of these blends. This effect is due to the differences in the nature of the two blended phases (starch and LDPE) (Figure.1). Starch as the dispersed phase, has hydrophilic nature, in contrast, LDPE as the continuous phase, is a hydrophobic polymer (W. Liu et al., 2003; Samper-Madrigal, Fenollar, Dominici, Balart, & Kenny, 2015). In order to overcome this problem, some attempts have been carried out to improve their compatibility and increasing interfacial adhesion between LDPE and starch (Sabetzadeh, Bagheri, & Masoomi, 2016). In this regard, enhancing the compatibility between LDPE and starch through different strategies has been investigated in several studies; however, a comprehensive review regarding the application of developed techniques to overcome this problem in packaging systems was not published yet. Thus, in the present paper, for the first time, the factors affecting the mechanical properties of LDPE/starches blends will be discussed.

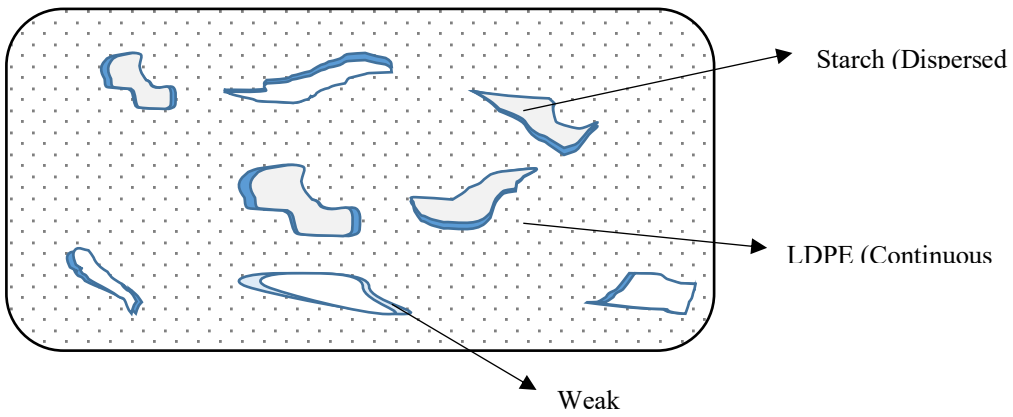


Figure 1. Schematic view of starch dispersion in the LDPE matrix

2.Effects of starch modification

Starch in natural forms without any modification could not be used in conventional methods of producing plastics. By the modification process, the heterogeneous structure of native starch granules is converted to a homogeneous structure (Matzinos, Bikiaris, Kokkou, & Panayiotou, 2001). Some techniques have been

introduced for modifying the structure of starches. In the following sections, an overview of the most used techniques will be provided.

2.1.Effect of thermo-plasticization of starch

The crystalline and semi-crystalline part of starches can be converted to amorphous forms by application of high temperature and pressure in an aqueous environment (Prinos, Bikiaris, Theologidis, & Panayiotou, 1998). In this condition, due to gelatinization, the native starches swell by absorption of water, and after heating, their crystalline structure is destroyed, and hydrogen interactions between glucose units are broken (Rodriguez-Gonzalez, Ramsay, & Favis, 2003). Gelatinized starch characteristics could be further improved by the addition of plasticizers. Plasticized and gelatinized starches are known as thermoplastic starch (TPS) (Phetwarotai, Potiyaraj, & Aht‐Ong, 2012).

TPS is a type of gelatinized starch with an amorphous structure that as opposed to dry starch, is capable of flow and thus when blended with synthetic polymers like LDPE, can behave similarly to conventional polymer-polymer mixture (Rodriguez-Gonzalez et al., 2003). By the application of heat and shear stress, TPS can be molded or shaped. This characteristic allows TPS to be processed with common techniques used in plastic manufacturing industries (Bastioli, 1998; Gomez & Aguilera, 1984; Kokini, Lih-Shiuh, & Chedid, 1992). The amount of starch in the LDPE/starch film formulation can be increased with thermo-plasticization of starch granules (BeMiller & Whistler, 2009).

Prinos et al. (1998) showed that LDPE-thermo-plasticized corn starch had superior mechanical properties than LDPE/native corn starches. Garg and Jana (2007) showed that the addition of native starch to the LDPE matrix, in comparison with other modified starches, could reduce the mechanical properties of the blends.In another study, one-step processing with a single screw extrusion system was employed to produce LDPE/thermoplastic starch film with unique characteristics (Rodriguez-Gonzalez et al., 2003). The authors indicated that the developed films had high elongation at break (EB), Young's modulus (YM), and tensile strength (TS) in comparison with LDPE/native starch blend. It also was found that despite the absence of any interfacial modifier, the film formulation containing LDPE/TPS with respective ratio of 55:45 retained 94 % of EB and 76 % YM of LDPE film. Surprisingly, when the film was fabricated by a composition level of 71:29 LDPE/TPS, the film maintained 96 % of EB and 100 % YM of LDPE film.

The utilization of native corn starch in film production is limited due to its low water solubility and brittleness (Eskandarinia, Rafienia, Navid, & Agheb, 2018). The mechanical performance of thermoplastic corn starch/LDPE at various ratios was evaluated by Oromiehie, Lari, and Rabiee (2013). It was reported that as the starch concentration increased, TS and EB showed a decreasing trend, but YM increased. The decrease of TS is related to the tough nature of LDPE and the brittle structure of starch. The addition of starch to LDPE film formulation leads to matrix embrittlement, due to the replacement of a tough matrix with a brittle polymer (Chiu, Lai, & Ti, 2009; Da Róz, Carvalho, Gandini, & Curvelo, 2006). The reduction of EB with the incorporation of thermoplastic corn starch into LDPE film formulation is also attributed to 1) the weakening of the London forces between LDPE layers and 2) the fact that elongation at break of starch itself is lower than LDPE (Matzinos, Tserki, Gianikouris, Pavlidou, & Panayiotou, 2002).

The effect of different compatibilizers on mechanical properties of starch/LDPE films was investigated by several scientists and will be discussed in the following sections (Bikiaris et al., 1998; Girija & Sailaja, 2006; Raquez et al., 2011).

2.1.1.Effect of type and content of plasticizer

During the production of biodegradable polymers from starch, due to the presence of strong H-bonds in granules, starches degrade before melting, but by using plasticizers, their melting point decreases (Lorcks, Pommeranz, Klenke, Schmidt, & Heuer, 2000; Radfar et al., 2019). Plasticizers reduce the strong hydrogenic interactions within the starch polymers and forming

new bonds between starch and plasticizers (Bikiaris, Prinos, & Panayiotou, 1997).

In the presence of a plasticizer, the dispersion capability of the starch in the polymeric matrix improves and subsequently interfacial adhesion between phases (Fang, Fowler, Tomkinson, & Hill, 2002; Garg & Jana, 2007; Kiatkamjornwong, Thakeow, & Sonsuk, 2001; Kim & Lee, 2002; Sabetzadeh, Bagheri, & Masoomi, 2012; Y. J. Wang, Liu, & Sun, 2004), flowability at elevated temperatures and ductility of the blends (Cerclé, Sarazin, & Favis, 2013) develop.

The type and quantity of plasticizers along with processing conditions are three of the most important factors that affect the physical properties of prepared TPS (Kalichevsky & Blanshard, 1993; Orford, Parker, Ring, & Smith, 1989; Poutanen & Forssell, 1996; Shogren, Swanson, & Thompson, 1992). Glycerol, sorbitol, glycol, urea, ethanolamine, and water are the main plasticizing agents which can be used in preparation of TPS (Fishman, Coffin, Konstance, & Onwulata, 2000; M. Huang, Yu, & Ma, 2005; Mazerolles et al., 2019; Shi et al., 2007; Smits et al., 2001).

In the gelatinization process low molecular weight plasticizers such as water and glycerol are the most active ones due to their high affinity to the starch molecules. Moreover, by application of high molecular weight plasticizers (e.g. polyglycerol), the gelatinization temperature of starches increases (Taghizadeh & Favis, 2013). The reason for this phenomena is not completely clear, but according to Perry and Donald (2002), the most important reason is the reduction in the rate of plasticizer penetration to granules, along with increasing plasticizers molecular weight (Perry & Donald, 2002).

According to Arvanitoyannis et al. (1998), in prepared blends of LDPE and two types of starch (rice and potato) as fillers, in the presence of different amounts of water (5 to 20 %) by a twin-screw extruder, due to the plasticization effect of water, percentage elongation improved and tensile strength decreased as the water content of blends increased.

The effect of glycerol on the mechanical and physical properties of LDPE and rice starch blends was investigated by Y. J. Wang et al. (2004). The tensile properties of these blends after the addition of glycerol and production of thermoplastic starch improved which is associated with better dispersion of rice starch in the matrix and strong interaction between two phases.

Taghizadeh, Sarazin, and Favis (2013) reviewed the effect of high molecular weight plasticizers from the polyol family, including glycerol, sorbitol, diglycerol, and polyglycerol in TPS/PE composites. They reported that starch could react with poly and di glycerols better than glycerol. This was due to the high number of hydroxyl groups in these compounds which are substituted by etheric bonds. By substitution of these functional groups, the conformational flexibility and molecular movements of chains during mixing improved. Consequently, with increasing molecular flexibility, the interaction between molecules during mixing increased and as a result, in comparison with glycerol, the miscibility of diglycerol and polyglycerol with starches improved. The prepared blend with polyglycerol and without compatibilizer had a higher EB; on the other hand, sorbitol-TPS/PE blend was rigid and had higher YM and TS. This effect is due to recrystallization ability of sorbitol at ambient temperature and forming rigid characteristics.

The effect of plasticizer concentration (glycerol in three concentrations of 25, 30 and 35 %) on the LDPE/corn starch film properties was investigated by Pushpadass, Bhandari, and Hanna (2010). Their finding showed that increasing glycerol content could significantly decrease TS and YM of composites, which can be attributed to the weak intermolecular interactions in the film matrix due to free volume increasing, and separation of starch and LDPE phases along with increasing glycerol content.

2.2.Effect of starch esterification

Another approach to incorporate starch in polyethylene and enhance the compatibility between two immiscible polymers is rendering its hydrophobic character by esterification or etherification (Seidenstücker & Fritz, 1999). In

these processes, hydroxyl groups of starches are replaced by hydrophobic functional groups (BeMiller & Whistler, 2009; Mohammadi Nafchi, Moradpour, Saeidi, & Alias, 2013; Seidenstücker & Fritz, 1999; Wesslén & Wesslen, 2002).

Evangelista, Sung, Jane, Gelina, and Nikolov (1991) investigated the mechanical properties of LDPE containing starch octenyl succinate aluminum complexes. Based on their results, the prepared films with octenyl succinate aluminum had better mechanical properties than the films containing native starches.

According to Thiebaud et al. (1997), esterification of starch with fatty acid chloride (octanoate starch with degrees of substitution 1.8 and 2.7) could improve the mechanical properties in comparison to LDPE/native starches blends. The ester groups serve as an internal plasticizer and can improve the EB of the LDPE/starch film. The increasing degree of substitution and length of fatty acid chains also improved the properties of the prepared blend.

In another study, the mechanical performance of acetylated and phthalated tapioca starch/LDPE blends with and without LDPE-co-glycidyl methacrylate copolymer as a compatibilizer was tested by Sailaja (2005). The authors observed that the esterification of starch significantly reinforced the mechanical characteristics of the films. Furthermore, they reported that the incorporation of compatibilizer into film formulation led to further improvement in the mechanical performance of the films. Likewise, Sailaja and Seetharamu (2009) showed that blends containing LDPE and esterified starches (acetate and phthalate) in the presence of LDPE-co-glycidyl methacrylate as compatibilizer demonstrated better mechanical properties than blends containing native starches. Their observation also revealed that starch esterified with phthalate performed better than starch esterified with acetate. However, TS and YM of the blends containing phthalate esterified starch were close to those of pure LDPE.

The processability and thermo-plasticity of starch blends are directly related to the size of ester groups. As mentioned above, ester groups act as internal plasticizers (Sagar & Merrill, 1995). The esterified starches with different kinds of ester groups have been blended with LDPE. The most frequently used ester groups are carboxy methylate (K. A. Kumar & Soundararajan, 2016), polyacrylate (Kiatkamjornwong et al., 2001), acetate (Thakore et al., 1999), hydroxypropylate (Swanson, Westhoff, & Doane, 1988), fatty acid esters (e.g. methyl oleate) (Sagar & Merrill, 1995; Thiebaud et al., 1997), adipate and acetylate (Nakamura, Cordi, Almeida, Duran, & Mei, 2005) and tert-butyl acrylate and methacrylamide (M. Raj, Savaliya, Joshi, & Raj, 2018).

In conclusion, the modification of starch by esterification improved the hydrophobicity and thermo-plasticity of the blends, and mechanical properties of the prepared mixtures were better than those of untreated starch.

2.3.Effect of starch modification by cross-linking

As mentioned above, the poor mechanical characteristics of starch-based films have restricted their industrial applications. Cross-linking of starches is another chemical modification process that commonly led to the improvement of mechanical properties of the starch-based films. It should be noted that although cross-linking is a useful strategy to reinforce the mechanical properties of films, the current starch cross-linking techniques are expensive and toxic. The citric acid (CA) is a non-toxic trifunctional additive that is used as a plasticizer, cross-linking and partial esterification agent. It also hydrolyzes starch polymers and produces low molecular weight chains as a side reaction (Olsson et al., 2013). Citric acid could improve fluidity of TPS by deterioration of the chain entanglement and weakening of the interactions in starch molecules (H. Liu, Xie, Yu, Chen, & Li, 2009).

N. Reddy and Yang (2010) used citric acid as a cross-linker to improve the mechanical properties of starch-based films. The possible crosslink reaction between citric acid and starch is presented in Figure.2. Citric acid cross-linked starch films had 1.5 fold TS when compared with non-cross-linked films. Furthermore, it was found that the

value of TS in the developed film was higher than those reported for most cross-linked starch and synthetic polymer blended films previously developed. In another research, Ghanbarzadeh, Almasi, and Entezami (2011) cross-linked potato starch to improve the mechanical properties of potato starch/LDPE blend film. The authors showed that following an increase in the citric acid concentration, TS increased and EB decreased. This effect has been attributed to hydrolyzing branched chains of starch molecule in the presence of citric acid that led to the formation of a highly linear structure. It resulted in increasing the number of H-bonds between the starch chains and increasing TS in the produced films.

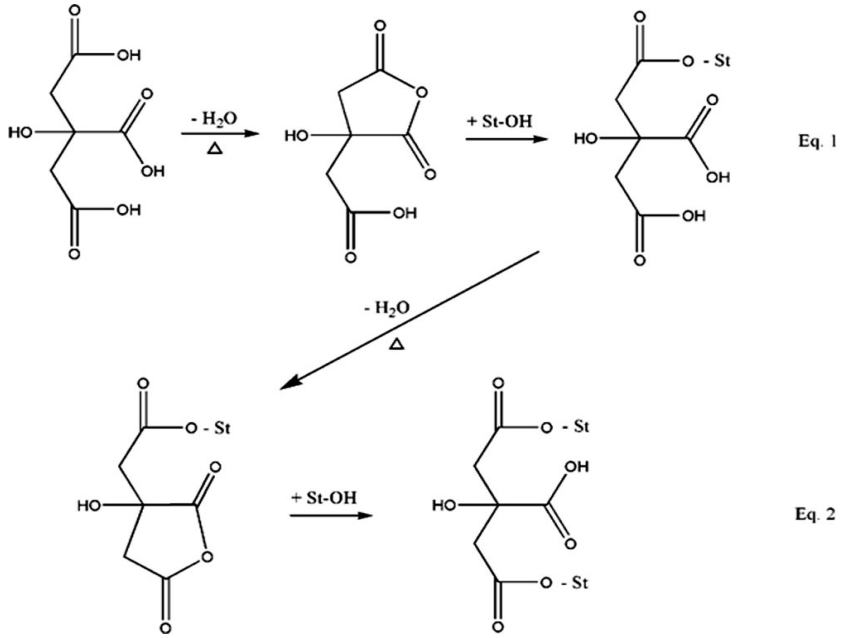


Figure 2. The possible crosslink reaction between citric acid and starch (Kahar et al., 2012).

Kahar, Ismail, and Othman (2012) reported that compatibilization of the PE/natural rubber /tapioca starch systems with citric acid could improve the tensile properties and decrease the surface tension of the blends. They concluded that low molecular weight TPS with better fluidity easily dispersed in the PE/ natural rubber system.

Ning, Jiugao, Xiaofei, and Ying (2007) studied the effect of citric acid on the properties of TPS/LLDPE blends. They used a one-step extrusion method for plasticization and blending of starch and PE. The obtained results showed that a small amount of citric acid (2%), could greatly enhance the TS and EB of the blends. This may be due to the improvement of interfacial adhesion between two polymers used to fabricate the film. Interestingly, the TS and EB of the developed film incorporated by 2% citric acid were similar to pure PE. Beyond this concentration, the mechanical performance of the films decreased. This is attributed to the acidolysis of starch in higher citric acid concentration, which led to the complete deterioration of the starch rigid structure. Fourier-transform infrared spectroscopy (FTIR) analysis also showed that in the presence of citric acid, hydrogen bonds between TPS and glycerol as plasticizer strengthened.

Commonly, in order to cross-linking of starch in polysaccharide industry, epichlorohydrin was used (Kuniak & Marchessault, 1972). In their study, Garg and Jana (2007) used epichlorohydrin as cross linker of corn starch to improve the mechanical properties of corn starch/LDPE film. The mechanical performance of the developed films was evaluated by different mechanical parameters such as TS, EB, melt flow index, and burst strength. It was found that when LDPE was mixed with 7.5% native starch, there was a reduction in TS, EB and melt flow index but burst strength increased. On the other hand, the values of

TS, EB and melt flow index of the films incorporated by cross-linked starch were notably greater than those containing native starch. From a morphological point of view, the films produced by cross-linked starch/LDPE were smoother than those obtained from the native starch blend films. The good mechanical properties of the films fabricated by cross-linked starch/LDPE films may be associated with the absence of pores and crakes in their structure. Furthermore, from a chemical perspective, this effect is related to decreasing the hydrophilic nature of native starch after cross-linking. But, in cross-linked starch, the OH- groups on starch molecules interact with epichlorohydrin and produce mono/di-glycerol ether starch (Hamerstrand, Hofreiter, & Mehltretter, 1960). The reduction of hydroxyl groups in starch structure decreases the hydrophilic nature of cross-linked starch, and as a result improves the homogeneity and compatibility of the starch/LDPE blend. Likewise, it has been reported that the crosslinking of potato starch with different concentrations of epichlorohydrin (0.1, 0.5, 1.0 and 2.0 g epichlorohydrin per 100 g of starch) improved TS, EB and strain energy of the films (Kim & Lee, 2002).

3.Effect of starch granules characteristics

3.1.Impact of granular structure

The granule size of starches is another important factor that affects starch/LDPE film properties. The effects of variation in granule size of different types of starch including corn, wheat, rice and potato starch on mechanical properties of starch/LLDPE films were examined by Lim, Jane, Rajagopalan, and Seib (1992). The lowest value of TS was found for the films produced with potato starch (highest granular diameter), while the highest TS was observed in case of rice and small particle corn starch produced by acidic hydrolysis of native corn starch with average granule size of 2 μm (the smallest one). Moreover, among unmodified and native tested starches including corn, wheat, rice, and potato starch, the films containing rice starch had the greatest TS. Taken together, there was a negative correlation between granular starch size and mechanical strength of the films. A similar trend was reported for the elastic modulus (EM) and yield strength of the studied films containing different granule sizes. In this regard, the prepared films with small particle corn starch and potato starch had the highest and lowest yield strength and EB, respectively. Due to the uniform dispersion ability of small particle starches in LDPE/starch matrixes, less discontinuity was observed in the produced films. As a result, it can be said that with using small particle corn starch, it is possible to increase the starch content in the film, without any decrease in film quality (Lim et al., 1992).

Willett (1994) studied the mechanical properties of starch/LDPE composites as a function of granule size by using unmodified corn starch and potato starch. The obtained results revealed that TS and tensile modulus of composites were more sensitive to granule diameter, while this factor had no significant effect on EB.

3.2.Amylose to amylopectin ratio

According to Mani and Bhattacharya (1998), in addition to granular structure (shape, size), the composition (amylose and amylopectin ratio) of starches could affect the mechanical properties of LDPE/starch blends. They prepared polyethylene blends with four different kinds of corn starches including unmodified waxy corn (involving 100% amylopectin), industrial corn (with approximately 70% amylopectin and 30% amylose), common corn, (containing approximately 50% amylopectin and 50% amylose) and an unmodified high amylose starch (consisting of approximately 30% amylopectin and 70% amylose). According to their findings, blends including waxy corns were characterized by higher EB and TS in comparison with other ones, which can be correlated with cross-linking between highly branched amylopectin and polymer. Their results also revealed that as amylose content of prepared blends increased up to 50%, TS and EB decreased and then again increased when amylose content reached 70%. Formation of the co-continuous phase with synthetic polymer had a great role in this regard.

4.Effect of starch content

The increase in the proportion of starch in LDPE/starch blends without any adverse effect on film mechanical properties attracted considerable attention. However, based on the previous investigations, increasing starch content above 30 % in the formulation, decreased EB and increased TS of the films (Arvanitoyannis et al., 1998; Nawang, Danjaji, Ishiaku, Ismail, & Ishak, 2001; Psomiadou et al., 1997).

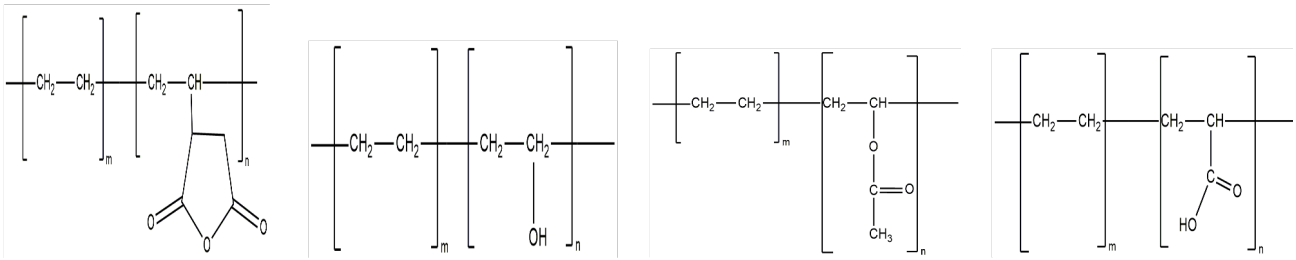
In this context, Sabetzadeh et al. (2012), prepared malleted LDPE/corn starch films containing different concentrations of thermoplastic corn starch (from 5 to 40%) and constant LDPE-g-MA (3 wt %). Within the investigated concentration range, the blends containing 25% (w/w) of thermoplastic starch had the best mechanical properties. The authors concluded that the uniformity of particle distribution in the matrix decreases as the concentration of starches increases.

5.Effect of modification of LDPE by compatibilizers

As previously mentioned, utilizing a plasticizer could improve dispersion of starch in polyethylene blends. However, the resultant blends due to limited interactions between hydrophobic PE and hydrophilic thermoplastic starch still are immiscible, have poor performance and tend to separate (B. Raj, Annadurai, Somashekar, Raj, & Siddaramaiah, 2001). The effective way for increasing compatibility between thermoplastic starch and polymer and also decreasing interfacial properties is the addition of a compatibilizer (W. Liu et al., 2003).

Compatibilizers could improve affinity and adhesion between starch and LDPE. These compounds are used in small quantities and treat surfaces of starch and polyethylene polymer; in another word, the interfacial tension of immiscible polymers is reduced by means of compatibilizers and thus morphology of blends would be finer and the degree of continuity increased (J. Davis, 1998; Mortazavi, Ghasemi, & Oromiehie, 2014). These compounds have chemical groups like carboxyl, anhydride, epoxy, urethane, or oxazoline, which react with functional groups of polymer and starch. The formed covalent and hydrogen bonds improve interfacial adhesion (Chen et al., 2016; Tran et al., 2011).

It has been shown that mechanical properties (such as TS, EB and impact behavior) of LDPE/starch blends improved significantly after addition of different compatibilizers (Chen et al., 2016; Mortazavi et al., 2014; Oromiehie & Rabiee, 2013).



polyethylene-grafted-maleic anhydride | Ethylene-vinyl alcohol (EVOH) | Ethylene-vinyl acetate (EVA) | Ethylene-co-acrylic acid (EAA)

Figure 3. Schematic structure of some compatibilizers

Various compatibilizers such as ethylene-vinyl acetate copolymer (EVA) (Prinos et al., 1998), poly(ethylene-co-acrylic acid) (EAA) (Bikiaris et al., 1997), poly(ethylene-grafted-maleic anhydride) (PE-g-MA) copolymers, poly(ethylene-co-glycidyl methacrylate) (PEGMA), poly(ethylene-co-vinyl

alcohol) (EVOH) and poly(LDPE-g-dibutyl maleate) copolymer have been reported in different studies (Taguet, Huneault, & Favis, 2009). Schematic structure of some of these compatibilizers is shown in Figure.3.

According to the literature, the most commonly used compatibilizer is PE-g-MA (polyethylene-grafted-maleic anhydride) (Radfar et al., 2020). The anhydride maleic groups of PE-g-MA interact with –OH groups of starches and on the other hand its PE part reacts with polyethylene matrix (Ramkumar, Bhattacharya, & Vaidya, 1997; S. Wang, Yu, & Yu, 2005).

One of the most important reasons for the popularity of PE-g-MA is the ease of grafting anhydride groups onto other polymers at regular temperatures during the melting process without any significant undesirable side reactions (Kalambur & Rizvi, 2006). The proposed mechanism of the reaction of starch and PE-g-MA is presented in Figure.4.

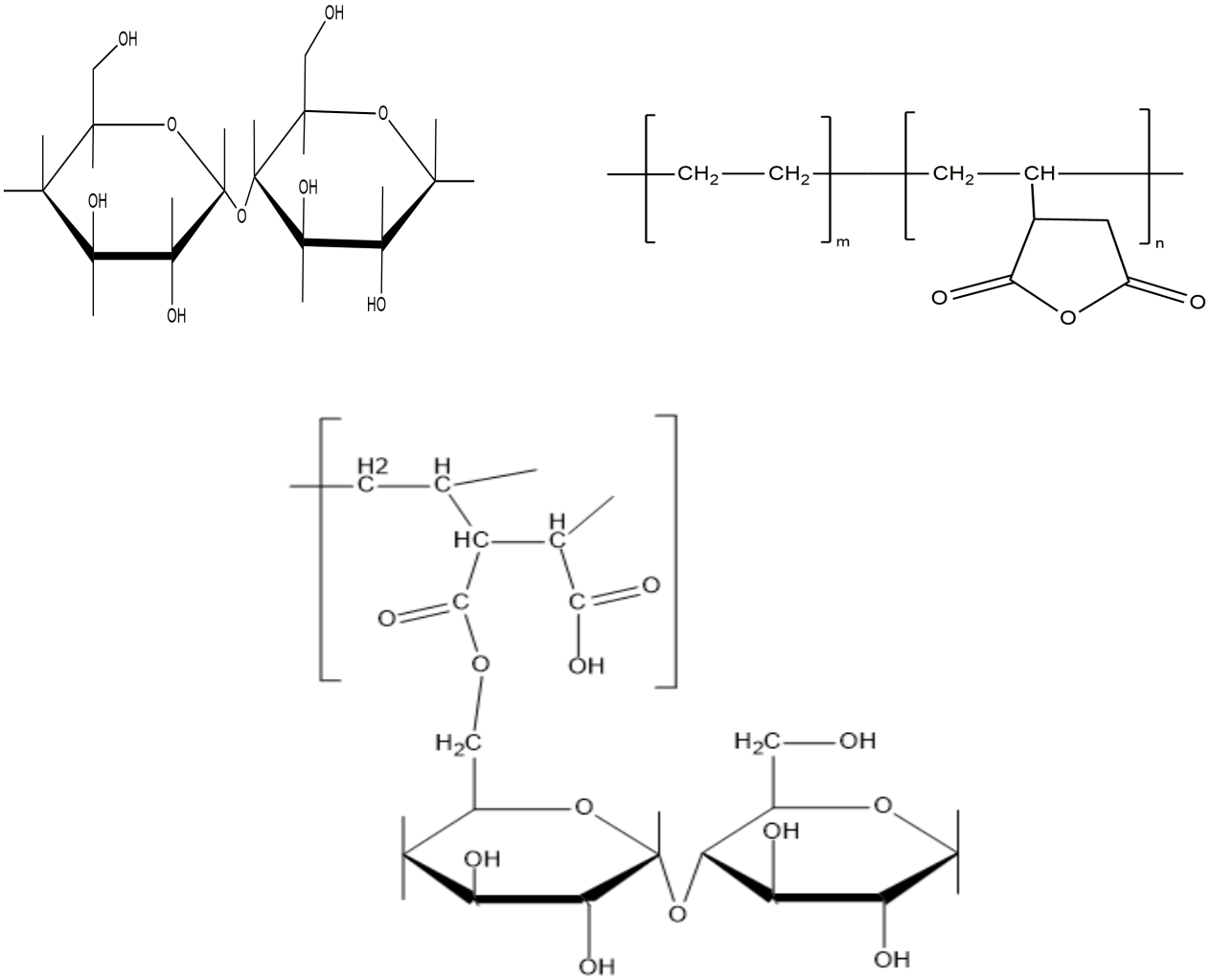


Figure 4. Proposed mechanism for the reaction of maleated PE with starch

Different studies have shown that 3-5 wt% addition of PE-g-MA improved the mechanical properties of low-density polyethylene and starch blends (Oromiehie & Rabiee, 2013; Sabetzadeh, Bagheri, & Masoomi, 2017; Y. J. Wang et al., 2004). It has been proven that compatibilized blends with PE-g-MA had a high elastic network in their structures compared with uncompatibilized ones (Mortazavi et al., 2014).

Ghafoori, Mohammadi, and Ghaffarian (2007) studied the effect of three compatibilizers including poly(ethylene-co-vinyl acetate) (EVA),

polyethylene grafted with maleic anhydride (PE-g-MA) and styrene-ethylene-butadiene-styrene grafted with maleic anhydride (SEBS-g-MAH) on the properties of LDPE/starch blends. Their results showed that, by the addition of 3.3 wt% of these compounds, the adhesion energy of the blends improved. PE-g-MA in comparison with other compatibilizers showed the best results.

Bikiaris et al. (1998) observed that compatibilized LDPE/starch blends prepared with a poly(ethylene-grafted-maleic anhydride) copolymer in comparison with uncompatibilized ones had better mechanical properties.

C.-Y. Huang, Roan, Kuo, and Lu (2005) , prepared high starch/LDPE blends using two compatibilizers (maleic anhydride grafted plasma-treated LDPE and acrylic acid grafted plasma-treated LDPE) after plasma treatment. Their results revealed that MA-g-PLDPE had the best compatibilization effect.

W. Liu et al. (2003) proved that addition of PE-g-MA, especially at higher starch contents (20 and 25%), could improve interfacial properties of corn starch–LDPE–PE-g-MA blends in comparison with those without compatibilizer. They also showed that the critical interfacial concentration for the addition of PE-g-MA was around 10 % of starch weight in the LDPE/starch blend. At this concentration, compatibilizer saturated the interface between two components (starch and LDPE) and the interfacial tension was reduced at the maximum content. In another research, Raquez et al. (2011) also used the same PE-g-MA concentration (10 wt %) as compatibilizer to improve the interfacial properties of pea starch/LDPE film. They also found that the use of cross-linked pea starch in LDPE film formulation improved the mechanical strength of the film. Scanning electron microscope (SEM) analysis confirmed the improvement of interfacial adhesion between both layers after addition of MA-g-PE. The effect of MA-g-PE on mechanical properties of plasticized tapioca starch blends with the LDPE was examined by Girija and Sailaja (2006). As expected, the authors observed that MA-g-PE could improve mechanical performance of the film. The same results have been reported by other researchers who evaluated the influence of MA-g-PE on the mechanical properties of LDPE/starch based films (Sailaja, 2005; S. Wang, Yu, & Yu, 2004).

6.Effect of nanoparticles

Typically, a composite is made from a continuous phase (like polymer) and two or more dispersed phases or fillers (Meeks, Smith, Clark, & Pantoya, 2017; Nielsen, 1977). If one of the dispersed phases or fillers has at least one dimension less than 100 nanometers, the composite is called nanocomposite (Schadler, Brinson, & Sawyer, 2007). Incorporation of nanoparticles is considered as an effective technology for improving mechanical and barriers properties of the films. The mechanical and physical properties of nanocomposites usually are different from their constituents. It has been shown that nanocomposites have better mechanical strength and barrier properties in comparison with conventional polymers (Rhim, Park, & Ha, 2013). Higher aspect ratio and greater surface areas are distinctive features of nanoparticles (De Azeredo, 2009).

Organic or inorganic nanoparticles that are used in food packaging include nanoclay particles or layered silicates (e.g. montmorillonite), metal nanoparticles (e.g. silver), metal oxide nanoparticles (e.g. Tio_2) and natural biopolymers (e.g. cellulose and chitosan) (Othman, 2014). Among mentioned nano-fillers, the most commonly used materials are clay minerals. This is because they are natural, cheap, available, non-toxic, and relatively processable with significant reinforcement effect. Nanoclays usually have layered structures (nanoplatelets) with dimensions in nanometric range (de Azeredo, 2013; M. M. Reddy, Vivekanandhan, Misra, Bhatia, & Mohanty, 2013).

Clay minerals could be classified into three groups including kaolinite group (e.g. kaolinite and halloysite), illite group (e.g. hydrous micas, brammalite and celadonite), smectite group (e.g. montmorillonite, hectorite, laponite, bentonite and saponite) (Ismadji, Soetaredjo, & Ayucitra, 2015).

During the preparation of nanocomposites from clays, polymeric chains diffuse between silicate layers and form three main arrangements including phase-separated or conventional composite (microcomposite), intercalated and exfoliated (J. Liu, Boo, Clearfield, & Sue, 2006). The detailed morphology of each structure is given in Figure 5.

Most of the natural nanoclays are hydrophilic, thus it is difficult to mix them homogeneously with hydrophobic polymers. For this reason, nanoclays are modified by cation exchange reactions (Kampeerapappun, Aht-ong, Pentrakoon, & Srikulkit, 2007; Ludueña, Vázquez, & Alvarez, 2013). A lot of suppliers commercially provide different kinds of modified and also intact clays (Table 1). Most of the above-mentioned nanoparticles are modified with quaternary ammonium salts (Ahmed & El-Shishtawy, 2010). Incorporation of nanoparticles, such as clay minerals, especially montmorillonite (MMT), can improve the mechanical properties of polymer blends (Bagdi, Müller, & Pukánszky, 2006).

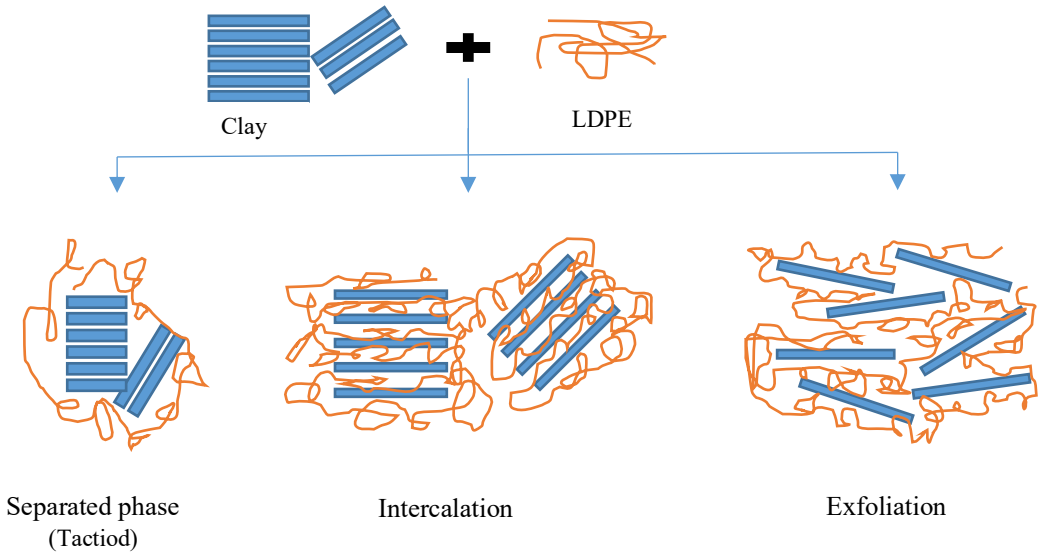


Figure 5. Morphology of three main types of polymer/clay nanocomposite

Table 1-some of most famous commercial clays[97-99]

Country	Name of company	Series name	Examples
USA	Southern clay products (now is a part of BYK Additives Ltd)	Cloisite®	Cloisite® Na^{+}, 10 A, 20A and 30B
USA	Nanocor, Inc	Nanomer®	Nanomer® 1.30P & 1.44P
Italy	Laviosa Chimica Mineraria	Dellite®	Dellite®43B & Dellite® 72T
Germany	Süd-Chemie	Nanofil®	Nanofil®15 & Nanofil® SE3000

Montmorillonite, sepiolite, halloysite, feldspar, bentonite, and nano-cellulose were the most common nanoparticles that have been used in various studies to date as reinforcing agents for producing LDPE/starch films (Musa, Bashir, Ahmad, & Buntat, 2015). The summary of reported

results about LDPE/starch nanocomposites is shown in Table 2.

Table 2 - Summary of reported studies using nanoparticles in the LDPE/ starch blends

Filler type	Method of preparation	% Loading	The best concentration(%)	References
Cloisite®15A	Montmorillonite is modified with quaternary ammonium salts	1-5	5	[20]
Halloysite Nanotubes	Halloysite nanotubes were hybridized with kenaf core fibers	0-15	12	[101]
Bentonite	Nanoparticles used without any modification	3-15	12	[102]
Feldespar		3-15	-	
Montmorillonite	MMT modified with sorbitol via solid state method	2-6	2	[103]
Sepiolite	Prepared with hydrophobic silane	2-6	6	[2]
Extracted cellulose nanofibers	Isolated CNFs from wheat straws directly used without any modification	6–14	6	[104]
Nanosilics	Synthesized from rice husk ash through acid leaching	0.5 -2	1.5	[105]

Chuayjuljit, Wiriyasoonthorn, Jiratumnukul, and Limpanart (2009) studied the mechanical properties of cassava starch, montmorillonite (MMT) and low-density polyethylene (LDPE) nanocomposites. They modified MMT with sorbitol through a solid-state method. Their results indicated that the addition of 2 phr (part per hundred) of MMT and 10 phr of starch to polymer blends resulted in higher mechanical properties of the blends, but these effects decreased with increasing MMT concentration, due to aggregation of MMT in the blends and weak adhesion with polyethylene. Inceoglu and Menceloglu (2013) prepared starch/LDPE films containing compatibilizer, plasticizer, and a Na-montmorillonite as a reinforcement filler (7.5 wt %). Solution intercalation method was used for making nanocomposites. Their results showed that produced films in comparison with clay-free composites had superior physical and mechanical properties. Increasing the reinforcement effect of clay nanoparticles due to uniform distribution of clay in plasticized starch–LDPE matrix, as well as prevention of plasticizer evaporation, because of barrier properties of clays, were suggested possible mechanisms for these observations.

In another study, Sabetzadeh, Bagheri, and Masoomi (2014) investigated the effect of organomodified montmorillonite (Cloisite15A) contents on mechanical properties of LDPE/corn starch blends. They prepared LDPE-corn starch mixtures, using a twin-screw extruder, with different nanoclay contents (0.5, 1, 2 and 3 phr). In samples containing 1 phr of nanoclay, ultimate tensile strength and Young's modulus increased compared with samples without nanoclay. The main mentioned reason was the good interaction of nanoparticles with maleic anhydride-grafted low-density polyethylene (LDPE-g-MA) segments, in the form of physically cross-linked networks. They also indicated that the possibility of phase separation and also agglomeration of nanoparticles increased in the presence of higher nanoclay contents.

In a complementary study by the same authors, the effect of nanoclay content on properties of ternary LDPE/LLDPE/TPS blends were studied in the presence of PE-g-MA (3 wt.%) and Cloisite®15A (1, 3 and 5 phr). The prepared films had intercalated and to some extent exfoliated structures. This study also showed that, along with increasing of nanoclay content from 1 to 5 phr, the tensile strength and impact strength of the prepared films increased. The observed results were due to the finer dispersion of nanoparticles and partially formed exfoliated platelets in the matrix. Whereas, elongation at break of the films slightly decreased with the increasing nanoparticle contents of the blends. This behavior was related to stiffing effect of nanoclays in the matrix and thus limited mobility of polymeric chains (Sabetzadeh et al., 2016).

Bio-nanofibers, like cellulose-based nanofibers, recently have gained a lot of interest because of their renewability, low density, high firmness and strength features (Gray, Hamzeh, Kaboorani, & Abdulkhani, 2018; A. P. Kumar & Singh, 2008). Alidadi‐Shamsabadi, Behzad, Bagheri, and Nari‐Nasrabadi (2015) studied the effects of wheat cellulose nanofibers (6–14 wt %) on rheological and mechanical behaviors of LDPE/starch nanocomposites. The extracted nanofibers first were blended with starch by using an internal mixer and then the obtained blend was mixed with low-density polyethylene in a single screw extruder. Tensile strength and elongation at break of composites were decreased after addition of nanofibers. This decrease was more marked for elongation at break compared with blank samples. This was mainly due to the incompatibility of cellulose nanofibers and polyethylene mixture. As almost all of the waxy parts of nanofibers were removed during the extraction process, the resultant nanofibers had hydrophilic nature.

7.Effect of recycled polyethylene

It is possible to blend starch with recycled LDPE, instead of virgin polyethylene. There is little research in this area. Pedroso and Rosa (2005b) studied the properties of virgin and recycled polyethylene blends with corn starch. Their results showed that the Young's modulus and elongation at break of blends containing recycled polyethylene were significantly higher than those having virgin polyethylene. This was probably due to the higher interaction between recycled polymer and starch. During reprocessing and preparation of recycled polyethylene, because of radical chain reactions, carboxylic and ketone groups are formed, and subsequently, crosslinks and branches along the polymer chains take place. The samples containing recycled polyethylene also had a lower tensile strength than virgin ones.

Similar results were observed by Pedroso and Rosa (2005), who also used reprocessed LDPE (Pedroso & Rosa, 2005a).

8.Effect of processing technique

Various techniques have been used for the preparation of starch/LDPE blends and production of the films (Rodriguez-Gonzalez et al., 2003; Rodriguez‐Gonzalez, Virgilio, Ramsay, & Favis, 2003). St-Pierre, Favis, Ramsay, Ramsay, and Verhoogt (1997) proved that it is possible to control the morphology of the dispersed starch (especially size and shape) in the blends by processing technique. Matzinos et al. (2001) prepared LDPE/starch blends by melt compounding in a twin screw extruder and then processed the obtained LDPE/TPS pellets to films, by injection molding and film blowing techniques. Their findings revealed that films produced by injection molding method had higher tensile strength and Young's modulus compared with blown films. Finer dispersion of starch particles in the matrix and formation of the continuous phase in injected molded films due to the smaller size of thermoplastic starch was the proposed reason for the described observation. They concluded that with injection molding technique it is possible to produce films with up to 40 % wt. of starch.

In another study, B. Raj, K, and Siddaramaiah (2004) investigated the effect of two different processing methods for production of films. In the first method they employed a solution casting technique followed by thermo-pressing, and in the second method they used extrusion process by means of a twin screw extruder for preparation of blends and finally they converted them to films by blowing. Their finding showed that extruded films had better mechanical properties compared to solution casted films due to better molecular alignment.

9.Conclusions

The blending of starch with LDPE as a synthetic polymer could increase the biodegradability of obtained films. However, due to the different polarity and weak interaction of the two phases, mechanical properties of the produced films are not satisfactory.

This review shows that LDPE/starch blend mechanical properties are influenced by various factors, such as starch granule characteristics

(amylose to amylopectin ratio, size and shape), the quantity of starch in the formulation, nature of polyethylene, type of plasticizers.

To improve the mechanical properties of these blends, many approaches have been proposed by researchers. These approaches are mainly based on the reduction of hydrophobicity of starch molecules by processes such as thermoplasticization, cross-linking and esterification and/or surface modification of polymer by the use of a compatibilizer. Beside the above-mentioned factors, utilization of nanoparticles was shown to be an effective strategy for the improvement of film properties. The most important point is that, in many cases, the best results can be achieved by application of a combination of approaches, e.g. thermo-plasticization, compatibilization and addition of nanoparticles.

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OCTACOSANOL EXTRACTION, SYNTHESIS METHOD AND SOURCES: A REVIEW

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ABSTRACT

Octacosanol are straight chain aliphatic fatty alcohol which consist of 28-carbon chain, which is basically found in epicuticular region of plant like sugarcane, wheat germ oil, rice bran oil etc. and animal source like krill. Octacosanol is waxy in nature and insoluble in water but sparingly soluble in low molecular weight alkanes, chloroform, ethyl acetate etc. Octacosanol used as a nutritional supplement and functional food. Octacosanol under investigational reported for enhanced stamina endurance, cholesterol lowering effect, Parkinson disease, platelets antiaggregatory properties, amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), cytoprotective use, and atherosclerosis. Octacosanol extracted and prepared by various methods like Soxhlet extraction, Supercritical fluid extraction and synthetically synthesis.

1.Introduction

Stephen De Felice has coined the term Nutraceutical for the food product or its functional components exhibiting nutrition and pharmaceutical properties both. Nutraceuticals can be functional food ingredients or dietary supplements, obtained from natural sources (mostly plants origin). (Taylor et al., 2003) It is taken by many people at worldwide for getting the health promotion or benefits and disease risk reduction. Infinite numbers of bioactive compounds (individually or collectively) are reported with the expected beneficial effects and it has provided many benefits, depending on mechanisms occurred at varied level with their positive effects. (Rapport & Lockwood, 2000) Few decades people more health conscious but their fast, hectic & modern lifestyle which change diet schedule and diet pattern. They move towards junk food and ready to eat food's system in which they lack of essential and non-essential nutrients, mineral, vitamins etc. such types of health-conscious population shift towards Functional food, dietary supplements and Nutraceuticals products. This is obtained from herbal source, marine sources or synthetically derived. Octacosanol, one of the most abundant alcohols in policosanols, is taken as an alternative to aspirin for patients suffering from gastric irritation due to its cytoprotective effects (Varady et al., 2003).

There are various such products are available in market and many more in pipeline to introduce in health market. They available in three stages like extract form, intermediate formulation and finished dosage forms. All stages have their own merits and demerits. Like herbal products are highly unstable towards light, moisture, oxygen and temperature. Herbal products are less bioavailable due water insoluble property. So intermediate formulation performed on initial extract form to improve solubility, stability and bioavailability in finished products.

Octacosanol is one of the popular functional foods. Octacosanol is being used to prepare various kinds of dietary supplements, health foods, and pharmaceuticals (Janikula, 2002).

Cholesterol is an important component in the body as it is a major component of cell membranes, but high levels can cause hypercholesterolemia and eventually atherosclerosis leading to coronary heart diseases. (Gouni & Berthold, 2002) In various human studies, a daily supplement of 5–20 mg policosanol decreased the low-density lipoprotein (LDL) cholesterol concentration between 19 and 31% and the total cholesterol (TC) concentration between 13 and 23%. Long-term studies have shown that high-density lipoprotein (HDL) cholesterol levels increased in the range of 8–29%. (Francini et al., 2008) A daily dose of 40 mg policosanol seems to be effective in reducing the serum triglycerol concentration. Octacosanol improves oxygen usage by strengthening the heart, supporting low LDL and high HDL levels there by maintaining healthy heart function (Norris et al., 1986). Therefore, octacosanol is an important nutraceutical for the future because of increasing problem of obesity and chronic heart diseases (Snider, 1984). Pure octacosanol has been investigated as a possible treatment for Parkinsonism and amyotrophic lateral sclerosis (Beltz & Doering, 1993) it has also been used by athletes to enhance their performance by enhancing stamina (Irmak et al., 2006).

Policosanol, a mixture of long chain fatty alcohols (C24– C34 alcohols) is obtained from plant waxes and beeswax as a solid waxy substance and insoluble in water but Soluble in organic solvents (Ou et al. 2012). Several times the word "policosanol" is being used for labelling the enriched octacosanol for commercial purposes. Octacosanol is isolated from sugar cane wax consisting of a mixture of 1-octacosanol (60–70%), 1-triacontanol (10–15%), 1-tetracosanol (<2%), 1-hexacosanol (3–10%), 1-heptacosanol (<3%), 1-dotriacontanol (5–10%) and 1-tetratriacontanol (<5%) (Kawanishi et al., 1991). Sugar cane wax (6.85 g) was separated from sugar cane juice filter mud (100 g) and 22.52 g of octacosanol was extracted from 100 g of sugar cane wax (Chen et al., 2006). Octacosanol is also isolated from rice bran wax, but its content was never reported to be more than 15–20% in the policosanol mixture (Vijaya et al., 2013). The solvent extraction processes of policosanol from natural substances result in mixtures of fatty alcohols from which it is possible to obtain a single compound of interest only after expensive purification operations (Cravotto et al., 2010).

2.Various Sources, Extraction and Synthesis Process for Policosanol (Octacosanol)

An efficient synthetic method was developed for the preparation of 1-octacosanol (C_{28}-alcohol) from commercially available lipid-based intermediates namely sebacic acid (decanedioic acid) and stearyl alcohol. The key step in the synthesis is the preparation of tert-butyl dimethyl octacos-10-enyloxy silane from 10-tert-butyl dimethyl silanyloxy decanal and octadecyl triphenyl phosphonium bromide salt employing Witting reaction as per Fig.1.

This product on simultaneous hydrogenation of double bond and deprotection of tert-butyldimethylsilyl protecting group in a Single step on treatment with Pd/C and H_2. In methanol at ambient temperature resulted octacosanol in 95% yield. The products were characterised by IR, ^{1}H NMR, and GC–MS analysis. (Cravotto, 2005) Another synthesis of octacosanol from 10-undecenoic acid (UDA) and stearyl bromide. According to their methodology, conversion of UDA to methyl 10-oxo-decanoate was carried out by two routes,

(i) Dihydroxylation of UDA epoxide followed by oxidation and esterification or
(ii) Esterification of UDA, followed by oxidation using osmium tetra oxide.

Octacosanol was obtained from Wittig product after two steps namely, lithium aluminium hydride reduction of ester group to alcohol and double bond hydrogenation using Pd/H_2 under 30 bar pressure. Both the methods employed either ultra sound and micro-wave irradiation methods or expensive reagents like

osmium tetra oxide, m-chloroperoxybenzoic acid (Shin & Kim, 1994).

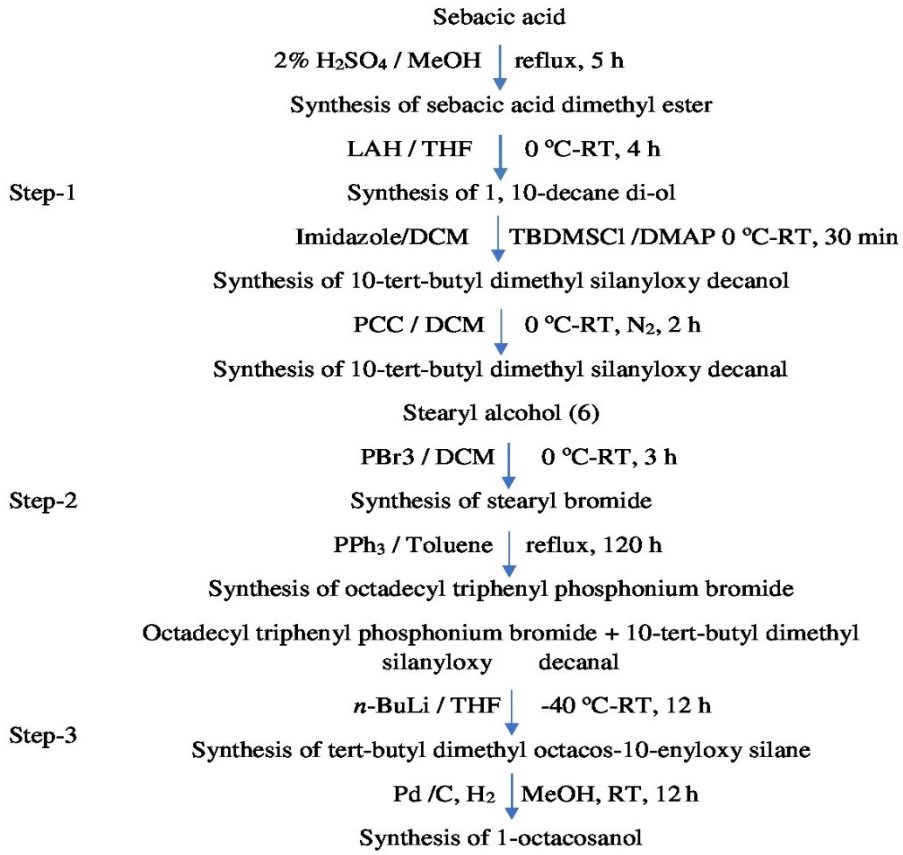

Figure 1. Synthetic route for 1-octacosanol

3.Extraction of Policosanols from Perilla seeds (*Perilla frutescens*)

Perilla seeds (*Perilla frutescens*) are a traditional source of oils produced in Korea, India, China, and other Asian countries. Annual production of perilla seeds in Korea is about 40000 metric tons and expelled perilla oil production is the third largest among edible oils in Korea (Prakash et al., 2006). Perilla seed oil contains considerably high levels of linoleic acid (Hwang et al., 2002a). Despite its nutritional benefits, perilla seeds are largely unknown in Western society. Therefore, the extraction of oils and oil-based products from such plant seeds would provide a new supplement in the world market.

Perilla and sesame seeds (800 g) were washed with tap water and placed on an absorbent towel. Immature seeds were removed by hand. Remaining seeds were dried at room temperature for 24 h. The uncrushed seeds were refluxed with 800 mL of hexane for 30 min in a round-bottom flask (2 L) at 70 °C. The warm mixture of seeds and hexane was filtered through a coffee filter paper to remove seeds. The filtrate was then passed through Whatman No. 2 filter paper to remove impurities and stored at -18 °C for 8 h to precipitate the Policosanols. The filtrate was prefiltered through Whatman No. 42 filter paper. Precipitate on the No. 42 filter paper was vacuum-dried at room temperature and collected as waxy materials (Hwang et al., 2002b).

4.TLC of Policosanols

TLC analyses of Policosanols extracted from the seeds were performed. Waxy material solution in hexane (about 10 μg/10 μL) was

spotted on a 20 x 20 cm, silica gel 60, 250 μm TLC plate. The developing solvent was a mixture of hexane, diethyl ether, and acetic acid (85:15:2, v/v/v). Developed bands were visualized by dipping the plate in 10% cupric sulfate solution containing 8% phosphoric acid for 5 second. Then the TLC plate was dried for 5 minute and kept in an oven at 150 °C until the developed bands were charred. (Hwang et al., 2004**)**

5.Compositional Analysis of Policosanols

Components were determined as per below method, using an HPLC equipped with a 250 mm x 4.6 mm i.d., 5 μm Luna silica column connected with a 4 x 3 mm i.d. guard column. The detector was an all tech Evaporative Light Scattering Detector 800, operated at 40 °C with nitrogen pressure of 3.5 bars. Two Waters 510 HPLC pumps were operated in gradient mode at a flow rate of 1 mL/min. Elution solvent consisted of a gradient of hexane (solvent A) and 0.2% acetic acid in methyl *tert*-butyl ether (solvent B), with the following profile: 0-2 min, 100% A; 3-10 min, 95% A; 14 min, 55% A; 23-26 min, 0% A; and 27-40 min, 100% A. The column and guard column were heated to 38-40 °C using a Waters Column Heater Module. Exposed lines from injection loop to detector connection were maintained at 38-40 °C wrapped with a heating tape. Samples were prepared in hexane (2 μg/20 μL), and 20 μL of each sample was injected for the analysis (Dixit & Khosa, 1971).

6.Compositional Analysis of Policosanols Fractionated from Waxy Materials

The composition of policosanols in the waxy materials was analysed using GC as mention below method. The policosanol fraction (2 mL) collected from HPLC (20 μg of policosanol) was derivatized to trimethylsilyl (TMS) ethers (10 min at 60 °C) using 0.05 mL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide and 0.2 mL of chloroform. A standard solution of alcohols (docosanol, tricosanol, and tetracosanol and hexacosanol, heptacosanol, octacosanol, and triacontanol was prepared in 0.2 mL of chloroform (1-8 μg of each) and derivatized as above for the identification of retention times and the calculation of their response factors. The TMS ether derivatives (2 μL) were injected into a 6980 Series GC equipped with a 30 m x 0.25 mm i.d., 0.25 μm, DB-5 column, flame-ionization detector, and helium as a carrier gas. Injector and detector temperatures were both set at 315 °C. The oven was programmed to start and hold at 150 °C for 1 min before increasing to 210 °C at 20 °C/min, increasing to 310 °C at 4°C/min, holding at 310°C for 1 min, increasing to 315 °C at 25 °C/min, and holding for 5 minute (Morris et al., 2000).

7. Preparation, Purification and Octacosanol from the Leaves of Sabicea grisea

Powder of the dried leaves of *S. grisea* was extracted at room temperature with 90% ethanol. The solution was filtered using a Whatman N° 1 filter paper under suction and concentrated to dryness at 50 °C under reduced pressure. The obtained crude ethanol extract was partitioned between hexane, chloroform and hydro-alcoholic solution (7:3). The hexane fraction was further fractionated on a silica gel column using hexane, containing increasing amounts of ethyl acetate. Fractions with similar thin layer chromatography profiles were pooled. The remaining fractions were subjected to washings with hexane that resulted in the isolation of octacosanol ($C_{28}H_{57}OH$) (45 mg), melting point 81–82 °C. The concentration of octacosanol was 88.23 µg per 1 g dry weight of leaves. Verification of the purity of octacosanol was carried out by gas chromatography. The GC data revealed that a major peak was eluted at 38.79 min. However, minor impurities (especially hexacosanol at 36.08 minute—$C_{26}H_{53}OH$) were also detected as per the GC profile indicating that the compound was about 90% pure (Pollard et al., 1931).

This compound was characterized based on its GC-MS, and 1H and 13C NMR analyses together with available literature. GC-MS analysis of octacosanol was carried out with instrument GC–MS system under the following conditions: DB-5 fused silica column (30 m

length, 0.25 mm i.d., 0.25 mm film thickness); injector temperature, 220 °C; temperature programmed at 60–270 °C at 3 °C/min;

Injection type, splitless (1 μL of a 1:1000 *n*-hexane solution); carrier gas, helium, adjusted to a linear velocity of 32 cm/s (measured at 100 °C); mass spectra, 70 eV, in EI mode; ion source temperature, 180 °C; scan mass range, 25–700 u (Chibnall et al., 1931).

8.The Isolation of n-Octacosanol from Wheat Wax

The isolation of the longer-chain primary alcohol constituents of waxes from the blades of wheat (Triticum vulgare) which air dried in a room at 40°C and powdered material (14 kg.) was extracted with ether and from the material thus obtained (400 g.) the crude wax (62 g.) was prepared (Bleyberg and Ulrich, 1931). The yield was therefore 15-5 % of the ether extract or 0.44 % of the dried wheat. On saponification the wax gave 40 g. of unsaponifiable material, which was a hard-yellow wax, 12.4 g. of crude fatty acids. As a preliminary stage the unsaponifiable wax when treated by the phthalate (Backmann and Clark, 1927). Gave only a primary alcohol M.P. 82-83°C and material was dissolved in warm chloroform which was poured into a dish and left exposed to the air. On evaporation of the solvent a yellow friable powder was obtained, which was then shaken for ten minutes at room temperature with light petroleum. Twelve successive extractions removed all material (10 g.) readily soluble in this solvent, leaving 30 g. of insoluble crude primary alcohol.

9.Constitution of the primary alcohol

The crude primary alcohol was crystallised from carbon disulphide to remove the last traces of paraffin, giving 26.6 g. of white granular crystalline powder, M.P. 82-82.5°. 26 g. were acetylated by boiling gently for 16 hours with acetic anhydride and fused sodium acetate (20 g.). The dark brown solution was poured into water and crushed ice and the mixture stirred for 15 minutes. (Piper et al., 1931) The brown powder was collected, again stirred with iced water and finally dissolved in benzene-methyl alcohol, leaving a tarry residue. From the solution (charcoal) 27.5 g. of crystalline acetate were obtained, M.P. 63-64°. This material was fractionally distilled in vacuum from a Willstatter flask of 100 cc. capacity. The temperature of the metal-bath was about 280°C and several fractions were collected at 185-195°C/0.03 mm. the melting-points of which ranged from 63.5° to 64.5°C. The higher-melting fractions were then collected and redistilled (Ramaswamy et al., 1980).

The principal component of the wax from blades of young wheat is a long chain primary alcohol which has been identified as n-octacosanol (M.P. 83.2-83.4°C) by reduction to n-octacosane (M.P. 61.3-61.5°C) and by oxidation to n-octacosanoic acid (M.P. 90.8-91.1°C). The purity of all three products has been confirmed by X-ray analysis. The wax also contains mixed fatty acids, the composition of which has not yet been determined, and paraffin, M.P. 66°C, which has been shown to be a complex mixture (Cravotto et al., 2004).

10.Extraction of policosanol from rice brain oil by supercritical fluid extraction (SFE)

160 g of freshly obtained rice bran, stabilized using an automated microwave oven at 110°C for 200 seconds and stored at 4°C was extracted using an SFE machine for 3 hours at 60°C, 600 bars pressure, with a carbon dioxide flow rate of 25 g/minute.38 The extracted oil was collected and percentage yield calculated with respect to the rice bran sample weight.

The RBW in the SFE-extracted RBO sample was separated as acetone insoluble (Dunford et al., 2010). Acetone chilled to approximately 4°C was added to 5 mL RBO (1:1, volume/volume [v/v]), and the mixture was centrifuged at 4,000 rpm for 25 minutes. Supernatant oil was decanted carefully, and the insoluble portion was washed with 5 mL chilled acetone and centrifuged. The wax obtained was blown with nitrogen, using a nitrogen generator, and then dried in an oven at 60°C. The dried wax was then weighed and stored at 4°C until further analysed.

Policosanol was extracted from RBW. (Kazuko et al., 1991) About 10 g RBW was

placed into a 200 mL conical flask and hydrolysed with 100 mL 0.2 M NaOH by sonication with a Power sonic 505 ultra sonicator 50 Hz, 350 W, at 60°C for 90 minutes. The hydrolysed mixture was then extracted with an equal volume of petroleum ether, cooled down to 2°C, and then divided into 50 mL centrifuge tubes and centrifuged at 4,000 rpm for 10 minutes. The upper petroleum ether layer and the lower NaOH layer were removed by carefully decanting; the middle yellowish layer (policosanol) was collected and freeze dried.

11.Determination of policosanol content

Policosanol contained in the RBW was determined by gas chromatography mass spectrophotometry (GC-MS). The GC-MS determination of policosanol was done. Policosanol standards docosanol, tetracosanol, hexacosanol, and octacosanol. (Tolloch, 1976). A 5mM concentration mixture of these standards in chloroform was prepared, 0.5 mL of this mixture was derivatized with 0.2 mL N,O-Bis(trimethylsilyl)trifluoroacetamide by incubating at 60°C for 20 minutes, and then the volume was made up to 1 mL by adding more chloroform after cooling to room temperature. RBW policosanol extracts were derivatized the same way. Policosanol standards and RBW policosanol extracts were first injected into the GC-MS machine.

The GC oven temperature was programmed from 150°C to 300°C with a heating rate of 4°C/minute and maintained at this temperature for 15 minutes. Initial flow rate of the carrier gas, helium, was 1.0 mL/minute. Inlet temperature was 300°C. GC-MS parameters were as follows: the MS transfer line temperature was 280°C, the ion source was kept at 230°C, and the MS quadrupole temperature was kept at 150°C. The ionization energy was 70 eV with 2 scans/second and a mass range of 100–1,000 amu. The standards/samples (2 μL) were injected into GC-MS with a 1:10 split ratio.

12.The purification of crude octacosanol extract from rice bran wax by molecular distillation

Octacosanol has been found in many plants, e.g. in leaf, bark and stem waxes of rye grass, apple peel, and wheat germ**.** Rice bran wax (RBW) has been reported to be one of the best sources containing Octacosanol**.** To extract and purify octacosanol from RBW, column chromatography and recrystallization are popular methods (Steve et al., 1963).

Molecular distillation (MD) can avoid using any organic solvents in the purification, even if it can remove any harmful solvent residues in the products, resulting in the generation of a much smaller waste and higher safety (Christensen & Reineccius, 1995 a; Wu & Zhang, 2000 b). Molecular distillation is a special case of short-path distillation in which the distance between evaporating and condensing surfaces is less than the mean free path of the molecules involved in high vacuum**.** (Feng et al., 2002 a; Ridway, 1956 b). This technology is considered as one of the best methods separating and purifying natural product, especially for substances with high-molecular mass, high viscosity and high melting point (Armando et al., 1994 a; Ooi et al., 1994 b). Meanwhile response surface methodology (RSM) is effective for responses that are influenced by many factors and their interactions, which was originally described (Box and Wilson, 1951). Previous research indicated that it is useful for developing, improving and optimizing processes (Atkinson & Donev, 1992 a; San Martin et al., 2003 b). In the this research, MD is used as a main method for the purification of crude octacosanol extract from trans esterified RBW, and its working conditions such as distilling temperature and vacuum degree is optimized by RSM in order to obtain the highest octacosanol content and parallelly to maintain the yield in the purified product as large as possible. The detailed process and the effect of MD conditions on the purification are elaborated by Octacosanol mathematical model; there some of references

have referred to purification of crude octacosanol extract by MD (Feng et al., 2002).

RSM was applied to determine the working conditions of MD for the purification of crude octacosanol extract from RBW. A central composite rotate design (CCRD) was used to investigate effects of two independent variables (purification conditions), distilling temperature and vacuum degree, on dependant variables of the purification.

The Trans-esterification reaction of the RBW was carried out in a round-bottomed flask equipped with a temperature controller and a stainless-steel double-arm blender. The RBW was added to the flask, and heated until it completely melted. An n-butanol solution containing 0.2% KOH was added to the melted RBW with continuous stirring. The above solution was refluxed for 8h. The reaction mixtures were cooled to 0°C, and parallelly washed with distilled water to neutral. The resulting dried solid was extracted with ethanol at 70°C to obtain the crude octacosanol extract.

The MD was used to further purify crude octacosanol extract obtained from Trans esterified RBW. the distilling temperature and vacuum degree were two major factors responsible for the further purification of crude octacosanol extract, while the flow rate of feed, temperature of condensing surface, and rotate rate of scraper were not included as CCRD factors, and process were set at 3ml/min, 90°C, and 50 rpm respectively. The feed was heated to melt, after setting the parameters, feeding valve was turned on, and the degassed feed liquid was introduced subsequently down the evaporating surface and spread with a very thin film by scraper. Heated walls and high vacuum drive the more volatile components had been closely positioned internal condensing surface as the less volatile components continue down the cylinder. The obtained fractions, which separated, collected with individual discharge outlets. The distillates were collected to calculate the yield and determine octacosanol content.

By the reported methods of determine octacosanol content (Kazuko et al. (1991) and Gonzalez et al., 1996), 0.200 g 1, 3, 5-triphenylbenzene (TPB) was dissolved in 100.00ml cyclohexane. The TPB concentration in this solution was 2.000 mg/ml. Calibration curve were obtained by injecting standard solutions with concentrations of octacosanol ranging from 100 μg/ml to 900μg/ml. 0.025 g of sample, which was obtained by trans esterification in conjunction with MD from RBW, was dissolved in 3.0ml cyclohexane under the help of ultrasonic wave at 40°C. Then 1.5ml of 2.000mg/ml TPB solution was added to the above solution as internal standard. The resulting solution was made up to 5.0ml with cyclohexane before being subjected to GLC analysis. GLC was performed with a Hewlett Packard 6890A and a HP-5 (column 30m · 320lm · 0.25lm). The gas flow rates for N_2, H_2 and air were 45, 40, 450ml/min, respectively. The operating temperatures were set as follows: injector, 320°C; detector, 330°C; initial oven temperature 230°C, keeping 6 min., with a ramp rate 10°C/min to 280°C, keeping 20min., then with a ramp rate 20 °C/min to 300°C.

13.Preparation of octacosanol from filter mud by SCFE

Sugarcane is one of the major crops in the world and in China. Which is an ideal source of octacosanol, as its bagasse contains a higher amount of policosanol than sugarcane leaves and other materials, and has a high and stable content of octacosanol (Irmak et al., 2006; Oliaro-Bosso et al., 2009). After cane harvesting and processing, every 1000 kg of cane would produce 33 kg press mud or filter mud (Almazan et al., 1998) that contains 7% of crude wax, in which octacosanol amounts to 81% (Nuissier et al., 2002).

Extractions were performed on a SCFE extractor. 100 g of filter mud was suspended in 500 ml of absolute ethanol in a 1 L stainless extraction vials and extracted with 99.99% CO_2 at a flow rate of 30 L/h. The waxes were collected in a cooled separator at 25°C. The content of octacosanol in the extract was analysed as per prescribed procedure below. Extractions were performed in triplicate. 1kg. of

filter mud was suspended in 8 L of absolute ethanol in a 20 L reactor and refluxed at 80°C, 120 rpm for 4 hrs. After extraction, the processed solution was filtered through 300-mesh filter, the filtrate was cooled to 4°C, after centrifugation the green flocculates obtained, the sediments kept in the open air for 4 hrs to evaporate ethanol and dried in an oven at 60°C. The content of octacosanol in the waxes was determined by GC/MS.

The waxes were subsequently purified by the reported procedures. 10.0 g of the waxes was extracted using 200 mL of acetone in a Soxhlet extractor to remove chlorophyll and fat. The residue was placed in a 250 ml of flat bottom flask containing 100 mL of 95% ethanol and 4 g powdered sodium hydroxide and was refluxed at 80°C for 6 h; the mixture was cooled to 50°C and extracted with 200 mL of petroleum ether three times. The combined petroleum ether phase was cooled to 4°C and then was filtrated using filter paper, the filtrate cake was air-dried.

Policosanols in sugarcane bagasse and filter mud were extracted, for determination of octacosanol in the raw materials (Irmak et al., 2006). Octacosanol in the extracts was analysed (Chen et al., 2007).

1. Octacosanol was analysed on a GC/MS system, equipped with an HP-5 (30 m x 0.25 mm x 0.25 μm) capillary column. The conditions used for the GC measurement were as follows: Oven temperature programmed from 80°C to 320°C, at 10°C/min, and maintained at 320°C for 15 min.
2. Oven temperature programmed from 80°C to 320°C, at 10°C/min, and maintained at 320°C for 15 min.
3. Helium was used as carrier gas at a flow rate of 1.0 mL/min.
4. The inlet temperature was 300°C.

GC/MS operating temperatures were as follows:

1. MS transfer line 280°C, ion source 230°C, and MS quadrupole 150°C.
2. The ionisation energy was 70 eV.
3. The scan range and rate were 50-600 amu and 2 scans/s, respectively.
4. The injection volume was 10 mL.

The calibration curves were obtained by injecting the standard solutions with concentrations ranging from 100 to 900 μg/mL.

14.Octacosanol isolated from Tinospora cordifolia

T. cordifolia, generally known as guduchi, is broadly used in veterinary medicine and Ayurvedic system of medicine for its common tonic, antiperiodic, antispasmodic, anti-inflammatory, antiarthritic, anti-allergic and antidiabetic properties (Singh et al., 2003). Guduchi has been reported to be active against throat cancer in man and it has been reported to be non-toxic in acute toxicity studies in vivo, with almost no side effects (Chauhan, 1995). It has been shown that the polysaccharide fraction from guduchi was found to be very effective in reducing the metastatic potential of B16F-10 melanoma cells (Leyon and Kuttan, 2004a, b). The antiangiogenic and proapoptotic potential of T. cordifolia crude extract or hexane fraction (Leyon and Kuttan, 2004 a, b; Thippeswamy and Salimath, 2007). The pure compound responsible for this activity and its molecular mechanism of action has not been hither to investigate. A long long-chain aliphatic alcohol from T. cordifolia by activity-guided purification and shown that it inhibits tumour-induced angiogenesis in vivo by inhibiting VEGF gene expression. The mechanism of down regulation of VEGF gene expression in is shown to be involving inhibition of nuclear translocation of NF-<kappa>B and its binding to NF-<kappa>B consensus sequence. Octacosanol is a new antiangiogenic and antitumor agent that may lead to more selective and less toxic antineoplastic therapy.

The dried plant powder of T. cordifolia was extracted sequentially from non-polar to polar solvents namely hexane–benzene–chloroform–ethyl acetate and methanol. The solvents were evaporated by rotary evaporator and all the fractions were tested for antiangiogenic activity by peritoneal angiogenesis and Chicken chorioallantois membrane assay. Column was packed with hexane using silica gel 100–200 mesh size as a matrix and the hexane fraction

was loaded as dried slurry of silica gel. The ratio of material loaded and silica gel was 1:20. Elution was performed by hexane/chloroform/acetone (7:2:1) as mobile phase and all the eluted fractions were subjected to thin layer chromatography analysis. All the fractions eluted from the column were tested for bioactivity. One of them, fraction F 4, which showed antiangiogenic activity was further purified using HPLC Vydac C18 column in a Shimadzu LC-10AVP system with dual wavelength detector. The column was equilibrated with HPLC grade water and the loaded compound was eluted using linear gradient of 100%methanol at a flow rate of 1ml/min. The active compound was subjected to structure elucidation by ^{1}H, ^{13}C and $^{1}H–^{13}C$ COSY NMR spectroscopy and mass spectroscopy. The NMR experiments were done in $CDCl_3$ solution. Octacosanol (10mg) was dissolved in 100 µl of chloroform and 900 µl of DMSO and diluted 10 times with sterile distilled water to make final concentration of 1 µg/µl and used for subsequent experiments.

15.Extraction of Sugarcane Wax from Press Mud Solvent Extraction

Sugar cane press mud waste was extracted with different solvent such as Toluene and Benzene under a reflux system for 4 – 6 hr. at a stretch. The extract was filtered under mild vacuum and solvent recovered by distillation. After recovering the solvent, the solid mass containing wax mixtures and resins thus obtained was dissolved in hot isopropyl alcohol and filtered. The resin portion was separated and the total wax portion obtained which yellow or light cream was in color. The Physico-chemical properties of wax were analysed by Saponification, Iodine and Acid value, which were determined using standard methods of BIS (Lamberton et al., 1960).

The physico-chemical properties of press mud before and after extraction of wax were analysed. It includes pH, Moisture content, Total Nitrogen, Phosphorus, Potassium, Organic matter, organic carbon, Calcium, Magnesium and C: N ratio. All the procedures were followed described in APHA (*American Public Health Association)*.

16.Supercritical Extraction of Policosanol from Sugar Cane Wax

Sugar Cane Wax is obtained by heptane extraction from the sugar cane filter mud, a residue resulting from the sugar cane production containing 75% water, a large variety of fats, waxy esters, free alcohols, sterols and a resinous fraction mainly composed of calcium salts of heavy polyesters (Garcia et al., 1988). The industrial process for the separation of these alcohols consists of different successive and multiple steps, permitting a first fractionation of resin compounds, and the separation in a second step, of fats and a refined wax. Further solvent processing of the refined wax is focused on the isolation of a natural mixture of high molecular weight aliphatic alcohols of the series $C_{24}–C_{34}$, which once purified, have medical application like a medicant called Policosanol. This long current process has several problems related to both, complexity and the use of organic solvents (toxic, expensive, generation of residues, low selectivity) requiring solvent recovery, as well as being energy-intensive operation.

The use of CO_2 under supercritical conditions has been expanded to the isolation of bioactive compounds from natural materials like lanolin, jojoba esters and popolis (Lagunas et al., 1992). Due to CO2's properties, selectivity of triglycerides and waxes has also been achieved for substances containing a high level of lipid material (Stahl et al., 1988). This procedure has been used for the separation of natural waxes (Stahl et al., 1985) and aliphatic primary alcohols from the sugar cane wax (Fragernas, 1986) and rice bran (Garcia et al., 1994). All these results indicate that supercritical CO_2 technology is a promising alternative to the actual organic solvent extraction of the long chain alcohols from sugar cane wax.

Supercritical fluid extraction (SFE) as a technically viable alternative process for the extraction of high molecular weight *n*-alcohols from the sugar cane wax (Furukawa et al., 1987). Analysed the effect of pressure (300–350 bar),

temperature (50–100°C) and the amount of KOH used in the previous saponification (1–20%, w/w) on extraction yield of long chain material alcohols. A Response Surface Methodology (RSM) based on the statistical analysis (Lucas et al., 1997) of the experimental data was used to obtain mathematical expressions relating the operational variables and extraction yield.

17.Synthesis of 1-octacosanol

Policosanol was described by Cuban researchers as a mixture of eight higher aliphatic primary alcohols obtained from the wax of sugarcane (Saccharum officinarum L.). It contained: 1-tetracosanol (C_{24}), 1-hexacosanol (C_{26}), 1-heptacosanol (C_{27}), 1-octacosanol (C_{28}) – the most Abundant (60–70%), 1-nonacosanol (C_{29}), 1-triacontanol (C_{30}), 1-dotriacontanol (C_{32}) and 1-tetratriacontanol (C_{34}). The relative abundance of these alcohols and their total content defines the identity of policosanol. These mixtures are analysed by gas chromatography coupled with mass spectrometry (GC-MS) (Sierra et al., 2002)

The main step was a Wittig reaction between an octadecyl triphenyl phosphonium ylide and a methyl 10-oxodecanoate. In agreement with a flow-chart study of our synthetic process Fig. 2 (Cravotto & Cintas, 2007; Palmisano et al., 2007) (Cravotto et al., 2008).

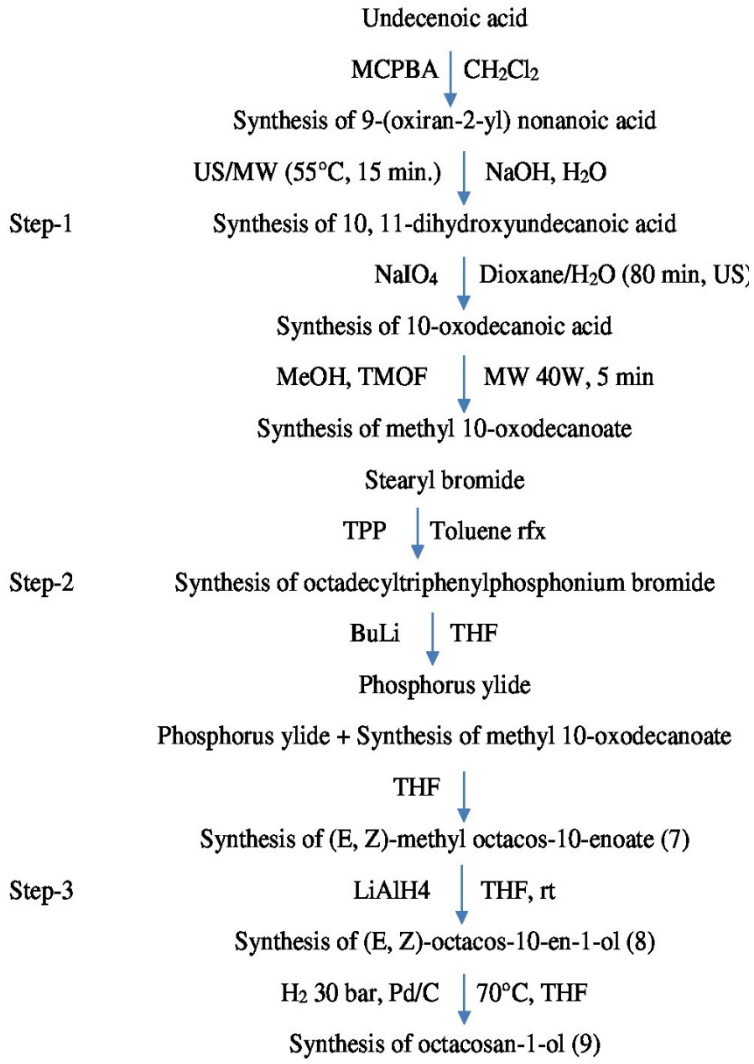

Figure 2. Synthesis of 1-octacosanol.

18.Extraction Methods for Policosanol from Rice Bran Wax

The effectiveness of different extraction methods for extracting Policosanol, especially Octacosanol, five extraction methods (A-E) were applied to the raw rice bran wax from manufacturer.

Method A. Saponification in alcohol was performed on the basis of the previously described procedure (Jiao & Wang, 2002) with some minor modifications. Briefly, 5 g of rice bran wax was mixed with 25 mL of 95% ethanol and 0.5 g of NaOH in a 100 mL four-hole flask and subsequently hydrolysed by refluxing in a water bath for 8 h with continuous stirring. Ten millilitres of alcoholic $CaCl_2$ solution (12 g of $CaCl_2$ plus 200 mL of 95% ethanol) was added, and the mixture was filtered while it was hot. After two washings with 95% ethanol, the mud cake was discarded. The collected filtrates were combined, cooled, and filtered again. The mud cake obtained in this step was dissolved with 3 times the volume of acetone preheated at about 50 °C. The solution in acetone was filtered after cooling. The resultant white PC product was naturally dried at ambient temperature.

Method B. Saponification in water (non-neutralized) was performed on the basis of a previously described procedure (Li & Qian, 2003) with some minor modifications. Briefly, 5 g of rice bran wax and 3 times the volume of water were placed in a 100 mL four-hole flask. After the wax was melted at 85 °C in a water bath, 0.5 g of NaOH was added. The mixture was boiled for 12 h followed by heat preservation for 36h. One millilitre of saturated $CaCl_2$ in water was added, and the reaction was continued for an additional 3 h at 80 °C. After filtration, the mud cake was washed to neutrality with hot water (80 °C) and dried at 65 °C. Subsequently, the solid was loaded to a Soxhlet apparatus and was extracted with 6-8 times the volume of acetone (Liu et al., 2001) as solvent for 16h. Once the extract solution had cooled, the Policosanol product was crystallized. Then it was filtered and dried at 65 °C.

Method C. Saponification in water (neutralized) was performed on the basis of the previously described procedure (Xu, 2002a) with some minor modifications. Briefly, 5 g of rice bran wax, 35 g of water, and 0.75 g of NaOH were placed into a 100 mL four-hole flask followed by continuous stirring for 20 h at 98 °C in a water bath. Ten grams of 10% HCl solution was subsequently added to neutralize the resultant. After 10 g of 8% $CaCl_2$ solution had been added, the reaction was kept for an additional 3 h. The resultant was cooled and filtered. After drying, the solid was refluxed with 10 times the volume of acetone for 12 h. The extract solution was cooled and filtered again. Policosanol product was obtained after drying.

Method D. Dry saponification was performed on the basis of the previously described procedure (Xu, 2002b) with some minor modifications. Briefly, 10 g of rice bran wax and 3 g of 50% $Ca(OH)_2$ soliquoid in water were added to a 100 mL four-hole flask and heated at around 100 °C in a water bath with continuous stirring for 5 h. The brown resultant was solidified once cooled. After the solid, which is the mixture of APAs and Ca-SFAs was weighed and ground, 5 g of the powder and 75 g of 95% ethanol were transferred into another flask and refluxed for 2h under stirring. The mixture was filtered while it was hot. After the filtrate had cooled, the Policosanol product was crystallized from the filtrate and was filtered and dried at 65 °C.

Method E. Trans esterification was performed on the basis of the previously described procedure (Chen et al., 2003) with some minor modifications. Briefly, 5 g of rice bran wax and 50 mL of 0.1% KOH solution in *n*-butanol were placed into a 100 mL four-hole flask. After 8 h of refluxing with continuous stirring, the reactant was cooled and substantially filtered. The mud cake was washed with hot water until it was neutral. After drying, the mud cake was loaded into a Soxhlet apparatus and extracted with acetone as the solvent for 12 h. The extract solution was cooled and filtered. The PC product was obtained after drying.

19.Conclusions

The major compound in Policosanol is 1-Octacosanol which is a long chain primary alcohol which has been obtained by various sources like animal, plant and synthetic route also. For isolation and purification various techniques used like supercritical fluid extraction, solvent evaporation extraction etc. and octacosanol quantification performed by gas chromatography.

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AMINO ACID PROFILES OF FIVE COMMONLY CONSUMED INSECTS IN SOUTHWESTERN NIGERIA

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ABSTRACT

The amino acid (AA) content of *Apis mellifera* (AB), *Macrotermes bellicosusI* (WT), *Imbrasia belina* (MoW), *Oryctes boas* larva (SB), and *Sitophilus zeamais* (MaW) were investigated. The total amino acid values were high at (g/100g crude protein, cp): *A. mellifera* (89.6), *M. bellicosus* (89.3), *I belina* (89.7), *O boas* larva (95.6) and *S. zeamais* (89.0). Glutamic acid (Glu) had the highest concentration in all the samples ranging from 12.3-15.0 g/100g crude protein, cp. The least concentrated amino acid was tryptophan (Trp) (1.22-1.33 g/100g cp) across board. Leucine (7.74-8.42 g/100g cp) was the most abundant essential amino acid (EAA) in all. The total essential amino acid (TEAA) (with His) ranged between 44.9-46.0 g/100g cp. Leu/Ile range was 1.09-1.47. P-PER$_1$ and P-PER$_2$ ranges were 2.73-2.99 and 2.65-2.96 respectively. The essential amino acid index (EAAI) range was 36.1-38.7 while the biological value (BV) range was 27.6-30.5. The isoelectric point (pI) range was 5.15-5.54, showing the samples to be in acidic medium of the pH range. In amino acid scores based on whole hen's egg, serine (Ser) had the least scores range (0.500-0.613). On provisional amino acid scoring pattern, the limiting AA was threonine (Thr) in *A. mellifera* (0.864), *M. bellicosus* (0.820) and *S. zeamais* (0.920); lysine (Lys) in *I. belina* (0.952); valine (Val) in *O. boas* larva (0.848). On pre-school child requirements, Lys (0.903-0.941) was limiting except in *O. boas* where all the parameters were higher than 100% requirement. Generally, no significant difference existed among the samples in most of the parameters determined.

1.Introduction

Insects are group of animals found in nearly all environments including the oceans. They form a class of animals within the arthropod group. More than one thousand four hundred edible insects have been recorded (FAO, 2008). A number of insects and their products were used in the past and are to a certain extent still eaten by some West African tribes, especially by children. Insect consumption is all over the regions of the world as substitutes for conventional proteins. Winged termites (*Macrotermes bellicosus)* are the commonly eaten termites specie especially in south western Nigeria. They are usually collected while on their nuptial flight or picked from the ground after they have shed their wings. A honey bee (*Apis mellifera*) is any member of the genus *Apis*, primarily distinguished by the production and storage of honey and the construction of perennial, colonial nest from wax. Currently, only seven species of honey bee are recognized with a total of forty-four subspecies (Michael, 1999). Today's honey bees constitute three clades: drones (males) produced from unfertilized eggs, i.e. have only a mother; workers and queens (both females) result from fertilized eggs (i.e. have both a father and a mother) (Maria and Walter, 2005). Mopane

worm (*Imbrasia belina*) is arguably the most popular among the moths. About 9.5 billion mopane caterpillars are harvested annually in southern Africa (Ghazoul, 2006). Vast number of people partakes in the mopane harvest and are willing to travel hundreds of kilometers across the mopane woodlands in search of the insects (Kozanayi and Frost, 2002). Though the caterpillars are important sources of nutrition in lean times, they also form a regular part of the diet (Stack *et al.,* 2003). Maize weevil (*Sitophilus zeamais*) is found in all warm and tropical parts of the word. It is a pest in stored maize, dried cassava, yam, common sorghum and wheat. Both adults and larvae feed on maize grains. Eggs, larval and pupal stages are all found within tunnels and chambers bored in the grains and are thus not normally seen. Adults emerge from the grain and can be seen walking over the grain surfaces (CABI, 2010). Scarab beetles larvae (*Oryctes boas)* are widely distributed throughout Africa, southern Asia and south America. They are typically collected, washed and fried for consumption (Fasoranti and Ajiboye, 1993). It is unusual to add oil because the larvae exude enough oil during the frying process. Their delicious flavour is credited to their elevated fat content (Fasoranti and Ajiboye, 1993).

There is need for people to consume adequate calories and nutrients to overcome the problem of protein-energy malnutrition. There is a link between malnutrition in Africa and inadequate and poor quality food supply (Kent, 2002). Most of these insects are nutritionally underutilized. Many developing regions consume insects as protein supplement in their diets. The objective of this study is to determine the amino acids composition and calculate the nutritional quality parameters of commonly consumed insects in southwestern Nigeria.

2. Materials and methods

2.1. Materials

2.1.1. Sample collection and preparation

Insect samples were obtained from farms and markets around Ekiti and neighbouring states and were later identified in the Zoology Department of Ekiti State University, Ado-Ekiti. Samples were screened, washed and rinsed with distilled water. Samples were then dried at 45^{o}C, dry milled to fine powder, stored dried prior to use for various analyses.

2.2. Methods

2.2.1. Extraction and analysis

Extraction and the instrumentation analysis were carried out by following AOAC method (AOAC, 2005). The samples were dried to constant weigh. Ten grams was weighed into 250ml conical flask. Samples were defatted with 30ml of petroleum spirit (3x) using Soxhlet extractor. Samples were hydrolyzed thrice for complete hydrolysis. The defatted samples were soaked in 30ml of 1M potassium hydroxide solution, incubated for 48 hours at 110^{o}C in hermetically closed borosilicate glass. After the alkaline hydrolysis, the hydrolysate was neutralized to get pH in the range of 2.5–5.0. The solution was purified by cation – exchange solid-phase extraction. The amino acids from the purified solutions were derivatised using ethylchloroformate (fig. 1).

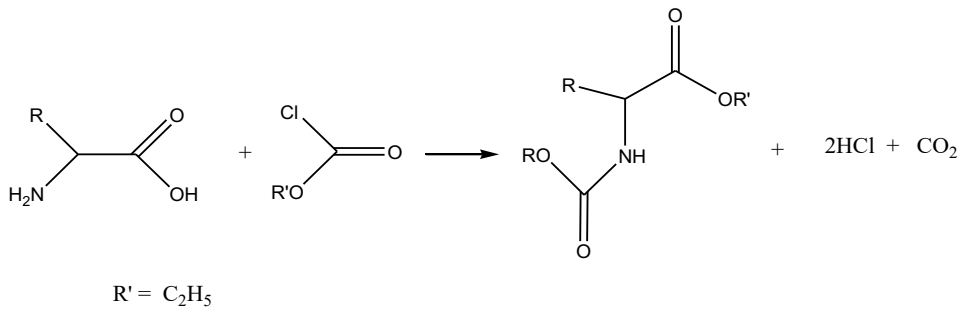

Figure 1. Derivatization process of amino acid

The derivatising reagent was removed by scavenge with nitrogen. The derivatized amino acid was made up to 1ml in a vial for gas chromatography analysis. The gas chromatographic conditions for the amino acids analysis were as follows: GC : HP6890 powered with HP Chemstation rev. A09.01 [1206] software; injection temperature: split injection; split ratio: 20:1; carrier gas: hydrogen; flow rate: 1.0ml/min; inlet temperature: 250^{o}C; column type: EZ; column dimensions: 10m x 0.2mm x 0.25µm; oven programme: initial @ 110^{o}C, first ramp @ 27^{o}C/min to 320^{o}C, second, constant for 5 mins at 320^{o}C; detector: PFPD; detector temperature: 320^{o}C; hydrogen pressure: 20 psi; compressed air: 35 psi.

2.3. Determination of Amino Acid Quality Parameters

2.3.1. Amino acid scores

Amino acid scores determination was first based on whole chicken's egg (Paul *et al*., 1976); the scoring included both essential and nonessential amino acids. The second determination was based on the provisional essential amino acid scoring pattern (FAO/WHO, 1973). The last score was based on pre-school child essential amino acid requirement for ages 2-5 years (FAO/WHO/UNU, 1985).

2.3.2 Other determinations

Other determinations such as total amino acid (TAA), total essential amino acid (TEAA), total non-essential amino acid (TNEAA), total acidic amino acid (TAAA), total basic amino acid (TBAA), total aromatic amino acid (TArAA), e.t.c. and their percentages were carried out. Total sulphur amino acid (TSAA) and percentage cystine in TSAA (% Cys in TSAA) were also calculated. Leu/Ile, Lys/Trp and Met/Trp ratios were computed.

Predicted protein efficiency ratio (P-PER) was calculated using equations derived by Alsmeyer*et al*. (1974), i.e.

$$P\text{-}PER_1 = -0.468 + 0.454\ (Leu) - 0.105\ (Tyr). \quad (1)$$

$$P\text{-}PER_2 = -0.684 + 0.456\ (Leu) - 0.047\ (Pro) \quad (2)$$

The isoelectric point (p*I*) was calculated using the equation of the form (Olaofe and Akintayo, 2000):

$$IPm = \sum_{i=1}^{n} IPiXi \quad (3)$$

Where IP_m is the isoelectric point of the mixture of amino acids, IPi is the isoelectric point of the i^{th} amino acid in the mixture and Xi is the mass or mole fraction of the i^{th} amino acid in the mixture (Fiar, 1975).

The essential amino acid index (EAAI) and biological value (BV) were calculated by the method of Oser (1959).

$$BV = 1.09\ (EAAI) - 11.73 \quad (4)$$

Various amino acid groups into classes I-VII (Nieman *et al*., 1992) were also calculated. Data obtained were subjected to simple descriptive statistics and Chi-square analyses (Oloyo, 2001).

3. Results and discussions

3.1. Common and scientific names

The common and scientific names of five edible insects commonly found in southwestern Nigeria used for this study are shown in Table 1. The insects analysed were one specie of hymenoptera (*Apismellifera*), one specie of isoptera (*Macrotermesbellicosus*), one specie of lepidoptera (*Imbrasiabelina*) and two species of coleoptera (*Oryctesboas*larva and *Sitophilus zeamais*).

3.2. Amino acid composition

Amino acids composition of the insect samples is presented in Table 2. Among the amino acids analyzed, Glu had the highest concentration in the samples having values ranging between 12.3 g/100g cp in *M. bellicosus* and 15.0 g/100g cp in *O.boas* larva. Tryptophan (Trp) recorded the lowest concentration (1.22-1.33g/100g cp) among the amino acids in each sample. Leucine (Leu) was the most concentrated essential amino acid in four out of

the five samples (g/100g cp): *A. mellifera* (8.28), *M. bellicosus* (8.11), *I. belina* (8.22) and *S. zeamais* (8.42). In *O. boas* larva, Lys (8.28g/100g) recorded the highest EAA. Other EAAs of high concentrations were (g/100gcp): arginine (Arg) (5.26-6.06), valine (Val) (4.24-5.76), isoleucine (Ile) (5.27-7.54) and phenylalanine (Phe) (4.11-4.71)). Valine daily requirement is 23 mg/kg and its deficiency leads to locomotor dysfunction in young rats (Adeyeye, 2009). The presence of substantial amount of arginine in diets enhances Ca^{2+} absorption, but under most physiological circumstances, this is of little consequence (White *et al*., 1973). Threonine (Thr) in the insect samples ranged between 3.28-4.34 g/100g cp with the highest concentration recorded in *O. boas* (4.34 g/100g cp). The concentration of methionine (Met) ranged between 2.02-2.51 g/100g cp. Met contains sulphur in the thioether linkage. Administration of Met prevents fatty liver and causes resumption of growth (Adeyeye, 2009). Histidine (His) ranged between 1.92-2.22 g/100g cp. His is a precursor of histamine, a substance normally present in small amounts in cells (Ogungbenle *et al*., 2013).

Table 1. Common and scientific names of the insect species

Symbol	Order	Family	Local name	English name	Scientific name
AB	Hymenoptera	Apidae	Oyin	Honeybee	*Apis mellifera*
WT	Isoptera	Termitidae	Esunsun	Winged termite	*Macrotermesbellicosus*
MOW	Lepidoptera	Notodontidae	Kanyin	Mopane worm	*Imbrasia belina*
SB	Coleoptera	Scarabaeidae	Gongo	Scarab beetle	*Oryctes boas*
MaW	Coleoptera	Scarabaeidae	Kokoroagbado	Maize weevil	*Sitophilus zeamais*

Table 2. Amino acid content of the insect samples (g/100g)

Amino acid	AB	WT	MoW	SB	MaW	Mean	SD	CV%	
Lysine	5.42	5.40	5.24	8.28	5.46	5.96	1.30	21.8	
Histidine	2.22	2.15	1.92	2.18	2.02	2.10	0.125	5.95	
Arginine	5.46	5.26	5.34	6.06	5.32	5.49	0.328	5.97	
Threonine	3.45	3.28	3.88	4.34	3.68	3.73	0.412	11.0	
Valine	5.55	5.76	5.12	4.24	4.80	5.09	0.606	11.9	
Methionine	2.10	2.17	2.02	2.51	2.25	2.21	0.188	8.51	
Tryptophan	1.22	1.27	1.28	1.24	1.33	1.27	0.042	3.31	
Leucine	8.28	8.11	8.22	7.74	8.42	8.15	0.257	3.15	
Isoleucine	7.13	7.26	7.54	5.27	7.03	6.85	0.902	13.2	
Phenylalanine	4.12	4.36	4.35	4.11	4.71	4.33	0.244	5.64	
Aspartic acid	7.70	7.35	7.55	8.53	7.47	7.72	0.470	6.09	
Glutamic acid	12.9	12.3	12.8	15.0	12.4	13.1	1.10	8.40	
Serine	4.67	4.84	4.38	3.95	4.64	4.50	0.347	7.71	
Proline	4.61	4.55	4.44	4.13	4.27	4.40	0.199	4.52	
Glycine	5.37	5.45	5.49	5.52	5.41	5.45	0.060	1.10	
Alanine	4.68	4.51	4.86	7.35	4.73	5.23	1.16	22.8	
Cystine	1.70	1.86	1.63	2.12	1.59	1.78	0.216	12.1	
Tyrosine	3.03	3.43	3.66	3.04	3.47	3.33	0.280	8.41	
Totals	89.6	89.3	89.7	95.6	89.0	90.6	2.79	3.08	

SD = standard variation, CV% = coefficient of variation percent, χ^2 = Chi-square, $\alpha = 0.05$,

df= k–1, NS = not significant at α = 0.05 and df = k-1,TV = table value

It is worthy of note that the FAO/WHO/UNU (1985) standards for pre-school children (2-5 years) are (g/100gcp): Leu (6.6), Ile (2.8), Lys (5.8), Met + Cys (2.5) and His (1.9). Hence, the insects would be able to provide the required or even more than the required Leu, Ile, Met + Cys and His. In Lys, results of four out the five samples (5.40-5.46 g/100g cp) representing 80% were comparable with the standard while *O. boas* (8.28 g/100g cp) would provide more than the required 5.8 g/100g cp. Generally, comparison of results among the samples showed no significant variation as observed in coefficient of variation percent (CV %) levels.

3.2.1. Amino acid categories

Table 3 depicts concentrations of total amino acid (TAA), total essential amino acid (TEAA), total non-essential amino acid (TNEAA), total acidic amino acid (TAAA), total basic amino acid (TBAA), total neutral amino acid (TNAA), total essential aliphatic amino acid (TEAlAA) and their percentage values. Some other parameters such as Leu/Ile ratios, Lys/Trp, Met/Trp ratios, predicted protein efficiency ratio 1 and 2 (P-PER_1 and P-PER_2), isoelectric point (pI), essential amino acid index (EAAI) and biological value (BV) are also depicted in Table 3. The TAA values of the present study ranged between 89.0-95.6 g/100g cp. The TEAA values (g/100g cp) in this study fell within 44.9-46.0 (with His); 42.7-43.8 (no His). These values were much higher than the 33.9 g/100g cp FAO/WHO/UNU (1985) standard for pre-school children (2-5 years). The EAA requirements are (g/100g, with His): infant (46.0), pre-school (2-5 years) (33.9), school child (10-12 years) (24.1) and adult (12.7); (without His): infant (43.4), pre-school (32.0), school child (22.2) and adult (11.1). It also revealed TEAA as (g/100g cp, with His): egg (51.2), cow's milk (50.4) and beef (47.9); (without His): egg (49.0), cow's milk (47.7) and beef (44.5). The values in this report were comparable to the afore-mentioned standards. Values of %TEAA in this report (48.1-50.7%) compare well with that of egg (50%) (FAO/WHO, 1990). In all the samples, the TAAA (19.7-44.0 g/100g cp) were higher than the TBAA (12.5-16.5 g/100gcp). The %TNAA in the samples [except in *S. zeamais* (36.2)] ranged between 58.2-63.6. This indicates that the neutral amino acids formed the bulk of the amino acids.

3.2.2. Quality parameter ratios

The values of Leu/Ile ratios (1.09-1.47) in the insects were good as they were all lower than the required 2.36 (FAO/WHO, 1991) since it will not lead to concentration antagonism. Leu/Ile imbalance from excess leucine may lead to developing pellagra (FAO, 1995). Present study revealed Lys/Trp as 4.09-6.66 and Met/Trp as 1.70-2.02. Mammalian tissue pattern have Lys/Trp: muscle (6.3), viscera (5.3), plasma protein (6.2) (Mitchell, 1950). The utilization of dietary proteins increases as their Lys and Trp contents approach that of muscle tissues. The nutritional values of some protein products with low Lys/Trp values can be enhanced by small additions of Lys (Adeyeye, 2015). Lys supplementation of wheat gluten increases the nutritive values of that of milk protein product. In the present results, *O. boas* larva Lys/Trp (6.66) meet the muscle, viscera and plasma protein standards while others were lower. All the Met/Trp values (1.70-2.02) were lower than the muscle value of 2.5 but approached the plasma proteins of 1.1 (Adeyeye, 2015). The predicted protein efficiency ratios, P-PER_1 (2.73-2.99) andP-PER_2 (2.65-2.96) vary between 0.00 for a very poor protein food and a maximum possible of just above 4.00 for good protein food. In general, the better the protein, the lower the level the diet required to produce the highest protein efficiency ratio. This reflects the importance of nutritive balance of all the amino acids for metabolic efficiency.

Table 3. Classification of amino acids (g/100g crude protein) of the insect samples

Amino acid	AB	WT	MoW	SB	MaW	Mean	SD	CV%	χ^2	TV	Remark
TAA	89.6	89.3	89.7	95.6	89.0	90.6	2.79	3.08	0.343	9.49	NS
TEAA + His	44.9	45.0	44.9	46.0	45.0	45.2	0.472	1.04	0.020	9.49	NS
TEAA – His	42.7	42.9	43.0	43.8	43.0	43.1	0.421	0.977	0.016	9.49	NS
% TEAA+His	50.7	50.4	50.1	48.1	50.6	50.0	1.08	2.16	0.093	9.49	NS
%TEAA–His	47.7	48.0	47.9	45.8	48.3	47.5	0.996	2.10	0.084	9.49	NS
TNEAA	44.7	44.3	44.8	49.6	44.0	45.5	2.33	5.12	0.475	9.49	NS
%TNEAA	49.9	49.6	49.9	51.9	49.4	50.1	1.01	2.02	0.081	9.49	NS
TAAA	20.6	19.7	20.4	23.5	44.0	25.6	10.4	14.6	16.8	9.49	S
%TAAA	23.0	22.1	22.7	24.6	49.4	28.4	11.8	41.5	19.6	9.49	S
TBAA	13.1	12.8	12.5	16.5	12.8	13.5	1.67	12.4	0.825	9.49	NS
%TBAA	14.6	14.3	13.9	17.3	14.4	14.9	1.37	9.19	0.501	9.49	NS
TNAA	55.9	56.8	56.8	55.6	32.2	51.5	10.8	21.0	9.03	9.49	NS
%TNAA	62.4	63.6	63.3	68.2	36.2	56.7	11.7	20.6	9.63	9.49	S
TEAlAA	21.0	21.2	20.9	17.2	20.3	20.1	1.67	8.31	0.553	9.49	NS
%TEAlAA	23.4	23.6	23.3	18.0	22.8	22.2	2.38	10.7	1.02	9.49	NS
TSAA	3.80	4.03	3.65	4.63	3.84	3.99	0.383	9.60	0.147	9.49	NS
%Cys in TSAA	44.9	46.2	44.8	49.9	41.3	45.4	3.09	6.81	0.844	9.49	NS
Leu/Ile ratio	1.16	1.12	1.09	1.47	1.20	1.21	0.152	12.6	0.077	9.49	NS
Leu – Ile	1.15	0.849	0.685	2.47	1.39	1.31	0.704	53.7	1.51	9.49	NS
%(Leu –Ile)/TAA	1.28	0.951	0.764	2.59	1.56	1.43	0.717	50.1	1.44	9.49	NS
%(Leu –Ile)/Leu	13.9	10.5	8.33	31.9	16.5	16.2	9.30	57.4	21.4	9.49	S
Lys/Trp	4.45	4.26	4.09	6.66	4.12	4.72	1.10	23.3	1.02	9.49	NS
Met/Trp	1.72	1.71	1.58	2.02	1.70	1.75	0.163	9.31	0.061	9.49	NS
P-PER1[a]	2.97	2.86	2.88	2.73	2.99	2.89	0.100	3.46	0.015	9.49	NS
P-PER1[a]	2.87	2.80	2.86	2.65	2.96	2.83	0.115	4.06	0.019	9.49	NS
pI[b]	5.16	5.15	5.16	5.54	5.15	5.23	0.172	3.29	0.023	9.49	NS
EAAI[c]	97.5	93.3	87.8	92.0	92.4	92.6	3.46	3.74	0.518	9.49	NS
BV[d]	94.5	90.0	83.9	88.6	89.0	89.0	3.80	4.26	0.647	9.49	NS

[a] Predicted protein efficiency ratio, [b] Isoelectric point, [c] Essential amino acid index, [d] Biological value, S = significant at $\alpha = 0.05$ and df = k-1.

Table 4. Amino acid scores of the samples based on whole hen's egg amino acid profile

Amino acid	AB	WT	MoW	SB	MaW	Mean	SD	CV%	χ^2	TV	Remark
Lys	0.874	0.870	0.845	1.33	0.880	0.960	0.207	21.6	0.179	9.49	NS
His	0.935	0.898	0.980	0.908	0.842	0.911	0.050	5.49	0.011	9.49	NS
Arg	0.894	0.863	0.875	0.994	0.871	0.899	0.054	6.01	0.013	9.49	NS
Thr	0.677	0.643	0.761	0.851	0.721	0.731	0.081	11.1	0.036	9.49	NS
Val	0.740	0.769	0.683	0.565	0.640	0.679	0.081	11.9	0.039	9.49	NS
Met	0.655	0.768	0.631	0.784	0.703	0.708	0.067	9.46	0.026	9.49	NS
Trp	0.676	0.704	0.711	0.691	0.736	0.704	0.023	3.27	0.003	9.49	NS
Leu	0.997	0.997	0.991	0.932	1.01	0.985	0.031	3.15	0.004	9.49	NS
Ile	1.27	1.30	1.35	0.940	1.26	1.22	0.163	13.4	0.087	9.49	NS
Phe	0.808	0.854	0.852	0.805	0.923	0.848	0.048	5.66	0.011	9.49	NS
Asp	0.719	0.687	0.706	0.798	0.698	0.722	0.044	6.09	0.011	9.49	NS
Glu	1.07	1.03	1.07	1.25	1.03	1.09	0.092	8.44	0.031	9.49	NS
Ser	0.592	0.613	0.554	0.500	0.588	0.569	0.044	7.73	0.014	9.49	NS
Pro	1.21	1.20	1.17	1.09	1.12	1.16	0.052	4.48	0.009	9.49	NS
Gly	1.79	1.82	1.83	1.84	1.80	1.82	0.021	1.15	0.001	9.49	NS
Ala	0.866	0.835	0.900	1.36	0.877	0.968	0.221	22.8	0.201	9.49	NS
Cys	0.946	1.03	0.908	1.18	0.881	0.989	0.121	12.2	0.059	9.49	NS
Tyr	0.758	0.856	0.915	0.759	0.866	0.831	0.070	8.42	0.023	9.49	NS
Totals	0.897	0.894	0.898	0.957	0.891	0.907	0.028	3.09	0.003	9.49	NS

Table 5. Essential amino acid scores of the insect samples based on FAO/WHO (1973) standards

Amino acid	AB	WT	MoW	SB	MaW	Mean	SD	CV%	χ^2	TV	Remark
Lys	0.985	0.981	0.952	1.50	0.993	1.08	0.234	21.7	0.203	9.49	NS
Thr	0.864	0.820	0.970	1.08	0.920	0.931	0.100	10.7	0.044	9.49	NS
Val	1.11	1.15	1.02	0.848	0.960	1.02	0.121	11.9	0.057	9.49	NS
Leu	1.18	1.16	1.17	1.11	1.20	1.16	0.034	2.93	0.004	9.49	NS
Ile	1.78	1.82	1.88	1.32	1.76	1.71	0.224	13.1	0.117	9.49	NS
Trp	1.22	1.27	1.28	1.24	1.33	1.27	0.042	3.31	0.006	9.49	NS
Met+ Cys	1.09	1.15	1.04	1.32	1.10	1.14	0.108	9.47	0.041	9.49	NS
Phe + Tyr	1.19	1.30	1.33	1.19	1.36	1.27	0.080	6.30	0.020	9.49	NS
Totals	1.13	1.19	1.19	1.19	1.19	1.18	0.027	2.29	0.002	9.49	NS

For EAA scores based on provisional essential amino acid scoring pattern (FAO/WHO, 1973) (Table 5), the following had scores greater than 1.00 in all the samples: Leu (1.11-1.20), Ile (1.32-1.88), Trp (1.22-1.33), Met + Cys (1.04 -1.32) and Phe + Tyr (1.19-1.36).

Table 6. Essential amino acid scores based on requirements of pre-school child (2-5 years)

Amino acid	AB	WT	MoW	SB	MaW	Mean	SD	CV%	χ^2	TV	Remark
Lys	0.934	0.931	0.903	1.43	0.941	1.03	0.225	21.8	0.197	9.49	NS
His	1.17	1.20	1.07	1.21	1.12	1.15	0.059	5.13	0.012	9.49	NS
Thr	1.02	0.965	1.02	1.14	0.968	1.02	0.071	6.96	0.020	9.49	NS
Val	1.59	1.65	1.46	1.25	1.37	1.46	0.162	11.1	0.072	9.49	NS
Trp	1.11	1.11	1.16	1.13	1.20	1.14	0.038	3.33	0.005	9.49	NS
Leu	1.25	1.23	1.25	1.17	1.28	1.24	0.041	3.31	0.005	9.49	NS
Ile	2.55	2.59	2.69	1.88	2.51	2.44	0.322	13.2	0.170	9.49	NS
Met + Cys	1.52	1.61	1.46	1.85	1.53	1.59	0.153	9.62	0.059	9.49	NS
Met + Tyr	1.14	1.24	1.27	1.13	1.30	1.22	0.077	6.31	0.020	9.49	NS
Totals	1.27	1.33	1.32	1.33	1.32	1.31	0.025	1.91	0.002	9.49	NS

3.2.3. Other quality parameters

The p*I* in this study ranged between 5.15-5.54. The calculation of p*I* from AA enhances production of certain isolate of organic products. The EAAI of 87.8-97.5 and their corresponding BV of 83.9-94.5 revealed to a reasonable extent the quality of protein in the insect samples. The insect values are better than in poultry (chicken, muscle without skin, duck, muscle without skin) (Finar, 1975). EAAI is essential in evaluating food for protein quality (Nielson, 2002). BV is a scale used to evaluate what proportion of a given nutrient source is utilized by the body. It shows how fast and efficiently our body utilizes proteins we consume. Both EAAI and BV values were generally high in this report.

3.3. Amino acid scores

In the amino acid scores based on whole chicken's egg amino acid (Table 4), Glu, Pro and Gly had scores greater than 1.00 in each sample: Glu (1.03-1.25), Pro (1.09-1.21) and Gly (1.79-1.84). Ile had scores greater than 1.00 in *A. mellifera* (1.27), *M. bellicosus* (1.30), *I. belina* (1.35) and *S. zeamais* (1.26). Ser had the lowest scores (0.500-0.613) in all the samples. Val had scores >1.00 in *A. mellifera* (1.11), *M. bellicosus* (1.15) and *I.belina*(1.02). Thr had the least EAA scores in *A. mellifera* (0.864), *M. bellicosus* (0.820) and *S. zeamais* (0.920) and therefore, to correct for the limiting amino acid (LAA) of the samples if they serve as sole source of protein food, it will be 100/86.4 (or 1.16) × protein of *A. mellifera*, 100/82.0 (or 1.22) × protein of *M. bellicosus* and 100/92.0 (or 1.09) × protein of *S. zeamais* (Bingham, 1977). Lys (0.952) and Val (0.848) were the LAAs for *I.belina* and *O. boas* respectively and would require corresponding 100/95.2 (or 1.05) and 100/84.8 (1.18) correction factors. Table 6 depicts the essential amino acid scores on the suggested requirements for pre-school children (2-5 years). The insect samples would supply virtually all the required EAAs for the pre-school children as most of the EAA scores were above 1.00 (i.e. 100%) except Lys (0.934) in *A. mellifera*, Lys (0.931) and Thr (0.965) in *M. bellicosus*, Lys (0.903) in *I. belina* and Lys (0.941) and Thr (0.968) in *S. zeamais*. Generally, the EAAs most often acting in a limiting capacity are (a) Lys, (b) Met + Cys, (c) Thr, and (d) Trp (Bingham, 1977).

4. Conclusions

The results of the study showed that the insects investigated are moderately rich in amino acids especially essential amino acids. Concentrations of Leu, Ile, Asp and Glu were high in the samples. Percentage essential amino acids in the samples were comparable with the percentage of non-essential amino acids. Total EAAs met the FAO/WHO/UNU standards for all categories of human beings: infant, pre-school, school child and adult. The samples' proteins were within range of conventional animal proteins. Generally, the insect samples are good sources of amino acids and would be useful as food supplements and in food fortification particularly because the EAAI and BV are even much better in values than most conventional meat protein sources.

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NUTRIENT COMPOSITION, FUNCTIONAL, PHYSICAL AND PASTING PROPERTIES OF YELLOW YAM (*Dioscorea cayenensis*) AND JACK BEAN (*Canavalia ensiformis*) FLOUR BLENDS

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Abstract

The study therefore focused on the nutrient composition, functional, physical and pasting properties of yellow yam and jack bean flour blends. Yellow yam tubers and jack bean seed were processed into flour, blended together and D-optimal mixture design was used to generate the percentage of yellow yam and jack bean flour resulting to a total of nine experimental runs. The flour blends were analyzed for nutrient composition, functional, physical and pasting properties using standard methods. Data obtained were subjected to statistical analysis. Means, analysis of variance were determined using SPSS version 21.0 and the difference between the mean values were evaluated at $p<0.05$ using Duncan multiple range test. The effect of optimization procedure was investigated using Design expert version (8.0). Crude protein, total carotenoids, starch, amylose and amylopectin ranged from 9.97 to 16.72%, 5.24 to 6.65µg/g, 79.38 to 80.07%, 25.36 to 29.88% and 70.13 to 74.65% respectively. Addition of yellow yam and jack bean flour had no significant ($p>0.05$) effect on the antinutritional composition (saponin, trypsin inhibitor and total polyphenol). Range of values for bulk density, dispersibility, water absorption capacity, swelling power and solubility index ranged from 0.62 to 0.73g/ml, 75.5 to 80.4%, 4.19 to 6.54g/g, 4.52 to 5.70g/g and 6.53 to 6.77% respectively. The yellowness (b*) of yellow yam and jack bean flour blend were not significantly ($p>0.05$) affected while the peak viscosity, breakdown viscosity, final viscosity and setback value ranged from 206.0 to 572.0RVU, 5.0 to 17.0RVU, 263.0 to 9.11.0RVU and 279.0 to 372.0RVU respectively. The flour blends were optimized with respect to crude protein, total carotenoid, starch content, amylose, amylopectin, dispersibility, water absorption capacity, swelling power, solubility index, peak viscosity, break down viscosity and yellowness were maximized while bulk density, final viscosity and setback values were minimize. The optimum flour blend ratio was 94.11% yellow yam and 5.89% jack bean flour.

1. Introduction

Yams are the edible tubers of various species of the genus Dioscorea and are important staple foods of many tropical countries particularly West African countries such as Cộte d'Ivoire, Ghana, Togo, Burkina Faso and Nigeria (Kouakou *et al.,* 2010; Amanze *et al.,* 2011). The yam tuber, which is

the most important part of the plant, can be stored longer than other root and tuber crops, ensuring food security even at times of general scarcity. It is the third most important tropical root and tuber crop after cassava and sweetpotato (Fu *et al.,* 2005). Yam contains mainly starch, with some proteins, lipids, vitamins and minerals (Lasztity *et al.,*1998). Yam tubers have been used as traditional food in the home with little industrial use; however the traditional uses are diverse and the crop still has many more utilization potentials yet untapped. Yam is consumed in different forms, mainly boiled, fried, or baked (Baah, 2009). Tubers are often dried and milled into flour for various products. The genus Dioscorea contains a wide range of yam species used as food. The most economically important species grown are white yam (*Dioscorea rotundata*), yellow yam (*Dioscorea cayenensis*), water yam (*Dioscorea alata*), Chinese yam (*Dioscorea esculenta*) aerial yam (*Dioscorea bulbifera*) and trifolate yam (*Dioscorea dumentorum*) (Ike and Inoni, 2006). *Dioscorea cayenensis* has various varieties but the most popular ones are white and yellow varieties. The yellow variety derives its common name from its yellow flesh, due to the presence of carotenoids (Aseidu, 2010). It is cultivated across yam producing areas in smaller amounts compared to white yam.

Among the locally available under-utilized legumes with high protein content is Jackbean (*Canavalia ensiformis*) (Idowu *et al.,* 2017). Jackbean has its origin in the Western part of India and Central America (Akande, 2016). Jackbean contains significant amounts of niacin, thiamine, phosphorus, calcium and iron (Leon *et al.,* 1990). Jackbean seed, like other legumes showed nutritionally adequate levels of most essential amino acids (EAA) except sulphur amino acids (methionine and cysteine) (Nnamdi-Okani, 2005). Jackbean has been used in foods because of its good thickening and gelling properties (Akande, 2016). They are also a good texture stabilizer and regulator in food systems (Akande, 2016).

Yam is a starchy root crop and widely known and exploited for food such as snacks (Aseidu, 2010) and noodles (Akinoso *et al.,* 2016) with so many varieties. However, *Dioscorea cayenensis* is an underutilized yam variety that is yet to be fully exploited and is fast being driven to extinction. Hence, there is need to explore the use of *Dioscorea cayenensis* into food product such as flour. *Dioscorea cayenensis* has been reported to be a source of carotenoids including α-carotene, β-carotene and numerous xanthophylls and their esters which could help in combating micronutrient deficiency in the country (Champagne *et al.,* 2010; Ukom *et al.,* 2014). Hence, tubers of *Dioscorea cayenensis* when properly processed could be used in the production of instant yam flour, yam flakes and other food products. Jackbean is an underutilized legume and is rich in protein and have unique functional properties owed to its appreciable values of swelling power, solubility and high amylose content of its starch (Marimuthu and Gurumoorthi, 2013). Several information are available on the use of flour from root and tuber with legume such as water yam and distillers spent grain (Awoyale *et al.*, 2015); Greater yam and jackbean flour (Affandi *et al.,* 2016) and water yam and lima bean (Rohmah *et al.,* 2018).This study is therefore aimed to produce flour blends from yellow yam (*Dioscorea cayenensis*) and jackbean and to determine its nutrient composition, functional, physical and pasting properties. Then, the best flour blends based on nutritional composition, functional, physical and pasting properties would be determined.

2. Materials and Methods

2.1. Materials

Matured yellow yam tuber (*Dioscorea cayenensis*) was purchased from Kila in Oke-Ogun, Oyo state while jackbean was obtained from the Research Farm of Federal Polytechnic Offa, Kwara State, Nigeria.

2.2.Flour preparation

The modified method of Oluwole *et al.* (2013) was used for the preparation of yellow yam flour. Yam tubers were washed with clean water to remove adhering soil and other

undesirable materials. The yams were hand-peeled using kitchen knife and sliced into sizes of 2 to 3 cm thickness. The sliced yellow yam was blanched in a water bath at 70 °C for 2mins to reduce browning after which the yam was removed. The sliced yellow yams were dried in a cabinet dryer at 60ºC for 72 h. The dried yam slice was milled using laboratory hammer mill (Fritsch, D-55743, Idar-oberstein-Germany) and the milled sample was sieved (using 250µm screen) to obtain the flour. The yellow yam flour was packed and sealed in polyethylene bags until further analysis.

The method described by Doss *et al.* (2011) was used for the preparation of jackbean flour. The jackbean was weighed and boiled in water (100 ºC) for 10 minutes. The boiled seed was rinsed with distilled water, dehulled and dried in a cabinet dryer at 60ºC for 72 h. The dried boiled seed was milled using laboratory hammer mill (Fritsch, D-55743, Idar-oberstein-Germany) and the milled sample was sieved (using 250µm screen) to obtain the flour. The jackbean flour was packed and sealed in polyethylene bags until further analysis

2.3 Formulation of blends

D-optimal mixture design was used to generate the percentage of yam and jackbean flour blends to investigate the effect of interaction of two independent variables as shown in Table 1

Table 1. Formulation of Yellow Yam and Jackbean Flour Blends using D-Optimal Mixture Design

Runs	Yellow Yam Flour (YF)	Jackbean Flour (JF)
1	90.00	10.00
2	92.50	7.50
3	90.00	10.00
4	93.75	6.25
5	92.50	7.50
6	90.00	10.00
7	95.00	5.00
8	95.00	5.00
9	91.25	8.75

2.4. Nutrient composition of yellow yam and jack bean flour blends

2.4.1. Determination of crude protein content

This was determined using AOAC (2010) method. One gram of the samples was weighed into the digestion flask and Kjeldahl catalyst tablets was added, 20 ml of concentrated H_2SO_4 was also added and the flask fixed into the digester at 410ºC for 6 h until a clear solution was obtained.. The cooled digest was transferred into 100 ml volumetric flask, and made up to mark with distilled water. The distillation apparatus was set up and rinsed for 10 min after boiling. 20 ml of 4% boric acid was pipetted into a conical flask. 5 drops of methyl red was added to the flask as indicator and the sample was diluted with 75 ml of distilled water and 10 ml of the digested sample was pipetted into the Kjedahl distillation flask. 20 ml of 40% NaOH was added through the glass funnel into the digested sample and it wasdistilled, the distillate was collected in the boric acid for 15 min until pink colour changes to green. The content of the flask was then titrated against 0.05 N HCl.

Calculation:

%Nitrogen (W/W)

$$= \frac{14.01 \times (\text{Sample titre-blank titre}) \times \text{Normality of acid}}{10 \times \text{Weight of Sample}} \quad (1)$$

%Crude protein (W/W) = %Nitrogen x 6.25 (2)

2.4.2. Determination of total carotenoid content

Total carotenoid content of the flour blends and extrudates were determined spectrophotometrically as described in the Harvest Plus Handbook for Carotenoid Analysis and using slight modifications of the methods described by Lee (2001). During the extraction process, some precautions were taken, like working in a reduced luminosity room. This was determined using spectrophotometric measurements using T60 UV Visible spectrophotometer at 450 nm. Six millilitre of n- hexane, 3 ml of acetone and 3 ml of ethanol ((hexane: acetone: ethanol, 2:1:1) containing 0.05% butylated hydroxytoluene (BHT) was added to 2 g of flour samples. The sample was centrifuge. The top layer of hexane containing the color was recovered and transferred to a cuvette. For total carotenoid, the absorbance of the hexane extract was read at 450 nm.

$$\text{Formula } \mu g/g \text{ Carotenoid} = \frac{A \times VOL \times 10^4}{2505 \times W} \quad (3)$$

A= absorbance, W= weight of sample, VOL= volume of hexane used.

2.4.3. Determination of starch content

The method of Dubois *et al.* (1956) as modified by AOAC (2006) was used for the determination of the starch content of the flour blends. Hot ethanol was used to extract starch from the yam and jackbean flour sample. The extract (supernatant) and digest (from the residue) was quantified calorimetrically for starch, using phenol-sulphuric acid as the colour developing reagent; and absorbance read at 490 nm wave length. 20mg of the flour sample was weighed into a centrifuge tube and wetted with 1 ml of 95% ethanol. 2mL of distilled water was added followed by 10 mL of hot 95% ethanol. The content was vortexed and centrifuged (GALLENKOMP Centrifuge Model 90 - 1, USA) at 2000 rpm for 10 min. The supernatant was decanted while the sediment was hydrolyzed with perchloric acid and used to estimate starch content. The absorbance was read with a spectrophotometer (Milton Spectronic 601, USA) at 490 nm.

$$\%\text{Starch} = \frac{(\text{Absorbance} - 0.0044)4}{\text{sample wt x } 0.55} \quad (4)$$

2.4.4.Determination of Amylose and Amylopectin content

The amylose content of the yam starch was determined based on the iodine colorimetric method of Williams *et al.* (1958) and Juliano (1971) as described by Addy *et al.* (2014). About 0.1 g of the starch sample was solubilized with 1 ml of 95% ethanol and 9 ml of 1 N NaOH, and heated in a boiling water bath for 10 min; 1 ml of the extract was made up to 10 ml with distilled water. To 0.5 ml of the diluted extract was added 0.1 ml I N acetic acid and 0.2 ml iodine solution (0.2 g I2+2.0 g KI in 100 ml of distilled water)to develop a dark blue colour. The coloured solution was made up to 10 ml with distilled water and allowed to stand for 20 min for complete colour development. The solution was vortexed and its absorbance was read on a spectrophotometer at 620 nm. Absorbance of standard corn amylose with known amylose concentration was used to estimate the amylose content.

$$\%\ \text{Amylose} = \frac{\%\ \text{amylose of standard x Absorbance of sample}}{\text{Absorbance of standard}} \quad (5)$$

$$\%\text{Amylopectin} = 100 - \text{Amylose content.} \quad (6)$$

2.4.5. Antinutritional factors of yellow yam and jack bean flour blends

2.4.5.1.Determination of saponin

The Spectrophotometric method of Brunner (1984) was used for saponin analysis. 1 g of flour sample was weighed into a 250 ml beaker and 100 ml Isobetyl alcohol was added. The mixture was shaken on a UDY shaker for 5 h to ensure uniform mixing. Thereafter, the mixture was filtered through a Whatman No. 1 filter paper into a 100 ml beaker and 20 ml of 40% saturated solution of Magnesium carbonate added. The mixture obtained with saturated $MgC0_3$ was again filtered through a Whatman No 1 filter paper to obtain a clear colourless solution. Then 1 ml of the colourless solution was pipetted into 50 ml volumetric flask and 2

ml of 5% $FeCl_3$ solution was added and made up to mark with distilled water. It was allowed to stand for 30 min for blood red colour to develop.Then 0-10 ppm standard saponin solutions were prepared from saponin stock solution. The standard solutions were treated similarly with 2 ml of 5% FeCl solution. The absorbances of the sample as well as standard saponin solutions were read after colour development on a T60 UV- visible spectrophotometer, U. K. at a wavelength of 380 nm.

Percentage saponin was calculated using the formula:

$$\text{Saponin (\%)} = \frac{\text{Absorbance of sample} \times \text{Average gradient} \times \text{Dilution factor}}{\text{weight of sample} \times 10{,}000} \quad (7)$$

2.4.5.2. Determination of trypsin inhibitor activity

The method of Kakade *et al.* (1974) was adopted for the determination of trypsin inhibitor activity as modified by Oluwole *et al.* (2013). 1g of flour sample was extracted with50ml of 0.01N NaOH, for 1 hr. The pH of the suspension was determined. This suspension was diluted to the point where 1 ml produces trypsin inhibition of 40 to 60%. 2 ml of trypsin solution was added to the test tubes, the tubes were placed in a water bath at 37°C. 5ml of BAPA solution previously warmed to 37°C was added, exactly 10 min, later the reaction was terminated by adding 1ml of 30% acetic acid. After thorough mixing, the contents of each tube was filtered (Whatman No.3) and the absorbance of the filtrate was measured at 410 nm against a reagent blank. Trypsin inhibitor activity is expressed in terms of trypsin units inhibited (TIU).

2.4.5.3Determination of total polyphenol content

Total polyphenols content was determined using the method described by Jayaprakasha *et al.* (2001) using Folin-Ciocalteu reagent with a minor modification. In a 2ml of Eppendorf tube, 780µl deionized water, 20µl sample extract, and 50µl Folin-Ciocalteu reagent (1:1 v/v) with water were added and mixed. After 1 minute, 150µl Sodium carbonate (0.2g/ml) was added, and the mixture was allowed to stand at room temperature in the dark for 1h. Then, 300µlofthe mixture was carefully introduced into a 96 well plate using Eppendorf micropipette. The absorbance was read at 750nm. The total polyphenol concentration was calculated from a calibration curve, using Gallic acid (1mg/ml) as standard (200 – 1000mg/L).

2.5.Functional properties of yellow yam and jack bean flour blends

2.5.1. Bulk density

Bulk density was determined using the method described by Wang and Kinsella, (1976) and Onwuka (2005). Ten grams of sample was weighed into 50ml graduated measuring cylinder. The sample was packed by gently tapping the cylinder on the bench top. The volume of the sample was recorded.

$$\text{Bulk density (g/ml)} = \frac{\textit{Weight of sample}}{\textit{Volume of sample after tapping}} \quad (8)$$

2.5.2. Dispersibility

This was determined by the method described by Kulkarni (1991) and Adebowale *et al.* (2012) Ten grams of flour was poured into 100 ml measuring cylinder and distilled water added to reach a volume of 100 ml. The set up was stirred vigorously and allowed to settle for 3 h. The volume of settled particles was recorded and subtracted from 100. The difference was reported as percentage dispersibility.

2.5.3. Water absorption capacity

Water absorption capacity of the flour samples were determined using the method described by Beuchat (1977) and Onwuka (2005).1 g of the flour was mixed with 10 ml of water in a centrifuge tube and allowed to stand at room temperature (30 ± 2 ºC) for 1 h. It was then centrifuged at 2000 rpm for 30 min. The volume of water on the sediment water measured. Water absorption capacities were calculated as ml of water absorbed per gram of flour.

2.5.4. Determination of swelling power and solubility index

The swelling power and solubility index was determined using the method described by Takashi and Siebel (1988) and Adebowale *et al.* (2012). One grams of flour was weighed into a 50 ml centrifuge tube. 50 ml of distilled water was added and mixed gently. The slurry was heated in a water bath at 90^0C for 15 minutes. During heating the slurry was stirred gently to prevent clumping of the flour. On completion, the tube containing the paste was centrifuged at 3,000rpm for 10 minutes using a centrifuge machine. The supernatant was decanted immediately after centrifuging. The weight of the sediment was taken and recorded. The moisture content of sediment gel was thereafter determined to get dry matter content of the gel.

$$\text{Swelling Power} = \frac{\textit{weight of wet mass of sediment}}{\textit{weight of dry matter in the gel}} \quad (9)$$

$$\text{Starch solubility index \%} = \frac{\textit{weight of dry solids after drying}}{\textit{weight of sample}} \text{ X } 100 \quad (10)$$

2.6. Physical properties of yellow yam and jack bean flour blends

2.6.1. Colour

The method described by Feili *et al*. (2013) was used. To measure the flour blends, Minolta chroma meter (CR- 410, Japan) was used based on (CIE) L* a* b* scale. After calibrating the instrument by covering a zero calibration mask followed by white calibration plate. Flour blends were analyzed by placing them on the petri dish, and then the image was captured on the samples. The colour attributes such as lightness (L*), redness (a*) and yellowness (b*) were recorded.

2.7. Pasting characteristics of yellow yam and jack bean flour blends

Pasting characteristics were determined with a Rapid Visco Analyzer (RVA TECMASTER, perten instrument-2122833, Australia). Three grams of sample were weighed into a dried empty canister, and then 25ml of distilled water was dispensed into the canister containing the sample. The suspension was thoroughly mixed properly so that no lumps were obtained and the canister was fitted into the rapid visco analyzer. A paddle was then placed into the canister and the test proceeded immediately automatically plotting the characteristic curve. Parameters estimated were peak viscosity, breakdown viscosity, final viscosity and setback value.

2.8 Statistical Analysis

All experimental data obtained from nutrient composition, functional, physical and pasting properties were carried out in triplicate. Data obtained were subjected to statistical analysis. Means, analysis of variance were determined using SPSS version 21.0 and the difference between the mean values were evaluated at $p<0.05$ using Duncan multiple range test. The optimization procedure was investigated using Design expert version (8.0) software. Regression analysis and significant effects of the independent variables were determined at 5% confidence level.

3. Results and Discussion

3.1. Nutrient composition of yellow yam-jackbean flour blends

The effect of yellow yam-jackbean flour inclusion on nutrient composition of the blend is shown in Table 2. The protein of the flour blends ranged from 9.97 to 16.72%.The interaction effect of yellow yam and jackbean flour did not significantly ($p>0.05$) affect the protein content of the flour blend as shown in the regression coefficient for the protein content of yellow yam and jackbean flour blends in Table 3. The result of protein content obtained in this study was slightly higher (9.97 to 16.72%) than that reported in previous studied by Awoyale *et al.* (2015) on water yam and distillers spent grain flour blends (7.2 to 15.10%) but lower than the value of Oluwamukomi and Adeyemi (2015) on yam and soybean flour blends (4.21 to 19.50%) and Okorie *et al.* (2016) on yam flour and cowpea flour blends (1.85 to18.21%). This might be due to the differences in the species of the yam and the nutritional composition of the soil from

which the yam was harvested, the moisture content and the maturity of the crop (Osagie, 1992). The high protein content of yellow yam and jackbean flour blends reported in this study could also be attributed to the high protein content of jackbean. The high protein content in jackbean substituted with yellow yam flour will be of great nutritional importance in most developing countries such as Nigeria where there is an occurrence of protein malnutrition (Anuonye *et al.,* 2012; Okpala and Okoli, 2011).

The total carotenoid ranged between 5.24 and 6.65µg/g. Inclusion of yellow yam flour with jackbean at 7.50% had the highest value for total carotenoid while inclusion of yellow yam with jackbean flour at 10% had the lowest value. The regression table presented in Table 3 shows that the regression coefficient for total carotenoid content of yellow yam and jackbean flour blends were significantly ($p<0.05$) affected. Carotenoids are rich plants that contain antioxidant components which offer various health benefits such as reducing the risk of cardiovascular diseases, cancer and other degenerating diseases (Eleazu and Eleazu, 2012). In this study, the total carotenoid content of the yellow yam and jackbean flour were low this could be as a result of heat, temperature and drying methods employed during flour processing which could be responsible for the total carotenoid degradation (Olivera *et al.,* 2010). However, Hiane *et al.* (2003) and Thakkar *et al.* (2007) also reported that total carotenoid degradation also occurs in temperature close or superior to 40 °C, which was higher than the temperature (60 °C) used in this study. Other factor such as grinding /milling could influence the total carotenoid degradation of yellow yam and jackbean flour thereby exposing their cellular content to the environment (oxygen) in the passage through the milling machine thus facilitating the oxidative process could also contribute to the low total carotenoid content obtained in this work (Olivera *et al.,* 2010).

The starch content ranged from 79.38 to 80.07% with yellow yam-jackbean blends at 5% had the highest value for starch content while yellow yam-jackbean at 10% had the lowest value for starch. The high starch content of the flour blends obtained in this study might be attributed to the high starch content in yellow yam and jackbean flour. Wireko-Manu *et al.* (2011) reported that tuber crops such as yam are relatively rich in starch and within the range of 60.42 to 77.56%. The result obtained in this study were higher (79.38 to 80.07%) when compared with other species of yam such as greater yam and jackbean flour (67.34 to 69.22%) as reported by Affandi *et al.* (2016); Okorie *et al.* (2016) on water yam and cowpea flour (59.57 to 78.31%) and Rohmah *et al.* (2018) on water yam and lima bean (70.538 to 71.772%). According to Degras (1986); Muthukumarasamy and Pannerselvan, (2000) and Baah (2009), starch is well known to account for 80% on a dry weight basis of yam carbohydrate, hence, it is a key factor in determining the physicochemical, rheological and textural characteristics of yam food products. The interaction effect of yellow yam and jackbean flour had a significant ($p<0.05$) effect on the starch content of the flour blend as shown in Table 3.

The amylose and amylopectin content of the flour blends ranged from 25.36 to 29.88% and 70.13 to 74.65% respectively. Significant ($p>0.05$) effect were not observed in the amylose content of yellow yam and jackbean flour interaction as shown in Table 3. Amylose is a major component of starch which influences pasting and retrogradation behaviour (Zhenghong *et al.,* 2003) and impart definite characteristics to starch (Moorthy, 1994; Rosida *et al.,* 2017) while amylopectin ratio gives specific characteristics and functionality to starches by determining the texture and nature of their product (Moorthy, 1994; Scott, 1996; Ezeocha *et al.,* 2014).The result obtained for amylose (25.36 to 29.88) in this study were in agreement with the values of Rohmah *et al.* (2018) on water yam and lima bean (28.53 to 29.82%) but higher than the findings of Affandi *et al.* (2016) on greater yam and jackbean flour (17.23 to 20.38%) and other root and tuber crops such as sweetpotato and soybean flour blend (12.62 to 12.94%) as reported by

Omoniyi *et al.* (2016). This could be due to the activities of enzyme in the starch biosynthesis (Krossmann and Lioyd, 2000) and different species of yam in which the starch was isolated and analytical methods used to determine the amylose content (Aprianita *et al.,* 2014). However various researchers such as Moorthy (2002) and Rosida *et al.* (2017) reported the amylose content for *Dioscorea cayenensis* to be 21-25%, *Dioscorea rotundata* to be 27.48 to 31.55% (Addy *et al.,*2014), *Dioscorea alata* to be 20-36% (Harijono *et al.,* 2013) while Marimuthu and Gurumoorthi (2013) reported the amylose content of jackbean to be 33.24%. However, Rohmah *et al.* (2018) reported that food with high amylose content will be hard and firm while food high in amylopectin will stimulate puffing process, thereby making the product to be light, dry and crispy. Thus, the interaction effect of yellow yam and jackbean flour on the amylopectin content were not significantly ($p>0.05$) affected.

Table 2. Nutrient Composition of Yellow Yam and Jackbean Flour Blends

YF (%)	JF (%)	Protein (%)	Total carotenoid (µg/g)	Starch (%)	Amylose (%)	Amylopectin (%)
90.00	10.00	16.68^{d}	5.24^{a}	79.35^{a}	29.86^{d}	70.15^{a}
92.50	7.50	13.71^{c}	6.59^{bc}	79.91^{b}	28.75^{c}	71.25^{b}
90.00	10.00	16.72^{d}	5.24^{a}	79.38^{a}	29.88^{d}	70.13^{a}
93.75	6.25	9.97^{a}	6.16^{b}	79.94^{b}	26.68^{b}	73.32^{c}
92.50	7.50	13.75^{c}	6.65^{c}	79.93^{b}	28.73^{c}	71.27^{b}
90.00	10.00	16.68^{d}	5.24^{a}	79.43^{a}	29.83^{d}	70.18^{a}
95.00	5.00	10.42^{a}	6.27^{bc}	80.07^{b}	25.89^{ab}	74.11^{cd}
95.00	5.00	10.42^{a}	6.26^{bc}	79.97^{b}	25.91^{ab}	74.09^{cd}
91.25	8.75	11.81^{b}	5.42^{a}	79.67^{ab}	25.36^{a}	74.65^{d}

Mean values with different superscripts within the same column are significantly different ($p<0.05$); YF- Yellow Yam flour, JF- Jackbean flour

Table 3. Regression Coefficient of Nutrient Composition of Yellow Yam and Jackbean Flour Blends

Parameter	Protein	Total carotenoid	Starch content	Amylose	Amylopectin
A	10.35	6.24	80.01	26.08	73.92
B	16.45	5.19	79.40	29.47	70.54
AB	-3.97	2.45*	0.75*	-0.90	0.89
F-value	15.24	14.45	147.77	3.69	3.66
R^2	0.84	0.83	0.98	0.55	0.55

*Significant at ($p<0.05$): A- Yellow Yam flour, B- Jackbean flour, AB- Interaction effects of Yellow Yam and Jackbean Flour, R^2- Coefficient of determination

3.2. Antinutritional Factor of Yellow Yam and Jackbean Flour Blends

The effect of yellow yam-jackbean flour addition on the antinutritional factors of the blend is shown in Table 4. The saponin, trypsin inhibitor and total polyphenol ranged from 4.97 to 6.86%, 112.44 to 283.1% and 7.81 to 16.84% respectively. Saponins are considered important due to their toxicity in yams (Okwu and Ndu, 2006). The saponin content of yellow yam and jackbean flour blends were higher than 2.51 to 4.31% reported by Adelekan *et al.* (2013) on trifoliate yam and pumpkin seed flour. The high level of saponin (4.97-6.86) observed in the yellow yam and jackbean flour blends might be responsible for its

characteristic bitter after taste. Saponins have natural affinity to ward off microbes which are helpful for treating fungal infection (Okwu and Ndu, 2006). They inhibit growth of cancer cells and help to lower blood cholesterol hence useful in the treatment of cardiovascular disease and other health related problems (Del-Rio *et al.,* 1997). The interaction effect of yellow yam and jackbean flour had no significant ($p>0.05$) effect on the trypsin inhibitor and total polyphenol as shown in Table 5. Trypsin inhibitor in legumes has been reported to be a limiting factor for their effective and efficient utilization (Leiner, 1996; Bamigboye and Adepoju, 2015). The high level of trypsin inhibitor (112.44-283.10%) of yellow yam and jackbean flour can be attributed to the high level of trypsin inhibitor in jackbean flour. However, Doss *et al.* (2011) reported the value of trypsin inhibitor in jackbean seed to be 378.3%. Trypsin inhibitor may hamper protein digestibility, trypsin inhibitor is thermo liable and may be destroyed with application of heat (Ohizua *et al.,* 2016; Olatunde *et al.,* 2019).

Polyphenol oxidase activity plays a significant role in enzymatic browning (Asiyanbi-Hammed, 2016). Poly phenol oxidase detected in yam and jackbean flour confirmed to the report that major yam species contain polyphenol oxidase activity with variation among species (Sanni and Fatoki, 2017). Ukom *et al.* (2014) reported the value of total polyphenol content for fresh *Dioscorea cayenensis* to be 27mgCE/100g. The high level of total polyphenol (8.42- 16.84%) of yellow yam and jackbean flour observed in this study can be due to the high level of polyphenol present in yam (*Dioscorea cayenensis*) flour. The result observed in this study for yellow yam and jackbean flour blends are higher than the values of 0.79 to 1.58% reported by Affandi *et al*. (2016) on greater yam and jackbean. This could also be due to different species of yam used in this study. However, the high quantities of total polyphenol compounds indicate that the flour samples could act as immune enhancers, hormone modulators, antioxidants, anti-clotting and anti-inflammatory (Okwu and Omodamoro, 2005). However, the interaction effect of yellow yam and jackbean flour on the total polyphenol content was not significantly ($p>0.05$) affected.

Table 4. Antinutritional Factors of Yellow Yam and Jackbean Flour Blends

YF (%)	JF (%)	Saponin (%)	Trypsin inhibitor (%)	Total polyphenol (%)
90.00	10.00	5.04^{a}	283.05^{c}	16.82^{a}
92.50	7.50	5.33^{b}	244.41^{c}	13.02^{a}
90.00	10.00	5.08^{a}	283.10^{c}	16.82^{a}
93.75	6.25	6.16^{c}	214.25^{bc}	9.07^{a}
92.50	7.50	5.34^{b}	244.45^{c}	13.14^{a}
90.00	10.00	4.97^{a}	283.05^{c}	16.84^{a}
95.00	5.00	6.43^{d}	201.85^{ab}	8.42^{a}
95.00	5.00	6.41^{d}	201.87^{ab}	8.47^{a}
91.25	8.75	6.86^{e}	112.44^{a}	7.81^{a}

Mean values with different superscripts within the same column are significantly different ($p<0.05$); YF- Yellow Yam flour, JF- Jackbean flour,

Table 5. Regression Coefficient of Antinutritional Factors of Yam and Jackbean Flour Blends

Parameter	Saponin	Trypsin inhibitor	Total polyphenol
A	6.35	211.71	8.71
B	5.19	267.97	16.17
AB	0.31	-135.13	-6.05
F-value	2.33	1.20	6.32
R^2	0.44	0.29	0.68

A- Yellow Yam flour, B- Jackbean flour, AB- Interaction effects of Yellow Yam and Jackbean Flour, R^2- Coefficient of determination

3.3. Functional properties of Yellow Yam and Jackbean Flour Blends

The effect of yellow yam-jackbean flour substitution on some functional properties of the blend is presented in Table 6. The bulk density of flour blends ranged from 0.62 to 0.73g/ml. The interaction effect of yellow yam and jackbean flour significantly ($p<0.05$) affect the bulk density of the flour blend as presented in Table 7. Bulk density depicts the behaviour of the material in dry mixes and is a significant parameter that can determine packaging requirement of the product (Mohammed *et al.,* 2009). The bulk density of yellow yam and jackbean flour blend (0.62 to 0.73g/ml) from this study were slightly higher than 0.62 to 0.68g/ml reported by Adelekan *et al.* (2013) on trifoliate yam and pumpkin seed flour but lower than the values of 0.56 to 0.84g/ml reported by Malomo *et al.* (2012) on yam (*Dioscorea rotundata*) and soybean flour. This may be due to different species and composition of flour used in substitution. Bulk density is an index of the heaviness of flour materials and expresses the relative volume of packaging material required. Bulk density is usually affected by the particle size and density of flour blend and it has significant application in packaging, transportation and raw material handling (Adebowale *et al.,* 2008; Ajanaku *et al.,* 2012; Adegunwa *et al.,* 2015). However, the low bulk density of the flour blends observed in this study would be of an advantage in the formulation of complementary food (Akpata and Akubor, 1999).

The dispersibility of flour blends ranged from 75.5 to 80.4%. Inclusion of yellow yam with jackbean at 5% had the highest value for dispersibility while inclusion of yellow yam with jackbean at 6.25% and 10% had the lowest dispersibility value. Dispersibility is a measure of how individual molecules of food sample usually flour is able to reconstitute in water. The higher the dispersibility value of yam and jackbean flour, the better the flour reconstitutes (Kulkarni *et al.,* 1991). The dispersibility values obtained for yellow yam-jackbean flour blends are relatively high and this is an indication that the flour blends will easily reconstitute to give fine consistency dough during mixing (Adebowale *et al.,* 2008; 2012). The dispersibility of the blends were not significantly ($p>0.05$) affected as shown in Table 7.

The water absorption capacity ranged from 4.19 to 6.54g/g. The interaction effect of yellow yam and jackbean had a significant ($p<0.05$) effect on the water absorption capacity as shown in Table 7. Water absorption capacity is the ability of flour to take up water and swell for improved consistency in food. It is also advantageous in food systems to improve yield and uniformity and give shape to the food products (Osundahunsi *et al.,* 2003). High water absorption capacity is attributed to loose structure of the starch polymers while low value indicates the compactness of the molecular structure. The values obtained for water absorption capacity of yellow yam and jackbean flour blends in this present work were low and this could be due to the protein and carbohydrate content of the flour blend (Omoniyi *et al.,* 2016; Khuthadzo *et al.,* 2019). This agrees with the study of Afoakwa (1996) that reported the significance of protein and starch in water uptake of flour at room

temperature. The water absorption capacity of the yellow yam and jackbean flour blend could also be influenced by low solubility, thereby leaching out of amylose, and loss of molecular structure of the starch as well as the crystalline structure (Khuthadzo *et al.,* 2019). Therefore, low water absorption capacity of the yellow yam and jackbean flour blends obtained in this study has good ability to bind water and would useful in foods such as baked products which involved hydration to improve handling features (Oppong *et al.,* 2015).

The swelling power and solubility index ranged from 4.52 to 5.70g/g and 6.53 to 6.77% respectively. Interaction effect of yellow yam and jackbean flour had no significant effect on the swelling power and solubility index of the flour blend as shown in Table 7. Swelling power is the ability of the flour to absorb water and hold it in the swollen flour granule. Swelling power is influenced by amylose and amylopectin content. The higher the amylose content the lower the swelling power (Rosida *et al.,* 2017). The result obtained for the swelling power of yellow yam and jackbean flour blends were low and this might be attributed to the protein-amylose complex formation of the flour blend (Pomeranz, 1991; Oke *et al.,* 2013). According to Pomeranz (1991), formation of protein-amylose in flours may be the cause of a decrease in swelling power. The low value of swelling power of yellow yam and jackbean flour blends obtained in this study will be desirable for manufacture of value added product such as extruded product. The result of swelling power of yam and jackbean flour blends was slightly higher than 2.70 to 4.83g/g reported by Malomo *et al.* (2012) on other species of yam such as *Dioscorea rotundata* and soybean flour blends. The extent of swelling depends on the temperature, availability of water, species of starch and other carbohydrates and proteins (Sui *et al.,* 2006)

Solubility is an indication of the existence of strong bonding forces probably due to high amount of protein and fat that might form inclusion complexes with amylose (Pomeranz, 1991). The value of solubility (6.53-6.77%) observed in this study were moderately high, this could be as a result of starch degradation in the flour blends (Khuthadzo *et al.,* 2019). Sanni *et al.* (2001) reported that high solubility index could be due to high amount of amylose which leaches out easily during the swelling process. However, various factors such as characteristic of the flour granules (granule size and the size distribution), amylose and amylopectin ratio, mineral content and presence of other components may influence the solubility of starch index in a flour blends (Singh *et al.,* 2003). A higher value of solubility of flour signifies an improved digestibility. The high value of solubility index obtained in this study might be useful for food preparations especially for infants and the aged who needs more readily digestible food (Diah *et al.,* 2018).

Table 6. Functional Properties of Yellow Yam and Jackbean Flour Blends

YF (%)	JF (%)	Bulk density (g/ml)	Dispersibility (%)	WAC (g/g)	Swelling power (g/g)	Solubility index (%)
90.00	10.00	0.73^{b}	76.5^{a}	4.22^{a}	4.52^{a}	6.55^{a}
92.50	7.50	0.64^{a}	78.0^{a}	4.34^{a}	4.73^{a}	6.63^{ab}
90.00	10.00	0.73^{b}	75.5^{a}	4.22^{a}	4.59^{a}	6.56^{a}
93.75	6.25	0.64^{a}	75.5^{a}	6.02^{b}	5.60^{b}	6.62^{ab}
92.50	7.50	0.63^{a}	77.5^{a}	4.37^{a}	4.79^{b}	6.62^{ab}
90.00	10.00	0.71^{b}	76.0^{a}	4.19^{a}	4.60^{b}	6.59^{ab}
95.00	5.00	0.62^{a}	80.4^{a}	6.50^{c}	5.65^{b}	6.65^{ab}
95.00	5.00	0.62^{a}	80.1^{a}	6.54^{c}	5.70^{b}	6.77^{b}
91.25	8.75	0.63^{a}	78.0^{a}	4.49^{a}	5.70^{b}	6.53^{a}

Mean values with different superscripts within the same column are significantly different ($p < 0.05$); YF- Yellow Yam flour, JF- Jackbean flour, WAC- Water absorption capacity

Table 7. Regression Coefficient of Functional Properties of Yellow Yam and Jackbean Flour Blends

Parameter	Bulk density	Dispersibility	WAC	Swelling power	Solubility index
A	0.62	79.64	6.61	5.66	6.71
B	0.72	72.29	4.22	4.66	6.56
AB	-0.18*	-2.90	-3.02*	-0.095	-0.14
F-value	24.85	3.84	40.82	3.98	7.27
R^2	0.89	0.56	0.93	0.57	0.71

*Significant at ($p<0.05$): A- Yellow Yam flour, B- Jackbean flour, AB- Interaction effects of Yellow Yam and Jackbean Flour, R^2- Coefficient of determination, WAC- Water absorption capacity

3.4. Physical (Colour) Attribute of Yellow Yam and Jackbean Blends

The effect of yam-jackbean flour inclusion on the yellowness (b*) of the blend is presented in Table 8. There were no significant ($p>0.05$) effect on the yellowness of yellow yam and jackbean flour interaction as shown in Table 9.The yellowness (b*) of yellow yam and jackbean ranged from12.28 to 13.77 with inclusion of yellow yam and jackbean at 6.25% having the lowest value for yellowness while inclusion of yellow yam and jackbean at 8.75% had the highest value for yellowness (b*). Colour is an important characteristic in food product identification and acceptability (Adeola *et al.,* 2018). According to Szabo *et al.* (2016), the resulting b* of the flour blends signifies the yellowness of the flour blends. The result obtained for the yellowness of yam and jackbean flour in this study could have been caused by carotenoids content because *Dioscorea cayenensis* is a rich source of total carotene (Ukom *et al.,* 2014). Colour is the first property that consumers consider in food products (Kumar *et al.,* 2012)

Table 8. Colour Attributes of Yellow Yam and Jackbean Flour Blends

YF (%)	JF (%)	Yellowness (b*)
90.00	10.00	13.12^{d}
92.50	7.50	12.73^{c}
90.00	10.00	13.22^{d}
93.75	6.25	12.28^{a}
92.50	7.50	12.76^{c}
90.00	10.00	13.19^{d}
95.00	5.00	12.50^{b}
95.00	5.00	12.53^{b}
91.25	8.75	13.77^{c}

Mean values with different superscripts within the same column are significantly different ($p<0.05$); YF- Yellow Yam flour, JF- Jackbean flour,

Table 9. Regression Coefficient of Colour Attribute of Yellow Yam and Jackbean Flour Blends

Parameter	Yellowness (b*)
A	12.40
B	13.28
AB	0.11
F-value	4.38
R^2	0.59

A- Yellow Yam flour, B- Jackbean flour, AB- Interaction effects of Yellow Yam and Jackbean Flour, R^2- Coefficient of determination

3.5. Pasting properties of Yellow Yam and Jackbean Blends

The effect of yellow yam-jackbean flour substitution on the pasting properties of the blend is presented in Table 10. The peak and final viscosity of the flour blends ranged from 206.0 to 572.0RVU and 263.0 to 911.0 RVU. The peak and the final viscosity was significantly ($p<0.05$) affected by the interaction effect of yellow yam and jackbean flour blends as illustrated in Table 11. Peak viscosity is the ability of starches to swell freely before their physical breakdown and indicates the strength of the pastes formed during gelatinization (Sanni *et al.,* 2004). The differences in the peak viscosity of yellow yam and jackbean flour blend obtained in this study indicates that there were differences in the rate of water absorption and starch granule swelling during heating (Ragaee and Abdel-Aal, 2006). The high peak viscosity exhibited by yellow yam and jackbean flour indicates that the flour will be suitable for products requiring high gel strength and elasticity such as extruded snacks (Adebowale *et al.,* 2005). High peak viscosity is an indication of high starch content and it also indicates water binding capacity of the flour and is often correlated with final product quality as well as providing an indication of the viscous load likely to be encountered by mixing cooking (Ikegwu *et al.,* 2010).

The final viscosity is the ability of the starch to form a viscous paste and gel during cooking and after cooling respectively (Maziya-Dixon *et al.,* 2007). The high value obtained for the final viscosity (263.0-911.0RVU) of yellow yam and jackbean flour compared to the peak viscosity (206.0-572.0RVU may be due to high degree of association between starch–water and their ability to recrystallize. The high final viscosity obtained in this study indicates their high resistance to shear stress during cooking and cooling. The final viscosity of flour is affected by protein content which could bind water so that water availability is decreased

The breakdown and setback viscosity ranged from 5.00 to 17.0RVU and 279.0 to 372.0 RVU respectively. The interaction effect of yellow yam and jackbean flour had no significant ($p>0.05$) effect on breakdown and setback viscosity as shown in Table 11. The breakdown viscosity of flour is referred to as a measure of the degree of disintegration of starch granules or its paste stability during heat (Asaam *et al.,* 2018). A higher breakdown viscosity value indicates a lower ability of the flour blends to withstand heating and shear stress during cooking (Adebowale *et al.,* 2005). Therefore the result obtained for yam and jackbean flour blend in this study suggest, they might withstand heating and shear processes without major change in consistence. Low stability of starch paste is associated with high value of breakdown (Ikegwu *et al.,* 2010).

Setback involves retrogradation or re-ordering of the starch molecules and setback viscosity has been correlated with the texture of various products (Maziya-Dixon *et al.,* 2005). The high setback (279.0 to 372.0RVU) observed for yam and jackbean flour blends in this study suggests that the flours were relatively unstable when cooked and have higher tendency to undergo retrogradation during freeze/thaw cycles. The higher the setback, the lower the retrogradation during cooling and the lower the rate of staling of the products made from the flour (Adeyemi and Idowu, 1990).

Table 10. Pasting Properties of Yellow Yam and Jackbean Flour Blends

YF (%)	JF (%)	Peak (RVU)	Breakdown viscosity (RVU)	Final viscosity (RVU)	Setback value (RVU)
90.00	10.00	209.0^{b}	5.00^{a}	554.0^{c}	350.0^{e}
92.50	7.50	435.0^{f}	9.00^{b}	755.0^{a}	329.0^{b}
90.00	10.00	206.0^{a}	6.00^{a}	559.0^{e}	345.0^{d}
93.75	6.25	549.0^{g}	14.0^{d}	907.0^{f}	372.0^{g}

92.50	7.50	429.0^{e}	10.0^{b}	263.0^{b}	333.0^{c}
90.00	10.00	212.0^{c}	11.0^{bc}	556.0^{d}	350.0^{e}
95.00	5.00	568.0^{h}	10.0^{b}	908.0^{f}	350.0^{e}
95.00	5.00	572.0^{i}	12.0^{cd}	911.0^{g}	354.0^{f}
91.25	8.75	292.0^{d}	17.0^{e}	555.0^{cd}	279.0^{a}

Mean values with different superscripts within the same column are significantly different ($p < 0.05$); YF- Yellow Yam flour, JF- Jackbean flour,

Table 11. Regression Coefficient of Pasting Properties of Yellow Yam and Jackbean Flour Blends

Parameter	Peak	Breakdown viscosity	Final viscosity	Setback viscosity
A	578.56	16.34	956.59	361.49
B	203.16	6.55	561.95	340.88
AB	162.22*	2.17	-1334.96*	-100.99
F-value	221.09	26.44	6.24	1.14
R^2	0.99	0.90	0.68	0.28

*Significant at ($p<0.05$): A- Yellow Yam flour, B- Jackbean flour, AB- Interaction effects of Yellow Yam and Jackbean Flour, R^2- Coefficient of determination

3.6. Optimisation of yam and jackbean flour blends

The flour blends was optimized based on some important properties attributed to extruded snacks product. The flour blends were maintained within the range of the study while the desired goal for each parameter and response was chosen with respect to the following dependent factors and as well as their main quality parameters serving as the constraints to process optimization: Protein, total carotenoid, starch content, amylose, amylopectin, dispersibility, water absorption capacity, swelling power, solubility, peak, breakdown viscosity, yellowness were maximize, bulk density, final viscosity, setback value were minimize and saponin, trypsin inhibitor, total polyphenol were set at none. The optimum flour blend ratio was 94.11% yellow yam flour and 5.89% jackbean flour. The solution to the optimised yellow yam and jackbean flour is presented in Table 12.

Table 12: Solution to Process Optimisation of Yam and Jackbean Flour Blends

YF	JF	PROT	TC	SC	AMY	AMYL	BD	DIS	WAC	SP
94.11	5.89	10.85	6.41	80.10	26.55	73.45	0.61	78.63	5.75	5.47

SOL	PEAK	BRD	FINAL	SB	YEL	DESIRABILITY
6.66	535.6	14.92	691.6	343.0	12.57	0.744

YF-Yam flour, JF-Jackbean flour, PROT- Protein, TC-Total carotenoid, SC-Starch content, AMY-Amylose, AMYL-Amylopectin, BD-Bulk density, DIS-Dispersibility, WAC-Water absorption capacity, SP-Swelling power, SOL-Solubility, BRD- Breakdown viscosity, YEL-Yellowness.

4. Conclusions

The study showed that the use of jackbean has the advantage of improving the protein content. The amylose, amylopectin and the starch content of yellow yam increases while the carotenoid content of the blend decreases. Addition of jackbean flour had a pronounced effect on the functional and pasting properties leading to lower bulk density, water absorption capacity, swelling power, peak viscosity and final viscosity. Hence, flour blends from yellow yam and jackbean can be used in development of food product such as extruded snacks and cooked paste.

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EXPERIMENTAL COMPARATIVE STUDY OF DIFFERENT TREATMENT CHAMBERS FOR FOOD PROCESSING USING PULSED ELECTRIC FIELD

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ABSTRACT

The major challenge today lies in the application of PEF in the industry; it requires an ever-increasing processing rate by seeking ways to continuously reduce energy consumption. All treatment chambers (TC) currently used in industry and in research laboratories, the product to be treated is placed in one volume unit delimited between two electrodes energized by a pulsed voltage which the electric field lines which are perpendicular to the electrodes are monoaxial and oriented almost in only one direction. The objective of this paper is to study experimentaly a new TC model comprising one, two or three monoaxial PEF units in parallel (TC1, TC2, TC3) and another biaxial PEF units (TC4) consisting of several "alternate ground-HV electrodes," in order to increase the processing flow rate and save more energy. The treatment chambers made of Plexiglas have a square paralelipedic shape, in which are placed either two (TC1), three (TC2), five (TC3) vertical and parallel stainless steel electrodes. One, Two or three of these electrodes are grounded and placed on the side walls of the chamber, while the high voltage electrode is placed in the central plane at equal distance between the ground electrodes. These chambers have been compared to biaxial treatment chamber comprising four identical metal plates placed on the side walls of a square shape (TC4). The obtained results have shown that the proposed model TC3 and TC4 are better than the two others in terms of extracted juice mass and energy consumption.

1. Introduction

The food industry is continually increasing because of worldwide demand (Heinz et al. 2002; Rao and Lund. 1986). The mechanical expression is widely used in the processes of solid-liquid separation for extraction of fruit juices and vegetable oils, dewatering of fibrous materials, etc. On the other hand, cell membrane acts as a physical barrier in removing the intracellular substances (water, juices and solutes) from plant food tissues in solid-liquid extraction and drying. Thus, the permeabilization of the cell membrane in a plant food tissue causes the release of intracellular water and solutes (secondary metabolites) to migrate in an external medium (Aguilera and Stanley. 1999).

Presently, the rupture of the cell membrane can be obtained by means of several methods according to the desired degree of disintegration and to the particular application. It is possible to identify: thermal and non-thermal methods. High temperature is used in food preservation and in pre-treatment and complementary stages before extraction processes. In this way it is possible to achieve a high degree of cell membrane breakdown, but due to the thermal denaturation of the cell membrane induced by heating; this treatment

damages sensory properties (Ponant et al. 1988; Jemai and Vorobiev. 2006).

Pulsed electric field (PEF) treatment is an innovative and promising method for non-thermal processing of foodstuff. It is a good alternative to conventional cell membrane permeabilization methods such as thermal treatments and the addition of chemicals as well as of enzymes (Rauh et al. 2009).

Pulsed electric field treatment is an unconventional method for liquid and food products which is efficient for juice yield intensification and for improving the product quality in juice production (Kinga et al. 2019; Roman et al. 2013), processing of vegetables and plant raw materials (McLellan et al. 1991; Bazhal and Vorobiev. 2000), food stuffs processing (Aashish and Divya. 2018), winemaking (Elif et al. 2014) and sugar production (Lebovka et al. 2007; Kalmykova. 1993).

In the last few years, several studies have demonstrated the ability of intense treatments to obtain safe and shelf-stable liquid foods. Further, novel applications such as improvement of mass transfer processes or generation of bioactive compounds by using moderate field strengths are under current development (Muhammed and Gulsun. 2017; Jeyamkondan et al.1999).

One of emerging and promising method is the combined PEF and pressure application, which demonstrates significant yield intensification for juice extracted from apples and beets and clarification of the extracted juice. But the major problem arising from simultaneous application of mechanical expression and PEF treatment is the choice of optimal modes of treatment.

The difficulty of the problem is that PEF-treated juice extraction process depends on multiple factors. The list of factors influencing the outcome of the process includes the inter-electrodes interval, the high voltage level, the electric field, the pulse duration, the number of pulses, the average power, the pressure level and so on,(Qamar et al. 2017).

The aim of the present study is an experimental comparative study of the influence of treatment chamber efficiency in yield and quality beet.

2. Materials and methods

2.1. Pulsed electric field for electroporation

Exposing a biological cell (plant, animal and microbial) to a high intensity electric field (kV/cm) using very short pulses (µs to ms) induces the formation of temporary or permanent pores on the cell membrane (figure 1). This phenomenon, called electroporation, causes the permeabilization of cell membrane i.e. an increase of its permeability and if the intensity of the treatment is sufficiently high, cell membrane disintegration occurs.

The electroporation for permeabilization of the cell membrane is used in many fields such as biotechnology, cell biology, medicine and food industry (figure 2). Mass transfer processes such as solid-liquid extraction and drying as well as food preservation are important unit operations of the food industry requiring the electroporation of the cell membrane.

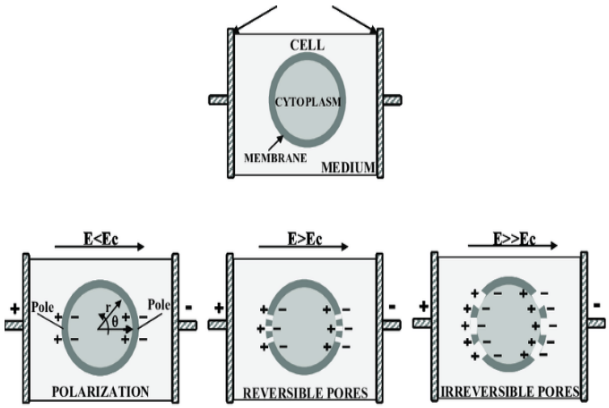

Figure.1. Schematic depiction of the permeabilization mechanism of a biological cell membrane exposed to an electric field E.

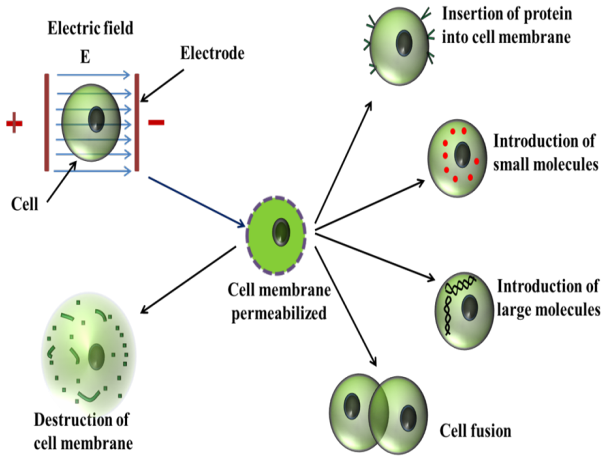

Figure.2. Electroporation applications of the cell membrane

2.2. PEF processing system

A PEF system for food processing in general consists of three basic components: a high voltage pulse generator, a treatment chamber and a control system for monitoring the process parameters (figure 3).

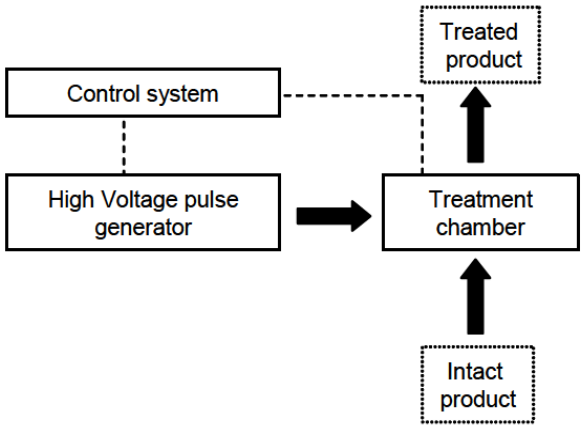

Figure 3. Descriptive representation of a pulsed electric field system for food processing.

2.3. Materials

Fresh beets, of average mass 60 g each one, were used. After sorting and cleaning operations, a homogenous mash was obtained using a domestic food processor. Before each experiment, the mash was properly mixed to obtain a homogenous mixture. A beet paw sample of mass 80 g was used for each experiment. After PEF treatment, an extraction step was achieved using an extraction chamber and a hydraulic pressing machine (Mega, 15 tons). The PEF treated extracted juice was then analyzed by measuring both its mass using an electronic balance of 0.1 mg precision and the betanine amount using a spectrophotometer (Optizen 200 plus) for λ= 530 nm.

All experiments were performed while maintaining following factors at constant values: pulse repetition frequency f = 1 Hz, extraction pressure P = 50 kg/cm2, total pressing duration t = 300 s and the inter-electrodes gap d= 60 mm.

The experimental setup used in the present work is composed of a number of components, comprising a high DC voltage source, an energy storage capacitor, a spark gap switch and a treatment chamber (Figure.4). A DC high voltage supply (Spellman 40 kV, 9 mA) charges the bank of capacitors until producing the spark gap's breakdown, causing an abrupt voltage (shock) applied to the load (treatment chamber where the sample is disposed). The storage element is composed of three sets of five series capacitors (2 µf, 2 kV), with the possibility to reach a maximum voltage of 10 kV and a total capacitance of 1.2 µF.

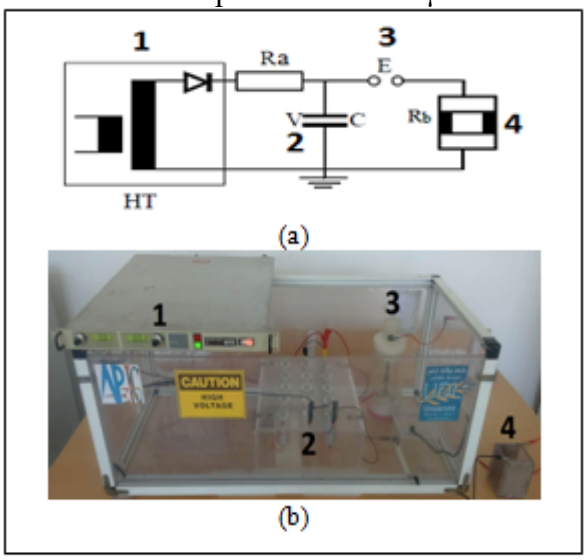

Figure.4. The pulse generator.
a) Descriptive schematic of the setup; b) The photography of the setup
1- HV DC power supply, 2-Set of capacitors, 3-Spark gap switch, 4-Treatment chamber

Four square paralelipedic treatment chambers made of Plexiglas, of dimensions 6x6x10 cm^3, in which are placed vertical stainless steel electrodes, were used in this work. The model TC1 comprises two parallel and opposite electrodes of dimensions 6x10 cm^2, the model TC2 is constituted of two units comprising three electrodes: one central HV electrode and two outside ground electrodes, the model TC3 is constituted of three units comprising five alternate HV and ground electrodes: two HV electrodes and three ground electrodes (Figure .5), while the model TC4 is constituted of four electrodes (Figure.6). For this latter, each pair of the adjacent metal plates form one electrode. The volume of both treatment chambers is 360 ml.

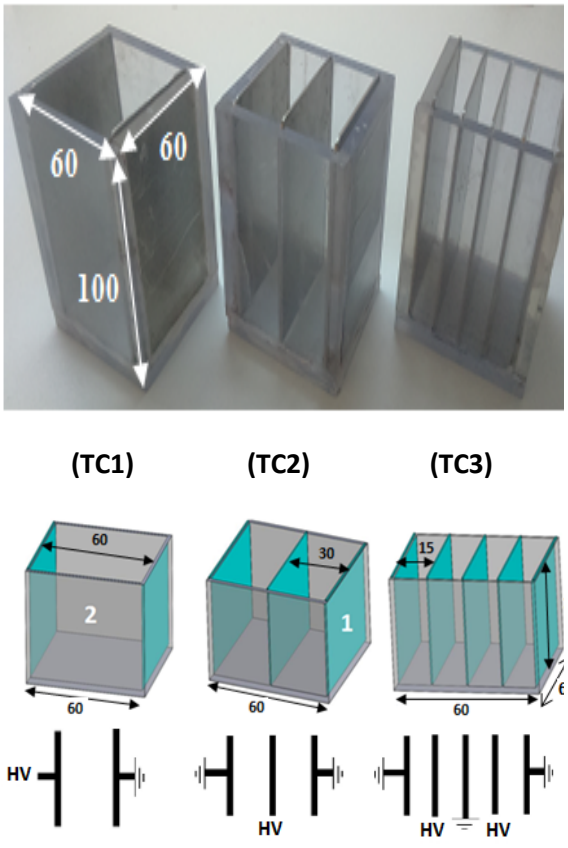

Figure.5. The treatment chambers (TC1, TC2 and TC3)
1: stainless steel electrodes; 2: paralelipedic treatment chambers (Dimensions in mm).

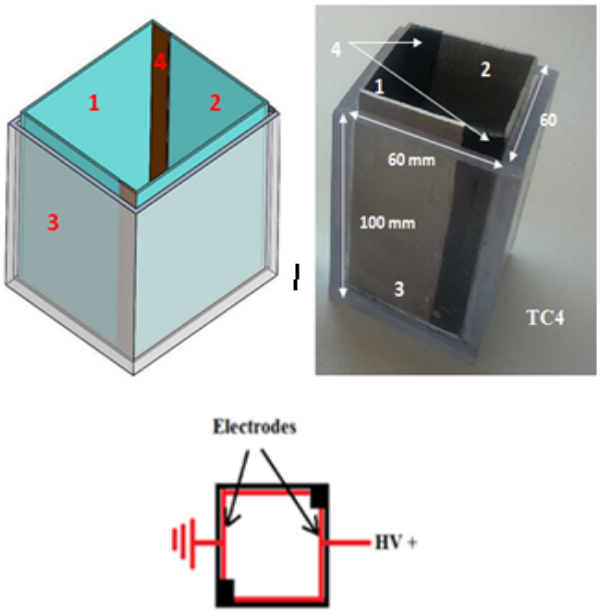

Figure.6. The treatment chambers TC4
1: Electrode HV. 2: Electrode ground.
3: Plexiglas. 4: Insulating.

2.3. Methods

An experimental investigation was performed to compare the PEF treatment efficiency between the three models. For each model, the influence of the applied voltage (V, kV), the pulses number (n) and the pulse duration (T, µs) was analyzed. The pulse duration is determined by the corresponding value of the capacitance, as follows:

For C = 0.2 µF, T=8 µs; for C=0.4 µF, T=20 µs, for C =0.8 µF, T= 40 µs and for C =1.2 µF, T= 60 µs (Figure.7).

Moreover, the mass of extracted juice m (g), the amount of Betatin expressed in terms of Absorbance Abs (%) and the energy $W = ½ n CV^2$ were considered significant to be considered as the response of the process.

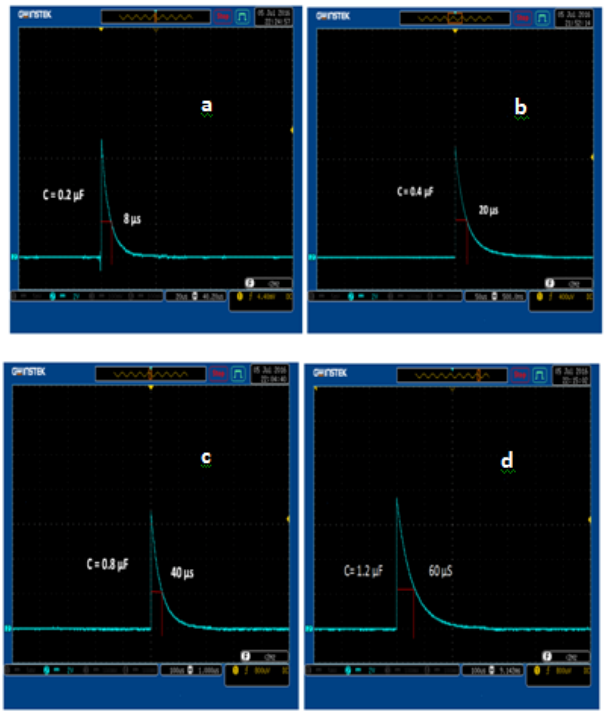

Figure.7. Current waveforms delivered by the pulse generator for different values of capacitance C
a. T= 8 µs (C = 0.2 µF), b. T= 20 µs (C = 0.4 µF), c. T= 40 µs (C = 0.8 µF), d. T= 60 µs (C = 1.2 µF)

3. Results and discussions

For all the experiments carried out in this section, for each TC model, one factor was varied while the two other factors were kept constant. Thus, figures 8-14 represent the variation of the PEF treatment efficiency, in terms of juice extracted mass m, absorbance Abs and Energy W, according to the voltage V, the pulses number n and the pulse width T, respectively.

The obtained results of the experiments are plotted in Figures.8-13, representing the variation of the extracted juice mass (m) and the absorbance (Abs) as function of the voltage V, the pulses number n and the pulse duration T respectively for the four models of treatment chambers.

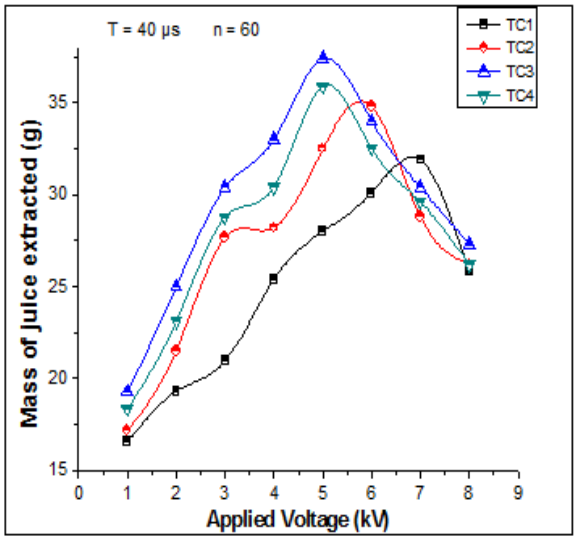

Figure.8. Mass of juice extracted according of the applied voltage for different TC model (n=60,T=40µs)

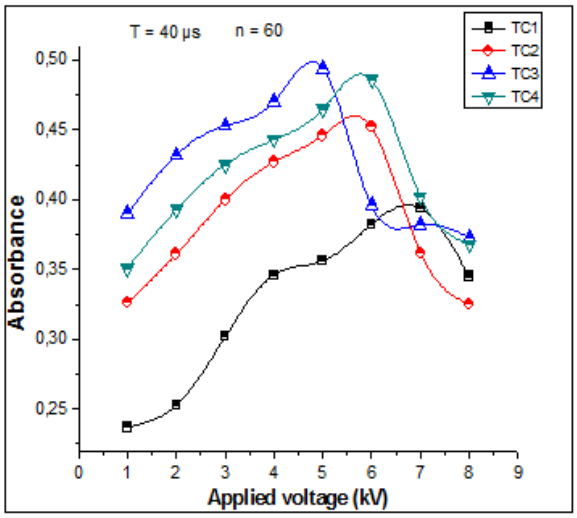

Figure.9. Absorbance of juice extracted according of the applied voltage for different TC model (n=60, T=40µs)

As expected, the mass of extracted juice and the quantity of Betanine obtained with a PEF treated sample increases according to the applied voltage (Fig.8 - Fig.9), for the four chambers. Beyond a determined value of the voltage, the effect of the PEF treatment is inversed due to "oxidation" of the product. However, the treatment is more efficient for models TC2, TC3 and TC4 compared with the

classic chamber TC1 comprising two electrodes. While for the model TC1, the optimal treatment was obtained for V = 7 kV (m= 31.9 g & Abs= 0.394), for others chambers greater values of (m) and (Abs) were obtained with smaller voltage.

However, comparing models TC2, TC3 and TC4 we see that the TC3 model gives better results in juice yield and betanine concentration due to significant value of energy provides for food processing at low voltages as a result of the better configuration of this chamber which promotes a high electric field.

Thus, for models TC3 and TC4, the optimal treatment was obtained for V = 5 kV (m= 37.4 g & Abs= 0.494), and V= 5 kV (m= 35.9 g & Abs= 0.465) respectively.

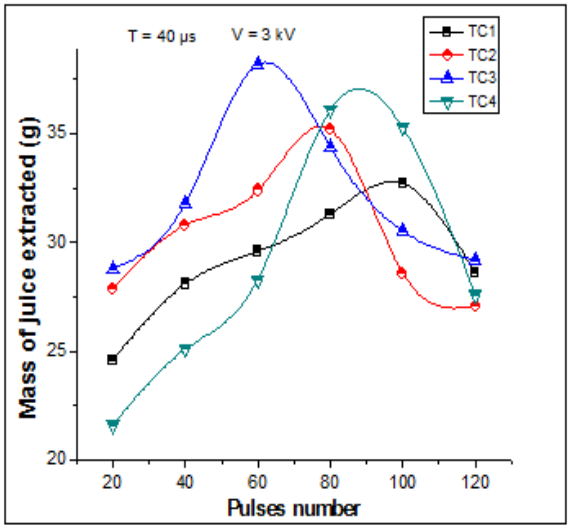

Figure.10. Mass of juice extracted according of the pulses number for different TC model (V= 3 kV,T=40µs)

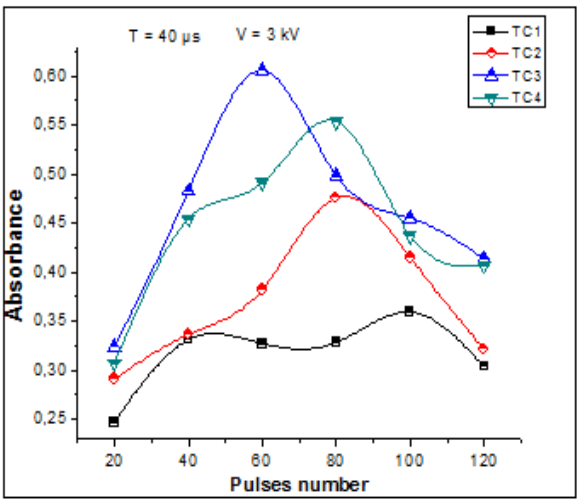

Figure.11. Absorbance of juice extracted according of the pulses number for different TC model (V= 3 kV, T= 40µs)

As seen in figures 10 and 11, the mass of extracted juice and the quantity of Betanine obtained with a PEF treated sample increases with pulses number, for the four chambers. The treatment is more efficient for models TC2, TC3 and TC4 compared with the classic chamber TC1. While for the model TC3, the optimal treatment was obtained for n= 60 pulses (m= 38.2 g & Abs= 0.606), for the other treatment chambers TC greater values of m and Abs were obtained with more pulses.

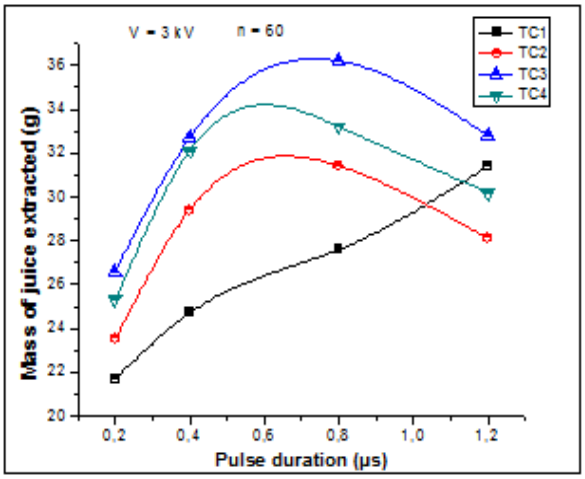

Figure.12. Mass of juice extracted according of the pulse duration for different TC model (V= 3 kV, n = 60)

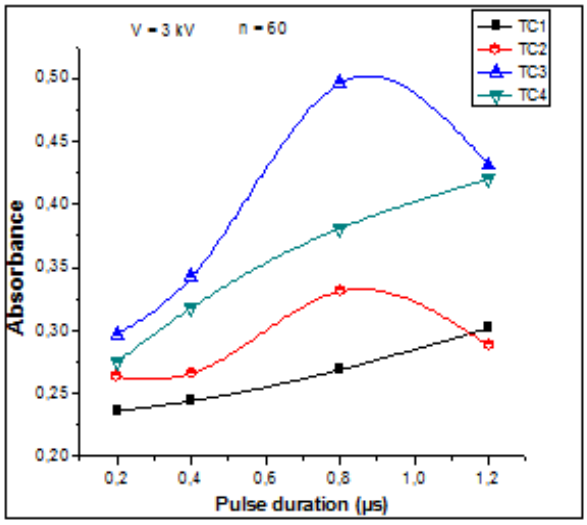

Figure.13 . Absorbance of juice extracted according of the pulse duration for different TC model (V= 3 kV, n = 60)

On the other hand, the mass (m) and absorbance (Abs) obtained with a PEF treated sample increases with the pulse width, for the four chambers (Figure 12 and 13), and decreases beyond a determined value. As for the previous factors, the treatment is more efficient for TC2, TC3 and TC4 models compared with the classic chamber. While for the model TC1, the optimal treatment was obtained for T = 60µs (m= 31.4 g & Abs= 0.302), for the models TC2, TC3 and TC4 greater values were obtained with smaller pulse width. The optimal treatment was obtained for T= 40 µs (m= 31.8 g & Abs= 0.331) using model TC2, for T = 40 µs (m= 36.2 g & Abs= 0.496) using model TC3 and for T = 40 µs (m= 33.2 g & Abs= 0.381) using model TC4. Indeed, the TC4 model represents a more efficiency treatment in comparison with the other models.

3.1. Energy saving during the PEF treatment

The following step is the comparing of the energy consumption and saving energy for four TC1, TC2, TC3 and TC4 chambers (Table 1 and figures 14 & 15).

Table 1. Energy consumption during the PEF treatment

TC1			TC2		
Mass(g)	**Abs**	**W(J)**	**Mass(g)**	**Abs**	**W(J)**
31.9	0.394	980	34.2	0.452	720
TC3			TC4		
Mass(g)	**Abs**	**W(J)**	**Mass(g)**	**Abs**	**W(J)**
37.4	0.494	500	35.9	0.486	500

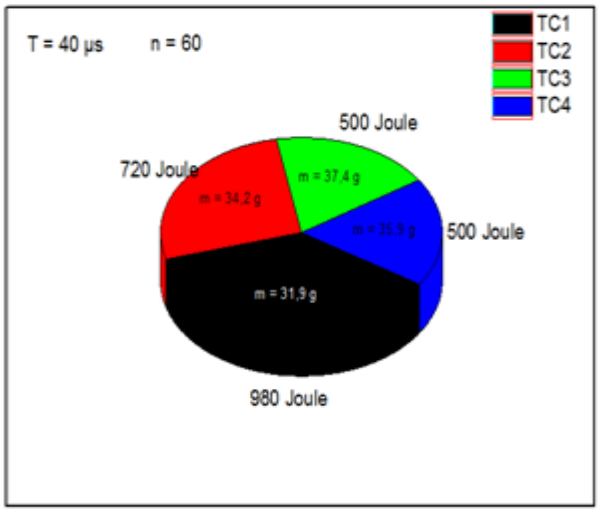

Figure.14. Energy consumption of optimal values for four TC models (T= 40µs, n= 60)

Saving energy for treatment chambers TC2, TC3 and TC4 compared to the TC1 treatment chamber can be calculated by the following equation:

$$W_{Saving} = \frac{W_{TC1} - W_{TCi}}{W_{TC1}}$$

TCi : Energy of TC2, TC3 or TC4 respectively.

An energy saving of 17.3%, 49 % and 49 % were achieved for models TC2, TC3 and TC4 respectively (Figure.15).

The advantage of the multi-units chambers (models TC2 and TC3) is that for the same voltage the electric field is increased compared to the "one-unit" chamber comprising two electrodes. For example, when a voltage of 6 kV is applied, the electric field of the models TC1, TC2 and TC3 is equal to 1, 2 and 4 kV/cm respectively.

In other hand, for the model TC4 the electric field in this case is distributed at all surface of the food during treatment winch gives better treatment and results. However, this model of treatment chamber presents possibility of breakdown between electrodes that affected on the treatment process and subsequently the juice yield and the Betanine concentration compared with treatment chamber TC3 model.

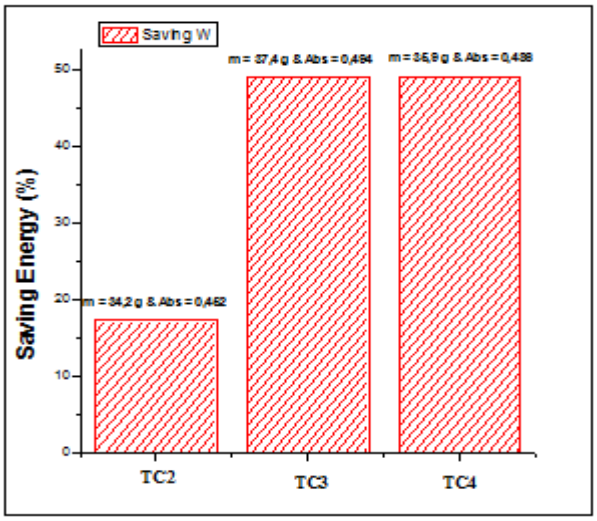

Figure.15. Saving energy consumption of optimal values for different TC model (T= 40µs, n= 60)

4. Conclusions

The present paper describes an experimental comparative analysis between four square treatment chambers of same dimensions but having either two three four or five metal electrodes placed on the side walls which is named TC1, TC2, TC4 and TC3 respectively. For TC4 which is constituted four electrodes, each pair of the adjacent metal plates form one electrode. The experimental analysis was made by measuring the mass of PEF pretreated extracted juice from beet and the amount of Betanine using a spectrophotometer. The obtained results, have shown that the model with four electrodes TC4 and the model with five electrodes TC3 are more efficient compared with TC1 and TC2 model, because higher quantities of juice and Betanine were obtained. On other hand, TC3 model which is constituted multi-units treatment chamber remains the best configuration because of its simplicity of realization and utilization to industrial level, so that there will be possible to increase the voltage and electric field treatment without a risk of breakdown between the electrodes.

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BACTERIOLOGICAL SAFETY OF SUYA, A READY-TO-EAT BEEF PRODUCT, AND ITS ASSOCIATION WITH ANTIBIOTIC-RESISTANT PATHOGENS IN NIGERIA

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ABSTRACT

The rapid development antibiotic-resistant food pathogens pose a heightened threat to public health. This study investigated the antibiotic resistance pattern of bacteria associated with suya meat, a ready-to-eat beef product, in Nigeria. Three hundred suya meat samples were cultured and pure isolates identified by API 20E and API 20NE. The resistance profile of isolates was determined using disc diffusion methods. Data were analysed by one-way analysis of variance and students' T-tests. The mean total plate counts (TPCs) of samples ranged from 1.0 x 10^5 to 3.7 x 10^5 CFU/g. There were no significant differences among the TPCs from zones A, B, C and D (P> 0.05). A total of 1014 isolates were obtained with *Pseudomonas aeruginosa* (13.51%) having the highest percentage occurrence and *Salmonella enteric* Typhimurium (1.48%), the lowest. A 92.90% portion of the isolates showed sensitivity to imipenem while 86.69% exhibited resistance to teicoplanin. This study revealed that the microbial quality of the ready-to-eat suya was at a borderline with reference to the microbiological guidelines for ready-to-eat animal food product. The study also revealed the presence of antibiotic-resistant bacteria in the ready-to-eat beef product which indicates a risk in food safety and a threat to public health. These findings will aid in the selection process of the right antibiotics in the treatment of food-borne infections while establishing the need for improvement on the microbial quality of the food product.

1.Introduction

Suya is a ready-to-eat spicy, barbecued, smoked or roasted meat. Its origin can be traced to the Hausa people of northern Nigeria, Sub-Saharan Africa, where their main occupation is rearing of cattle and growing of cash crops (Orogu and Oshilim, 2017). Thus, it is an important preoccupation and a major source of livelihood for the people. This generated the production of different types of beef products such as kundi, kilishi, balangu and suya, which are very popular protein-rich foods (Olayinka and Sani, 2014). However, suya is the most popular, as it is consumed in other parts of the country (El-Hassan *et al*., 2018). In the recent days, suya vendors are found in almost every nook and cranny of towns and cities with their grill stands, and are being patronized from midday to late at night. It has gradually made its way into elite circles where it has become a delicacy served at parties and other social events.

Spices such as ginger, salt, peanut cake, and other seasonings, are usually used to marinate the thinly spliced meat, and then barbecued (Egbebi and Seidu, 2011). Dried pepper mixed with spices, and sliced onions could also be added when served this delicacy.

The suya marinade is composed of complex mixture of additives and spices but there is no standard recipe for its production. The composition and types of ingredients vary from individual to individual, and according to regional preferences (Nwakanma *et al*., 2015; Amadi *et al*., 2016). However, the idea of requesting for only suya meat without the addition of the spices is becoming popular in the country. The reasons for this vary among individuals. Some simply prefer the taste of suya meat to having the spices sprinkled on it.

Barber *et al*. (2018) reported that there was an increased occurrence of disease outbreak caused by pathogenic and spoilage microorganisms in foods. The importance of foodborne diseases as a public health problem is often overlooked because their true incidence is difficult to evaluate and the severity of their health and economic impact is often not fully understood (Hassan *et al.,* 2014). Bacteria are considered the most common cause of foodborne illness representing two-thirds of foodborne disease outbreaks and wide variety of microbes with much common and less specific clinical symptoms (Bello *et al*., 2019).

The rapid development of multidrug resistance in microorganisms has become an increasingly emerging problem with serious consequences on public health (WHO, 2014). The resistance of bacteria to commonly prescribed antimicrobial agents are associated with increasing treatment failures, and which could be explained by the high frequency with which antimicrobials are used empirically to treat diseases (Ikechukwu *et al*., 2019). This implies that as resistant pathogens develop and spread, the effectiveness of the antibiotics diminishes. The aim of this study, therefore, was to investigate the antibiotic resistance profiles of bacteria of clinical importance associated with ready-to-eat suya in Nigeria.

2.Materials and methods

2.1.Study Area

Ogun State is a state in southwestern Nigeria. The estimated population is 5,217,716 according to the National Population Commission (NPC) and the National Bereau of Statistics (NBS) in 2013. The four geopolitical zones in Ogun State which include Yewa, Egba, Remo and Ijebu zones were the areas in which samplings were done.

2.3.Processing of suya meat

Sixty sticks of suya were personally prepared under aseptic conditions in accordance with standard procedures to serve as the control using beef. Putting all necessary precautions into consideration, meat samples were sliced into thin sheets and were inserted onto the suya sticks. Each stick of meat was pressed on the already prepared ingredient spread on a flat tray in order that the ingredient is evenly soaked into it. Groundnut oil was then sprinkled on the well-labeled sticks of meat before roasting was carried out.

2.2.Collection of suya meat samples

Two hundred and forty suya meat samples were purchased from different areas in the four geopolitical zones (Yewa, Egba, Remo and Ijebu zones) of Ogun State. Sixty suya samples from each of the four zones (60 x 4 = 240) were purchased from six different spots from ten different areas. The samples were collected and kept in refrigerator at 4 ^{o}C overnight to prevent contamination (as samples were purchased at night) and then transported to the laboratory for microbial analyses. Laboratory-based suya was prepared as a control each time suya meat samples were analysed in the laboratory, which summed up to 60 times. Invariably, a total of 300 suya samples were investigated in this study (Apata *et al*., 2013). For the purpose of this study, Yewa, Egba, Remo and Ijebu zones were labeled zones A, B, C and D, respectively while the samples prepared in the laboratory served as control.

2.4.Roasting of suya meat

Labeled stick meats were arranged round a glowing smokeless fire made from charcoal. The sticked meats were allowed to stay on the fire for 20 min with the distance of 22-23 cm from the centre of fire and intermittent turning

of the product. Groundnut oil was intermittently sprinkled while the meat was being roasted.

2.5.Cultivation of bacteria from suya meat

The microbiological analysis of suya meat was carried out as described by American Public Health Association (APHA, 1992) and Association of Official Analytical Chemist (AOAC, 2000). Ten grams each of suya samples were blended using a disinfected blender (model 242 NAKAI, JAPAN) with 90 ml of 0.1% (W/V) peptone water for 60 s and serial dilutions made in 0.1% peptone water (W/V) to obtain up to 10^{-5} dilution factor while 0.1 ml portion of each of the diluted samples was taken and dispensed in sterile Petri dishes containing appropriate agar medium using the spread plate method. Plate count agar (PCA) (Difco, USA) was employed for the determination of total bacterial count; violet red bile agar (VRBA) (Difco, USA) for total coliform.

Specific coliform organisms were differentiated by IMViC tests. Eosin methylene blue (EMB) agar (Oxoid, England) was used for the cultivation of *Escherichia coli* and *Enterobacter aerogenes*. For the isolation and enumeration of *Salmonella*, pre-enrichment was carried out using lactose broth incubated at 37 ^{o}C for 24 hrs. Ten (10) ml of pre-enriched medium was, under aseptic condition, pipetted and tranferred into 100 ml sterile Tetrathionate broth (Hi-media laboratories, India) incubated at 37 ^{o}C for 24 hrs. The resultant broth was streaked onto three *Salmonella* differential media which were brilliant green phenol lactose agar (Difco, USA), Bismuth sulphate agar (Difco, USA) and deoxycholate citrate agar (Oxoid, England).

For the identification of *Staphylococcus aureus* and micrococci, the sample was inoculated onto nutrient broth at 37 °C for 24 hours after which it was plated onto mannitol salt agar. Colony forming units were counted and were expressed in log10 cfu/g of samples. The microbiological guidelines for ready-to-eat food products are given in Table 1.

Table 1. Microbiological Guidelines for Ready-to-eat Food Products

Grades	TVC (total viable count)/g at 30°C	Description
I	$< 10^5$	Satisfactory
II	10^5- $< 10^7$	Borderline
III	$\geq 10^7$	Unsatisfactory

Center for Food Safety (2014).

2.6.Identification of bacterial isolates

Pure isolates on agar plates were characterized by initial morphological examination of the distinct colonies. The biochemical tests included catalase test, coagulase test, citrate utilization test, oxidase test, triple sugar iron agar, urease test, sugar fermentation test, methyl red test and indole production test (Cowan and Steel, 1985). *Salmonella* serotyping was by slide agglutination (Kauffmann-White-Le Minor scheme) based on the agglutination of bacteria with specific sera to identify variants of the somatic (O) and flagellar (H) antigens (Grimont and Weill, 2007; Guibourdenche *et al*., 2010). Specific identification of the other isolates was performed using the API 20E and API 20NE for confirmation of members of Enterobacteriaceae and non-Enterobacteriaceae, respectively.

2.7.Determination of antibiotic sensitivity of bacterial isolates

The disc diffusion assay was employed to investigate the sensitivity of isolates to the antibiotics (Clinical and Laboratory Standards Institute, CLSI, 2016). The standardized inocula of the test organisms were emulsified on the surface of Mueller Hinton Agar (Oxoid, England) using sterile cotton swab (220210 BD

SWUBE, India), and the plates was dried at room temperature for 5 min, and then incubated at 30 ^{o}C for 48 hours (Center for Food Safety, 2014). A total of 18 antibiotics representing 12 antibiotic classes were investigated against isolates from the suya samples.

The antibiotics investigated were Tetracycline (0.002–32 mg/L), Doxycycline (0.002–32 mg/L), Minocycline (0.002–32 mg/L), Erythromycin (0.016–256 mg/L), Colistin (0.064–1024 mg/L), Chloramphenicol (0.016–256mg/L), Trimethoprim/ Sulfamethoxazole (0.002–32 mg/L), Gentamicin (0.016–256 mg/L), Rifampicin (0.002–32 mg/L), Nalidixic acid (0.016–256 mg/L), Ciprofloxacin (0.002–32 mg/L), Penicillin G (0.002–32 mg/L), Ampicillin (0.016–256 mg/L), Imipenem (0.002–32 mg/L), Cefalotin (0.016–256 mg/L), Ceftriaxone (0.016–256 mg/L), Teicoplanin (0.016–256 mg/L) and Vancomycin (0.016–256 mg/L). A phase-contrast microscope with objective E.10 0.25 160/- (Nikon, France) was used (100× magnification) to read the limit of growth inhibition.

2.8.Data Analysis

Data were collated and statistically analysed using MedCalc statistical software, version 17.2. Simple means, percentages and frequencies from different locations were computed and compared using One-way Analysis of Variance (ANOVA) and independent T-test. Data were presented as mean ± standard error (SE) of triplicate data. The significance was determined at 95% level of confidence ($P \leq 0.05$).

3.Results and Discussions

3.1.Mean total bacterial counts from suya samples

Each value ($_{1} - _{10}$) in each zone represents mean of data obtained from six different suya spots in same area. The mean total plate count (TPC) from zones A, B, C and D ranged from 1.4 x 10^5 to 3.5 x 10^5 CFU/g, 1.1 x 10^5 to 3.5 x 10^5 CFU/g, 1.0 x 10^5 to 3.1 x 10^5 CFU/g and 1.0 x 10^5 to 3.7 x 10^5, respectively. The total Enterobacteriaceae counts (TECs) ranged from 1.0 x 10^3 to 2.3 x 10^3 CFU/g, 1.5 x 10^3 to 2.1 x 10^3 CFU/g, 1.1 x 10^3 to 2.5 x 10^3 CFU/g and 1.0 x 10^3 to 2.5 x 10^3 CFU/g as obtained in zones A, B, C and D, respectively. Total *Staphylococcus* count (TSCs) of suya samples ranged from 1.1 x 10^2 to 3.1 x 10^2 CFU/g, 1.1 x 10^2 to 3.0 x 10^2 CFU/g, 1.0 x 10^2 to 3.1 x 10^2 CFU/g and 1.0 x 10^2 to 2.2 x 10^2 CFU/g as encountered in zones A, B, C and D, respectively. There were no significant differences among the bacterial counts from zones A, B, C and D ($P > 0.05$) but values showed statistical differences from the control ($P < 0.05$) (Table 2).

Table 2. Mean total bacterial counts from suya samples in Nigeria

Zone	Area	Total plate count (TPC) (CFU/g)	Total Enterobacteria count (TEC) (CFU/g)	Total *Staphylococcus* count (TSC) (CFU/g)
A	A_1	1.9 x 10^5	2.3 x 10^3	1.2 x 10^2
	A_2	3.5 x 10^5	1.0 x 10^3	1.7 x 10^2
	A_3	2.3 x 10^5	2.2 x 10^3	2.1 x 10^2
	A_4	2.5 x 10^5	2.1 x 10^3	1.8 x 10^2
	A_5	2.1 x 10^5	1.8 x 10^3	3.1 x 10^2
	A_6	1.7 x 10^5	1.6 x 10^3	2.2 x 10^2
	A_7	1.4 x 10^5	1.8 x 10^3	2.1 x 10^2
	A_8	1.8 x 10^5	2.3 x 10^3	1.4 x 10^2
	A_9	2.6 x 10^5	2.1 x 10^3	2.3 x 10^2
	A_{10}	2.0 x 10^5	1.4 x 10^3	1.1 x 10^2
	Mean of means	2.18 x 10^{5a}	1.86 x 10^{3b}	1.90 x 10^{2c}

B	B_1	2.7×10^5	1.7×10^3	1.5×10^2
	B_2	1.3×10^5	1.5×10^3	3.0×10^2
	B_3	1.1×10^5	2.0×10^3	2.2×10^2
	B_4	1.9×10^5	1.8×10^3	1.5×10^2
	B_5	2.4×10^5	1.7×10^3	1.1×10^2
	B_6	2.9×10^5	1.5×10^3	1.3×10^2
	B_7	2.1×10^5	2.0×10^3	1.2×10^2
	B_8	3.5×10^5	2.1×10^3	2.1×10^2
	B_9	2.3×10^5	1.9×10^3	1.3×10^2
	B_{10}	2.2×10^5	1.5×10^3	1.2×10^2
	Mean of means	2.24×10^{5a}	1.77×10^{3b}	1.64×10^{2c}
C	C_1	3.1×10^5	1.7×10^3	1.2×10^2
	C_2	2.1×10^5	1.2×10^3	1.8×10^2
	C_3	1.2×10^5	2.3×10^3	1.6×10^2
	C_4	1.6×10^5	1.6×10^3	2.1×10^2
	C_5	1.0×10^5	2.1×10^3	2.2×10^2
	C_6	1.3×10^5	2.5×10^3	1.7×10^2
	C_7	1.7×10^5	1.3×10^3	2.1×10^2
	C_8	2.5×10^5	1.1×10^3	1.6×10^2
	C_9	2.7×10^5	1.8×10^3	1.1×10^2
	C_{10}	1.4×10^5	1.6×10^3	1.0×10^2
	Mean of means	1.72×10^{5a}	1.72×10^{3a}	1.64×10^{2b}
D	D_1	2.5×10^5	1.4×10^3	2.1×10^2
	D_2	2.3×10^5	1.9×10^3	1.9×10^2
	D_3	1.9×10^5	1.5×10^3	2.0×10^2
	D_4	1.0×10^5	2.5×10^3	1.3×10^2
	D_5	3.7×10^5	1.0×10^3	2.1×10^2
	D_6	1.2×10^5	1.6×10^3	1.4×10^2
	D_7	2.0×10^5	1.1×10^3	1.3×10^2
	D_8	1.6×10^5	1.6×10^3	2.2×10^2
	D_9	1.5×10^5	1.6×10^3	1.8×10^2
	D_{10}	1.1×10^5	1.2×10^3	1.1×10^2
	Mean of means	1.88×10^{5a}	1.54×10^{3b}	1.72×10^{2c}
Control	Cont.1	1.1×10^4	0	1.1 x 10
	Cont.2	1.5×10^4	1.0×10^2	1.0 x 10
	Cont.3	1.2×10^4	0	2.0 x 10
	Cont.4	1.6×10^4	1.1×10^2	1.3 x 10
	Cont.5	1.0×10^4	0	1.2 x 10
	Cont.6	1.3×10^4	0	1.7 x 10
	Cont.7	1.4×10^4	1.0×10^2	2.1 x 10
	Cont.8	1.2×10^4	0	1.0 x 10
	Cont.9	1.3×10^4	0	1.1 x 10
	Cont.10	1.4×10^4	1.6×10^2	1.5 x 10
	Mean	1.30×10^{4b}	0.47×10^{2c}	1.40×10^a

Each value ($_{1 - 10}$) in each zone represents mean of data obtained from six different suya spots in the area. Means of means with same superscript along same column showed no statistical difference.

Table 3. Morphological and biochemical characteristics of bacteria isolated from suya samples in Nigeria

Gram Reaction	Cellular morphology	Catalase	Oxidase	Coagulase	Indole	Motility	Methyl-Red	VogesProskauer	Urease activity	Citrate Utilization	Starch Hydrolysis	Gelatin Hydrolysis	Casein Hydrolysis	Spore test	NO_3 Reduction	Glucose	Sucrose	Arabinose	Maltose	Mannitol	Xylose	Galactose	Sorbitol	Invositol	Raffinose	Frauction	No of Isolates	Isolates per zone	Most Probable Identity
-ve	R	-	+	-	-	+	+	+	-	+	-	-	+	+	+	+	+	+	-	+	+	-	-	-	-	+	105	A=25,B=25,C=22,D=22,CT=11	*E. coli*
-ve	R	+	-	-	-	+	-	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	ND	+	55	A=11,B=11,C=13,D=13,CT=7	*E. aerogenes*
-ve	R	+	+	-	-	+	-	+	-	+	+	+	-	-	-	+	+	+	+	+	-	-	-	-	+	+	85	A=19,B=20,C=18,D=17,CT=11	*S. rubidaea*
-ve	R	+	+	-	-	+	-	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	137	A=30,B=28,C=32,D=32,CT=15	*P. aeruginosa*
+ve	C	+	-	+	-	-	-	+	+	-	-	+	+	-	+	+	+	-	+	+	-	+	ND	ND	ND	+	114	A=24,B=25,C=24,D=25,CT=16	*S. aureus*
+ve	R	+	+	-	+	+	+	-	-	+	-	-	-	-	-	+	+	-	+	+	-	-	+	-	+	+	54	A=12,B=11,C=11,D=12,CT=8	*C. freundii*
+ve	C	+	-	-	-	-	-	+	+	-	-	+	-	-	-	+	+	-	-	-	-	-	ND	ND	ND	+	125	A=20,B=25,C=30,D=31,CT=19	*S. epidermidis*
+ve	R	+	+	-	-	+	-	+	-	+	-	+	-	+	-	+	+	-	-	+	-	-	-	-	+	+	107	A=24,B=24,C=25,D=22,CT=12	*B. cereus*
+ve	C	+	-	-	-	-	-	-	+	-	-	+	-	-	-	+	+	-	+	+	-	-	-	-	ND	-	85	A=20,B=18,C=18,D=18,CT=11	*M. luteus*
+ve	R	+	+	-	-	+	-	+	+	+	-	+	-	+	-	+	+	-	-	+	-	-	-	-	-	+	26	A=7,B=6,C=5,D=5,CT=3	*B. subtilis*
+ve	R	-	-	-	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	27	A=8,B=17,C=0,D=2,CT=0	*C. butyricum*
-ve	R	+	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	-	+	47	A=11,B=7,C=12,D=13,CT=4	*K. pneumoniae*
-ve	R	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+	-	+	-	-	32	A=6,B=6,C=7,D=7,CT=6	*K. planticola*
-ve	R	+		-	-	+	-	+	-	+	+	-	-	-	+	+	-	-	+	+	-	-	-	-	-	+	15	A=4,B=3,C=3,D=5,CT=0	*S. enterica*Typhimurium

Keys: R = Rods; + = Positive reaction; - = Negative reaction; ND = Not determined; A = Zone A; B = Zone B; C =Zone C; D = Zone D and CT =Control.

Table 4. Percentage antibiotic susceptibility of bacterial isolates from suya meat samples in Nigeria

Antibiotic		*E. coli*	*Enterobacter aerogenes*	*Serratiarubidae a*	*P. aeruginosa*	*C. freundii*	*S. aureus*	*S. epidermidis*	*Bacillus cereus*	*Bacillus subtilis*	*Micrococcus luteus*	*Clostridium butyricum*	*K. pneumoniae*	*K. planticola*	*Salmonella* Typhimurium
	Status	n=105	n=55	n=85	n=137	n=54	n=114	n=125	n=107	n=26	n=85	n=27	n=47	n=32	n=15
Tetracycline	S	105 (100%)	55 (100%)	80 (94.1%)	5 (3.7%)	54 (100%)	80 (70.2%)	125 (100%)	76 (71%)	26 (100%)	85 (100%)	20 (74.1%)	29 (61.7%)	32 (100%)	15 (100%)
	I	0	0	5 (5.9%)	0	0	4 (3.5%)	0	0	0	0	0	4 (8.5%)	0	0
	R	0	0	0	132 (96.4%)	0	30 (26.3%)	0	31 (29%)	0	0	7 (25.9%)	14 (51.9%)	0	0
Doxycycline	S	105 (100%)	55 (100%)	85 (100%)	38 (27.7%)	54 (100%)	99 (86.8%)	125 (100%)	107 (100%)	26 (100%)	79 (92.9%)	27 (100%)	47 (100%)	32 (100%)	15 (100%)
	I	0	0	0	0	0	0	0	0	0	6 (7.1%)	0	0	0	0
	R	0	0	0	99 (72.3%)	0	15 (13.1%)	0	0	0	0	0	0	0	0
Minocycline	S	105 (100%)	55 (100%)	64 (75.3%)	57 (41.6%)	54 (100%)	103 (90.4%)	125 (100%)	107 (100%)	26 (100%)	85 (100%)	27 (100%)	47 (100%)	32 (100%)	15 (100%)
	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	0	0	21 (24.7%)	82 (59.9%)	0	11 (9.7%)	0	0	0	0	0	0	0	0
Erythromycin	S	77 (73.3%)	34 (61.8%)	25 (29.4%)	0	29 (53.7%)	114 (100%)	107 (85.6%)	38 (35.5%)	11 (42.3%)	71 (83.5%)	20 (74.1%)	15 (31.9%)	12 (37.5%)	4 (26.7%)
	I	5 (1.0%)	0	0	10 (7.3%)	0	0	0	0	0	0	0	6 (12.8%)	0	2 (13.3%)
	R	23 (21.9%)	21 (38.2%)	20 (23.5%)	127 (92.7%)	26 (48.2%)	0	18 (14.4%)	69 (64.5%)	15 (57.7%)	14 (16.5%)	7 (25.9%)	26 (55.3%)	20 (62.5%)	9 (60%)
Keys: S = Suseptible; I = Intermediately Susceptible and R = Resistance															

Antibiotic		*E. coli*	*Enterobacter aerogenes*	*Serratiarubidaea*	*P. aeruginosa*	*C. freundii*	*S. aureus*	*S. epidermidis*	*Bacillus cereus*	*Bacillus subtilis*	*Micrococcus luteus*	*Clostridium butyricum*	*K. pneumoniae*	*K. planticola*	*Salmonella* Typhimurium
	Status	n=105	n=55	n=85	n=137	n=54	n=114	n=125	n=107	n=26	n=85	n=27	n=47	n=32	n=15
Colistin	S	105 (100%)	55 (100%)	85 (100%)	137 (100%)	54 (100%)	11 (9.7%)	13 (10.4%)	0	0	14 (16.5%)	0	47 (100%)	32 (100%)	15 (100%)
	I	0	0	0	0	0	14 (12.3%)	0	11 (10.3%)	0	0	0	0	0	0
	R	0	0	0	0	0	89 (78.1%)	112 (89.6%)	96 (89.7%)	26 (100%)	71 (83.5%)	27 (100%)	0	0	0
Chloramphenicol	S	105 (100%)	49 (89.1%)	85 (100%)	38 (27.7%)	54 (100%)	107 (93.9%)	91 (72.8%)	84 (78.5%)	12 (24.3%)	79 (92.9%)	16 (59.3%)	47 (100%)	32 (100%)	11 (73.3%)
	I	0	6 (10.9%)	0	19 (13.9%)	0	0	0	0	0	0	5 (18.5%)	0	0	0
	R	0	0	0	80 (58.4%)	0	7 (6.1%)	34 (27.2%)	23 (21.5%)	14 (13.1%)	6 (7.1%)	6 (22.2%)	0	0	4 (26.7%)
Trimethoprim/ sulfamethoxazole	S	105 (100%)	55 (100%)	85 (100%)	58 (42.3%)	54 (100%)	109 (95.6%)	125 (100%)	107 (100%)	26 (100%)	78 (91.8%)	9 (33.3%)	47 (100%)	32 (100%)	15 (100%)
	I	0	0	0	0	0	5 (4.4%)	0	0	0	0	4 (14.8%)	0	0	0
	R	0	0	0	79 (57.7%)	0	0	0	0	0	7 (8.2%)	14 (51.9%)	0	0	0
Gentamicin	S	87 (82.9%)	55 (100%)	85 (100%)	73 (53.3%)	54 (100%)	80 (70.2%)	106 (84.8%)	81 (75.7%)	18 (69.2%)	85 (100%)	10 (3.7%)	39 (83%)	32 (100%)	15 (100%)
	I	0	0	0	0	0	9 (7.9%)	7 (5.6%)	0	0	0	5 (18.5%)	0	0	0
	R	18 (17.1%)	0	0	64 (46.7%)	0	25 (21.9%)	12 (9.6%)	26 (24.3%)	18 (69.2%)	0	12 (44.4%)	8 (17%)	0	0
Keys: S = Suseptible; I = Intermediately Susceptible and R = Resistance															

Antibiotic		*E. coli*	*Enterobacter aerogenes*	*Serratiarubidaea*	*P. aeruginosa*	*C. freundii*	*S. aureus*	*S. epidermidis*	*Bacillus cereus*	*Bacillus subtilis*	*Micrococcus luteus*	*Clostridium butyricum*	*K. pneumoniae*	*K. planticola*	*Salmonella* Typhimurium
	Status	n=105	n=55	n=85	n=137	n=54	n=114	n=125	n=107	n=26	n=85	n=27	n=47	n=32	n=15
Rifampicin	S	0	0	29 (34.1%)	0	29 (53.7%)	114 (100%)	125 (100%)	39 (36.5%)	9 (34.6%)	85 (100%)	0	0	0	0
	I	0	0	9 (10.6%)	0	5 (9.3%)	0	0	14 (13.1%)	0	0	0	0	0	0
	R	105 (100%)	55 (100%)	47 (55.3%)	137 (100%)	20 (37%)	0	0	54 (50.5%)	17 (64.5%)	0	27 (100%)	47 (100%)	32 (100%)	15 (100%)
Nalidixic acid	S	76 (72.4%)	48 (87.3%)	64 (75.3%)	0	31 (57.4%)	0	0	0	0	0	27 (100%)	36 (76.6%)	32 (100%)	12 (80%)
	I	8 (7.6%)	0	0	5 (3.7%)	0	0	0	8 (7.48%)	0	0	0	0	0	0
	R	21 (20%)	7 (12.7%)	21 (24.7%)	132 (96.4%)	23 (42.6%)	114 (100%)	125 (100%)	99 (92.5%)	26 (100%)	85 (100%)	0	11 (23.4%)	0	3 (20%)
Ciprofloxacin	S	83 (79.1%)	55 (100%)	85 (100%)	68 (49.6%)	54 (100%)	99 (86.8%)	106 (84.8%)	27 (25.2%)	20 (76.9%)	31 (36.5%)	10	41 (87.2%)	32 (100%)	15 (100%)
	I	0	0	0	0	0	4 (3.5%)	7 (5.6%)	0	0	0	5 (18.5%)	0	0	0
	R	22 (21%)	0	0	69 (50.4%)	0	11 (9.7%)	12 (9.6%)	80 (74.8%)	6 (23.1%)	54 (63.5%)	12	6 (12.8%)	0	0
Penicillin G	S	0	0	34 (40%)	0	37 (68.5%)	92 (80.7%)	102 (81.6%)	77 (72%)	22 (84.6%)	85 (100%)	27 (100%)	0	0	0
	I	0	0	0	0	2 (3.7%)	0	0	0	0	0	0	0	0	0
	R	105 (100%)	55 (100%)	51 (60%)	137 (100%)	15 (27.8%)	22 (19.3%)	23 (18.4%)	30 (28.7%)	4 (15.4%)	0	0	47 (100%)	32 (100%)	15 (100%)

Keys: S = Suseptible; I = Intermediately Susceptible and R = Resistance

Antibiotic	Status	*E. coli*	*Enterobacter aerogenes*	*Serratiarubidaea*	*P. aeruginosa*	*C. freundii*	*S. aureus*	*S. epidermidis*	*Bacillus cereus*	*Bacillus subtilis*	*Micrococcus luteus*	*Clostridium butyricum*	*K. pneumoniae*	*K. planticola*	*Salmonella* Typhimurium
		n=105	n=55	n=85	n=137	n=54	n=114	n=125	n=107	n=26	n=85	n=27	n=47	n=32	n=15
Ampicillin	S	71 (67.7%)	48 (87.3%)	64 (75.3%)	58 (42.3%)	50 (92.6%)	80 (70.2%)	97 (77.6%)	54 (50.5%)	19 (73.1%)	62 (72.9%)	9 (33.3%)	26 (55.3%)	23 (71.9%)	10 (66.7%)
	I	0	0	0	7 (5.1%)	0	4 (3.5%)	8 (6.4%)	8 (7.5%)	0	7 (8.2%)	0	0	0	0
	R	34 (32.4%)	7 (12.7%)	21 (24.7%)	72 (52.6%)	4 (7.4%)	30 (26.3%)	20 (16%)	45 (42.1%)	7 (26.9%)	16 (18.8%)	18 (66.7%)	21 (44.7%)	9 (28.1)	5 (33.3%)
Imipenem	S	101 (100%)	55 (100%)	85 (100%)	137 (100%)	54 (100%)	100 (87.7%)	125 (100%)	89 (83.2%)	26 (100%)	68 (80%)	27 (100%)	32 (68.1%)	32 (100%)	11 (73.3%)
	I	0	0	0	0	0	10 (8.8%)	0	6 (5.6%)	0	0	0	0	0	0
	R	0	0	0	0	0	4 (3.5%)	0	12 (11.2%)	0	17 (20%)	0	15 (31.9%)	0	4 (26.7%)
Cefalotin	S	23 (21.9%)	0	58 (68.2%)	39 (28.5%)	25 (46.3%)	95 (83.3%)	91 (72.8%)	66 (61.7%)	13 (50%)	68 (80%)	17 (63%)	14 (51.9%)	23 (71.9%)	9 (60%)
	I	0	9 (16.4%)	0	9 (6.6%)	6 (11.1%)	9 (7.9%)	10 (8%)	0	4 (15.4%)	0	0	5 (10.6%)	0	0
	R	82 (78.1%)	46 (83.6%)	27 (31.8%)	89 (65%)	23 (42.6%)	10 (18.5%)	24 (19.2%)	41 (38.3%)	9 (34.6%)	17 (20%)	10 (37%)	28 (59.6%)	9 (28.1)	6 (40)
Ceftriaxone	S	67 (63.8%)	10 (18.2%)	79 (93%)	0	54 (100%)	85 (74.5%)	107 (85.6%)	34 (31.8%)	19 (73.1%)	62 (72.9%)	9 (33.3%)	41 (87.2%)	32 (100%)	15 (100%)
	I	11 (10.5%)	0	6 (7.1%)	0	0	9 (7.9%)	0	0	0	7 (8.2%)	0	0	0	0
	R	27 (25.7%)	45 (81.8%)	0	137 (100%)	0	20 (17.5%)	18 (14.4%)	73 (68.2%)	7 (26.9%)	16 (18.8%)	18 (66.7%)	6 (12.8%)	0	0

Keys: S = Suseptible; I = Intermediately Susceptible and R = Resistance

Antibiotic	Status	*E. coli*	*Enterobacter aerogenes*	*Serratiarubi daea*	*P. aeruginosa*	*C. freundii*	*S. aureus*	*S. epidermidis*	*Bacillus cereus*	*Bacillus subtilis*	*Micrococcus luteus*	*Clostridium butyricum*	*K. pneumoniae*	*K. planticola*	*Salmonella* Typhimurium
		n=105	n=55	n=85	n=137	n=54	n=114	n=125	n=107	n=26	n=85	n=27	n=47	n=32	n=15
Teicoplanin	S	0	0	0	0	0	30 (26.3%)	31 (24.8%)	0	0	46 (54.1%)	19 (70.4%)	0	0	0
	I	0	0	0	0	0	9 (7.9%)	0	0	0	0	0	0	0	0
	R	105 (100%)	55 (100%)	85 (100%)	137 (100%)	54 (100%)	75 (65.8%)	94 (75.2%)	107 (100%)	26 (100%)	39 (45.9%)	8 (29.6%)	47 (100%)	32 (100%)	15 (100%)
Vancomycin	S	0	0	0	0	0	49 (43%)	42 (33.6%)	0	0	46 (54.1%)	19 (70.4%)	0	0	0
	I	0	0	0	0	6 (11.1%)	9 (7.9%)	5 (4%)	0	0	0	0	5 (10.6%)	0	0
	R	105 (100%)	55 (100%)	85 (100%)	137 (100%)	48 (88.9%)	56 (49.1%)	78 (62.4%)	107 (100%)	26 (100%)	39 (45.9%)	8 (29.6%)	42 (83.4%)	32 (100%)	15 (100%)

Keys: S = Suseptible; I = Intermediately Susceptible and R = Resistance

Higher incidence of microbial contaminants in suya had been previously reported in other places (Bakobie *et al*., 2017; Ribah and Manga, 2018; Ikechukwu *et al*., 2019). Amadi *et al*. (2016) also reported the occurrence of APC and TCC values of 1.39 x 10^5 cfu/g and 6.2x10^4 cfu/g in roasted suya meat samples. Poor water and personal hygiene qualities, traditional processing techniques and exposure of suya in unhealthy environment could be attributed to this phenomenon. Similar findings on microbial biodiversity in suya had been earlier reported (Hassan *et al*., 2014; Orogu and Oshilim, 2017; Riba and Manga, 2018) and these underscore the level of contamination of the ready-to-eat food product.

3.2.Identification of bacterial isolates

One thousand and fourteen (1014) bacterial isolates were obtained. Two hundred and twenty-one (221), 226, 220 and 224 isolates were encountered from zones A, B, C and D, respectively while 123 isolates were obtained from control samples. The isolates were characterized as *E. coli*, *Enterobacter aerogenes*, *Serratia rubidaea*, *P. aeruginosa*, *C. freundii*, *S. aureus*, *S. epidermidis*, *Bacillus cereus*, *Bacillus subtilis*, *Micrococcus luteus*, *Clostridium butyricum*, *K. pneumoniae*, *K. planticola* and *Salmonella enterica* Typhimurium (Table 3).

The presence of *Salmonella enterica* Typhimurium in suya is of tremendous public health concern as this puts the presumably large number of consumers at risk of gastroenteritis. *Salmonella*'s ability to grow in food is largely dependent on storage temperature. It was recently reported by dos Santos *et al.* (2019) that *Salmonella enterica Typhimurium* is a leading cause of food poisoning cases in several countries. It is a non-specifically categorized as a zoonotic bacterium associated with animals and humans, but some strains could be invasive because of the ability to cross the intestinal wall and reach the systemic circulation (Almeida *et al*., 2017). The bacterium's pathogenicity ability could be attributed to its virulence factors.

C. perfringens are found in dust, soils, vegetation among other environmental media. Its presence could be attributed to growth parameters like favourable temperature. Equipment and food handlers have also been associated with contamination of food with various types of etiologic agents. *Staphylococcus* spp are abundant in the nose and throat as well as the skin of humans. This study agrees with the report of Uzeh *et al.* (2006) who isolated *Pseudomonas* sp., *Bacillus cereus* and *Staphylococcus aureus* from tsire-suya, a Nigerian meat product. This was also buttressed by the findings of Manyi *et al.* (2014) who reported *Streptococcus* sp., *Escherichia coli*, *Bacillus* sp., *Staphylococcus aureus*, *Klebsiella* sp. and *Pseudomonas* sp. in suya samples. The existence of these organisms in the suya could be attributable to the filthy environment, poor personal hygiene of the processors and retailers, the use of contaminated utensils during processing, use of contaminated materials for packaging, activities of flies as well as the addition of spices and seasonings during processing.

3.3.Percentage frequency of bacterial isolates

Data showed that percentage contamination of the suya samples from zones A, B, C and D were 21.80%, 22.29%, 21.70% and 22.09%, respectively while the control was 12.13%. There were no statistical differences among the level of bacterial contamination from zones A, B, C and D ($P > 0.05$). The data from these zones, however, showed significant differences from the control ($P < 0.05$) (Figure 1). The most occurred bacterium from the suya samples in zone A was *P. aeruginosa* with percentage occurrence of 13.58% while the lowest was *Salmonella enteric* Typhimurium with 1.81% (Figure 2). In all, the highest occurred bacterial species was *P. aeruginosa* (137; 13.51%) while the lowest was *Salmonella enteric* Typhimurium (15; 1.48%) (Figure 3).

The results of this study differ from a study by Onuorah *et al*. (2015) who reported *Escherichia coli* (34.3%) as the most frequent

while *Streptococcus pyogenes* (8.6%) had the lowest. *P. aeruginosa* is widely spread in nature especially in the soil, water, on plants and can easily contaminate food products. This finding agrees with the study of Egbebi and Muhammad (2016) who reported *P. aeruginosa* as the most predominant organism in their study. Higher percentage of organisms had earlier been reported (Kigigha *et al*., 2017; Orogu and Oshilim, 2017). There may be a possible outbreak of food poisoning and/or food-borne infections due to the consumption of contaminated suya meat, if appropriate quality control measures are not put in place. This may lead to serious economic and public health problems.

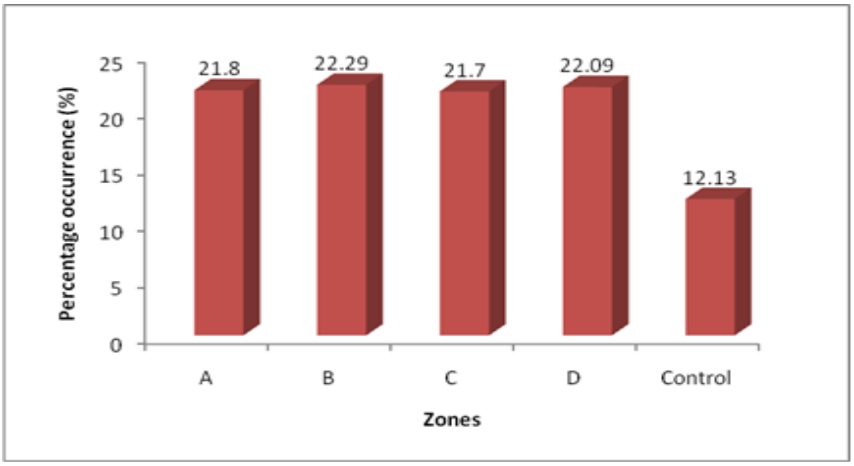

Figure 1. Percentage occurrence of bacteria in suya from different zones of Ogun State, Nigeria. Data were statistically analysed at 95% level of confidence (P<0.05) using ANOVA and paired wise sampling t-test.

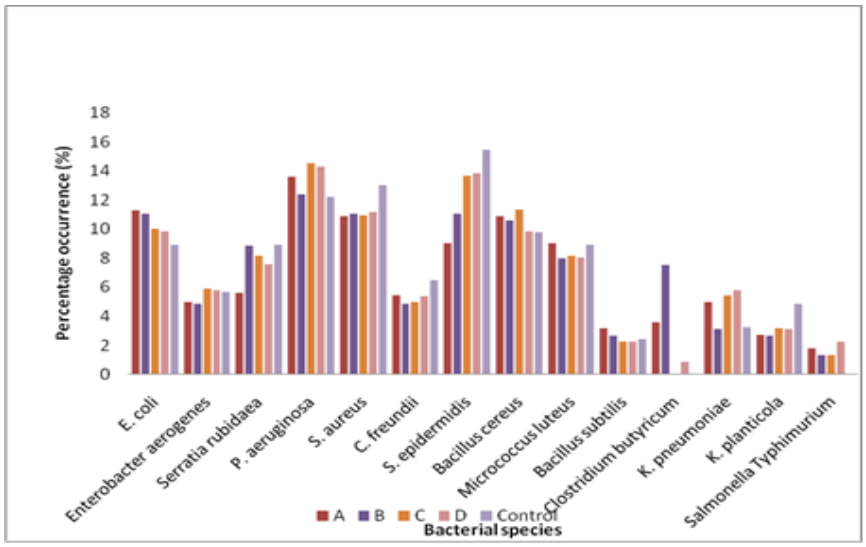

Figure 2. Percentage occurrence of individual bacterial species in suya from different geopolitical zones in Nigeria.

3.4.The antibiotic susceptibility patterns of bacterial isolates

For the purpose of simplification, a standardized, threshold-based assessment scheme has been introduced in which the degree of the effectiveness of the antibiotics investigated in this study is characterized as "susceptible (S)," "intermediate (I)," or "resistant (R)," based on their inbibition zones. The isolates showed varying degrees of

sensitivity to the antibiotics and are classified based on their zones of inhibitions (Table 4). Varying percentages including 11.20%, 15.09%, 9.86%, 18.05%, 21.10%, 34.81%, 30.57%, 36.19%, 41.50%, 36.70%, 43.49%, 52.86%, 54.83%, 68.64%, 82.15% and 86.69% of the isolated strains exhibited resistance to doxycycline, chloramphenicol, trimethoprim/sulfamethoxazole, gentamicin, tetracycline, ciprofloxacin, ampicillin, cefriaxone, colistin, erythromycin, cefalotin, penicillin G, rifampicin, nalidixic acid, vancomycin and teicoplanin, respectively (Figure 4).

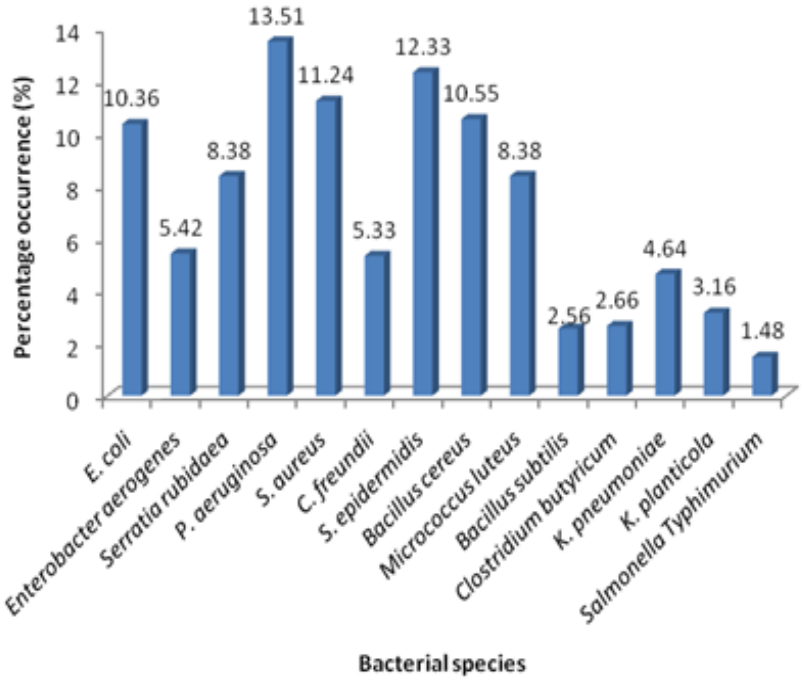

Figure 3. Cumulative percentage frequency of bacterial species from suya in Nigeria

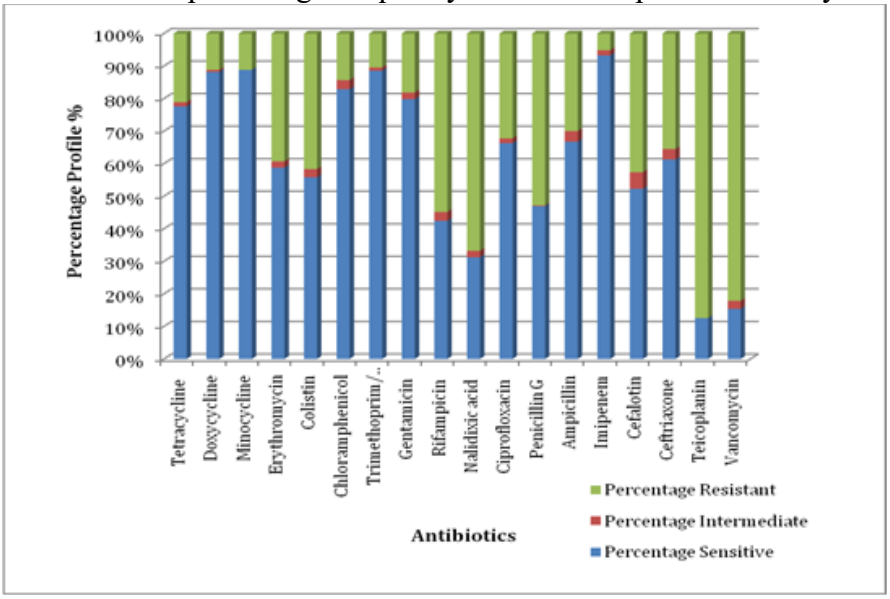

Figure 4. Cumulative percentage susceptibility and resistance profile of bacterial isolates from suya meat samples in Nigeria.

The findings on the antibiotic resistance of bacteria in this study deviated from the result of Barber *et al*. (2018) who reported that all *E. coli* was resistant to chloramphenicol and streptomycin. Nutanbala *et al*. (2011) reported the sensitivity of *E. coli* to ciproflaxain which is in line with the finding of this study. Ciprofloxacin belong to the fluoroquinolone class of antibiotics and has been known to have excellent activities against Gram-negative and Gram-positive bacteria such as *E. coli* and *S. aureus*, respectively (Cohen *et al*., 2017). The report of Sani *et al*. (2012) also buttressed the sensitivity of *S. aureus* to the fluoroquinolones. However, nalidixic acid exerted poor antimicrobial effects on the isolates as 68.64% of the bacterial isolates exhibited resistance to it in this study. The mechanism of action of the fluoroquinolones is the inhibition of bacterial DNA gyrase responsible for DNA replication and transportation (Moore, 2015). Ampicillin also inhibited the growth of 62.53% of the bacterial isolates in this study.

Minocycline, doxycycline and tetracycline exerted antimicrobial potency against 89%, 88.20% and 77.60% of the bacterial isolates. These antibiotics belong to the class tetracylines which inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site. Their high potency against bacterial isolates could be attributed to the fact that they are broad-spectrum agents. However, Mhondoro *et al*. (2019) reported high percentages of resistance to this class of antibiotics in their study. The penicillin-based antibiotics, such as the imipenem, act by binding to and inactivating penicillin-binding proteins (PBPs) located on the inner membrane of a bacterial cell wall. The strength and rigidity of the bacterial cell wall are affected by the inactivation of PBPs which interferes with the cross-linkage of peptidoglycan chains. This brings about the interruption of synthesis of bacterial cell wall which weakens the bacterial cell wall and results to cell lysis (Niwa *et al*., 2016).

Cefalotin and Ceftriaxone belong to the first and third generations of cephalosporins, respectively, and they possess the same mechanism of action like the penicillin-based antibiotics. The peptidoglycan layer of bacterial cell walls is disrupted by these antibiotics through competitive inhibition on penicillin-binding proteins (Moore, 2015). More than half (62.53% and 53.35%) of the isolates were sensitive to cefalotin and ceftriaxone, respectively. This is in line with the findings of Sani *et al*. (2012) and Page (2012) who also reported the sensitivity of similar bacterial isolates to the cephalosporins.

Trimethoprim/sulfamethoxazole and colistin inhibited the growth of 83.33% and 55.60% of the bacterial isolates, respectively. Cefalotin and ceftriaxone inhibit cell wall synthesis through the inhibition of β-lactamase (Bello *et al*., 2019). Erythromycin exerted antimicrobial potency against 54.90% of the bacterial isolates in this study which was also buttressed by the report of Hardman *et al*. (2017) where over half of the organisms isolated were sensitive to same class of antibiotics. Erythromycin is a macrolide-based antibiotic which reversibly binds to the 50s ribosomal subunit to inhibit synthesis of protein (Moore, 2015).

Gentamicin belongs to the aminoglycoside class of antibiotics. The high potency exerted by the gentamicin against bacterial isolates could be associated with the mechanism of action of this class of antibiotics which enables it to bind irreversibly to the 16S rRNA subunit of the 30S ribosome, resulting to inhibition of bacterial protein synthesis. This finding is supported by the reports of Barber *et al*. (2018), Mhondoro *et al*. (2019) and Breijyeh *et al*. (2020). A high percentage of the bacterial isolates (87.50%) were sensitive to chloramphenicol. Chloramphenicol belongs to the phenicol class whose mode of action is to interfere with bacterial protein synthesis.

The production of chloramphenicol acetyltransferase (CAT) is responsible for the resistance of bacteria to chloramphenicol while some resistance occur as a result of inability of certain bacteria to reach their target sites.

Only 15.39% and 12.43% of the bacteria isolated in this study were sensitive to vancomycin and teicoplanin, respectively. This is attributed to the fact that vancomycin and teicoplanin are narrow spectrum and exert very weak action against many Gram-negative bacteria. Vancomycin and teicoplanin belong to the glycopeptides and their modes of action are same as the β-lactam antibiotics. However, glycopepetides differ from β-lactams in that they interact with different molecular targets as they bind to acyl-D-alanyl-D-alanine in peptidoglycan and, hence, inhibit the function of glycosyltranfereases in susceptible bacteria. The hydrophilic antibiotics like β-lactams pass through porins, and glycopeptides cannot cross the outer membrane due to their structures that hinder it from using any of these passages (Breijyeh *et al*., 2020).

4.Conclusions

The microbial loads encountered in suya meat from this study were at the borderline based on the microbiological guidelines for ready-to-eat food products. The study revealed the presence and distribution of multidrug-resistant food pathogens in the food product which is of tremendous public health concern.

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THE APPLICATION OF MICRONIZATION TECHNOLOGY ON A FRUITING BODY EXTRACT OF *ANTRODIA CINNAMOMEA*

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ABSTRACT

Micronization has been applied in many drug preparations—including traditional Chinese medicine—to optimize oral bioavailability. The commonest technique used is mechanical commination (e.g., by crushing, grinding, and milling). Particles produced through the micronization process are quite easy to re-aggregate due to the Van der Waals effect, and therefore dispersants are added to maintain the stability of the micronized mixture and prevent them from aggregating into larger particles. In this study, the particles of *Antrodia cinnamomea* suspended in water extract are micronized by the impact between two currents of liquid generated by a liquid micronizer made specifically for the task. *Antrodia cinnamomea* fruiting bodies were extracted using 95% alcohol. The extraction was then diluted 20 times with water and underwent a micronization process for 240 minutes. Appropriate amounts of dispersant were then added to the micronized *Antrodia cinnamomea* extract, with each dispersant divided into three concentrations — 0.5%, 0.75%, and 1.0%. Three different dispersants were tested in this study, namely microcrystalline cellulose, silicon dioxide, and polyethylene glycol. Monitoring of the degree of aggregation over 56 days showed that a silicon dioxide concentration of 0.75% gives the best dispersion effect.

1.Introduction

Antrodia cinnamomea (Syn. *Antrodia camphorata*) is a fungal parasite found only on the inner cavity of the endemic species *Cinnamomum kanehirae* (Bull camphor tree) *Hayata* (*Lauraceae*) (Tzeng *et al.*, 2011). Due to its rareness, the price of *Antrodia cinnamomea* varies from tens of thousands to hundreds of thousands of US dollars per kilogram in the market, which is why it is regarded as the most expensive wild fungus in the world. Taiwanese indigenous people first found the fruiting body of this species in their search for wood, and after trying it and drink its boiled juice, they noticed that the fungus could relieve hangovers, eliminate fatigue, and improve liver disease. As a result, *Antrodia cinnamomea* has long been used as a medical treatment for food poisoning, diarrhea, hepatitis, high blood pressure, etc., by indigenous people in Taiwan. *Antrodia cinnamomea* is also a potential ingredient in modern health supplements and pharmaceuticals thanks to its antioxidant, nitrite scavenging, and antitumor properties (Cha *et al.*, 2009).

Antrodia cinnamomea contains various bioactive compounds, such as terpenoids, benzenoids, lignans, benzoquinone derivatives, and succinic and maleic derivatives, in addition to polysaccharides. Polysaccharides can

improve the human immune response and inhibit the hepatitis B virus (Lee *et al.*, 2002), while triterpenoids provide mainly anti-cancer and liver protection (Laszczyk *et al.*, 2009), and superoxide dismutase (SOD) eliminates free radicals. However, extracting bioactive compounds is not easy due to their low solubility, and the adsorption rate in the human body is not ideal. Therefore, to optimize the adsorption, many methods have been applied, such as through Self-Emulsifying Drug Delivery Systems (SEDDS) (Tang *et al.*, 2007), micronization (Xu *et al.*, 2004), etc. In this experiment, the micronization method was applied. In recent years, micronization has been applied in numerous fields, from foodstuffs to pharmaceuticals, and especially in traditional Chinese medicine. In addition to being effective in terms of improving its bioavailability, the micronization process can also reduce the side effects of traditional Chinese medicine (Fan *et al.*, 2019). Moreover, the amount of raw materials used can be reduced, thus achieving higher economic value. For example, the physiological effects of dietary fibers are related to their absorption, so increasing the specific surface area can improve the physiological activity of dietary fibers. By reducing the particle size of dietary fibers from 0.1 mm to 1 μm, the specific surface area can be increased by a factor of 100, and the recommended intake can be reduced to 1% of the original one. If the particle size is further reduced to 100 nanometers, the recommended intake can be reduced to one thousandth compared to the original (Lin *et al.*, 2012). However, the smaller the particles, the easier the re-aggregation. According to DLVO theory, there is a mutual attraction between colloidal particles (the Van der Waals force), and there is also a mutual repulsive force, which is the electrostatic repulsive force when the electric double layers overlap. These two opposing forces determine the stability of the colloid. When the attractive force between the particles is dominant, small particles are easy to aggregate and precipitate, because the attraction force causes the continuous aggregation phenomenon. When the electrostatic repulsion force is dominant, the particles can be prevented from aggregation and precipitation, so the colloids will be in a stable state. In many cases, in order to obtain substances with different stabilities, researchers will increase or decrease the attraction or repulsion via environmental changes. Common methods include changing the ionic environment, adjusting the pH value, or adding surface-active substances (polymeric dispersants), and so on (Alwadani *et al.*, 2018). The factors and parameters that affect aggregation need to be set according to different substances, such as temperature, humidity, concentration, pH value, pressure, electrical potential, etc. (Zapadka *et al.*, 2017). The empirical results of this study show that the original high-particle concentration with a particle size of less than 1 μm accounted for 69% of the suspension. After storage at 4°C for 24 hours, the average particle size increased from 0.79 μm to 9.35 μm. After thawing, the average particle size was 109 μm, reaching 132 μm after high-temperature sterilization. This means that there is an absolute correlation between temperature and the degree of reaggregation. The lower the temperature, the more severe the reaggregation. The concentration of particles is an important factor that affects the degree of re-aggregation and thus the stability of the liquid. The higher the concentration, the higher the probability of collision between the particles, meaning that the aggregation phenomenon is relatively proportional. To modify the suitable interface of the materials before applying it to the final product, dispersants can be added in order to make the scattered targets become well distributed, thus stabilizing them in the medium. Depending on the actual requirements and applications, each type of product has a different definition of stability. For example, in some fields, if a colloidal mixture is stable for two days, it is considered stable, but in others, it may need to be stable for more than two years without obvious changes in order to be accepted as such. In this study, three dispersants were used: microcrystalline cellulose (MCC), silicon dioxide (SiO_2), and polyethylene glycol. MCC

is a pure natural cellulose, a product made by converting fibrous cellulose into a re-dispersible gel or aggregate of crystalline cellulose. This material can be dried into pure fine particles and made into powder (Mikaela Börjesson, 2015). The color of MCC powder is white, and the powder is odorless and tasteless, which is why it is commonly used in pharmaceuticals, health foods, and cosmetics. Silicon dioxide (SiO_2)—an acidic oxide—exists in two main types as crystalline and amorphous. Amorphous silicon dioxide has been used in food applications for many years, such as in beer and wine, and as an anti-caking agent in powder (Guo *et al.*, 2018) and thick paste products. Polyethylene glycol is an oligomer or polymer of ethylene oxide, which vary in molecular weight. Different molecular weights in different applications often have different physical properties (such as viscosity, etc.) (Vidyasagar *et al.*, 2016), but most polyethylene glycols are similar in terms of their chemical properties. Polyethylene glycol 400 (PEG400) was chosen for this experiment due to its stability and due to the fact that it does not deteriorate easily (it has also been used widely in various industries).

2. Materials and methods

2.1. *Antrodia cinnamomea* extraction

Since the growth speed of *Antrodia cinnamomea* is slow, the most suitable cultivation conditions for it are artificial cultivation. Besides chemical factors (such as carbon and nitrogen sources in the medium), physical factors also play a crucial role in the cultivation of the fungus, especially temperature. Different strains will have different discrepancies, and the most suitable temperature for the mycelium in submerged culture is between 22 to 28℃. Exceeding this temperature can cause the mycelium to age more easily (Hadar and Dosoretz, 1991). Humidity is also a key factor affecting the cultivation of *Antrodia cinnamomea*, with a range between 40% and 80% being the most suitable. Physical wounds and the concentration of gas can also affect the growth rate of fruiting bodies. Furthermore, since a source of carbon and nitrogen is a very important factor in cultivating mushrooms, these elements are indispensable in the *Antrodia cinnamomea* culture medium. The carbon to nitrogen ratio (C/N) also affects the content of the components of the fungus. Commonly used sources of carbon are glucose, fructose, sucrose, maltose, etc. The concentration of glucose in the medium was 3%, which is the most suitable carbon source for mycelium. When the concentration of yeast extract reaches 3%, the biomass and the yield of triterpenes increase. The optimal conditions are 3% of yeast extract, 1% of peptone, and 2.5% of malt extract (Chang *et al.*, 2006). The source of nitrogen is the main source of mushroom protein and nucleic acid. Different carbon sources have different effects on mycelial growth (Sone *et al.*, 2014). Glucose (Wanglaichang Co., Ltd), malt extract (Sisco Research Laboratory Pvt), agar and peptone (Yihe Co., Ltd) were used to prepare the medium. *Antrodia cinnamomea* mycelium was obtained by isolating the mycelium in a solid medium (3% glucose, 2% malt extract, 2.5% agar). To prepare the submerged culture, 3% glucose, 2% malt extract, and 1% peptone were combined in an Erlenmeyer flask. After transferring the mycelium into the new medium, the flask mouth was wrapped with parafilm and the flask was placed on a shaker (TS520D model, Yihder Technology) for 14 days at 130 rpm. *Cinnamomum kanehirae* can be found on the Jiaoban Mountain in the Taoyuan region, in Nanzhuang of the Miaoli region, in Zhushan of the Nantou region, and in Liugui of the Kaohsiung region of Taiwan (Lin *et al.*, 2006). The wood of the *Cinnamomum kanehirae* is the sole host, mainly because its tolerance to the essential oil of *Antrodia cinnamomea* is relatively high, so other fungi cannot grow on it (Geethangili and Tzeng, 2011). A stout camphor log, used to culture the *Antrodia cinnamomea* fruiting body, was sterilized for two hours in an autoclave (HY-3005V model and HY230 model, Hungyi Instrument Co., Ltd) and then exposed to UV light for the next 24 hours. The culturing steps were performing in the laminar airflow bench (Hae Tian Science Co., Ltd): the mycelium—contained in the liquid medium—was spread throughout the chilled log and incubated inside a thermostatic chamber at 26°C to 28°C. The *Antrodia cinnamomea*

fruiting body formed after eight to ten months. To make the *Antrodia cinnamomea* aqueous extract, one kilogram of *Antrodia cinnamomea* fruiting body was cleaned thoroughly with sterile water and dried in the shade before being combined with 20 liters of 95% ethanol (Pingtung Brewery Co., Ltd) and settled in a glass bottle for one month at room temperature. Ethanol in the mixture was then evaporated completely. The *Antrodia cinnamomea* extract was diluted twenty times with distilled water to obtain a mixture, here called *Antrodia cinnamomea* liquid (ACL).

2.2. Micronizing the *Antrodia cinnamomea* liquid (ACL)

The micronizer used in this study was designed according to the impact principle, which states that particles suspended in liquid can be micronized by creating two contrary liquid flows. When the machine is operated at high speed, the interior of the machine will activate a gas circulation function. The machine consists of a round mixing tank with two stirring assemblies inside, a thermometer, a liquid inlet and outlet, and four stands [Fig. 1. (a)]. On the tank lid, there are three different observation holes as well as a pressure valve, an exhaust vent, and a liquid inlet [Fig. 1. (b)]. Each stirring assembly comprises a high-speed motor (which controls the flow direction), a shaft, and a cross-shaped stirring blade. The first motor operates clockwise while the other one does so counterclockwise, which causes the impact between two flows of liquid. Two stirring assemblies are arranged in a parallel and equidistant position inside of the mixing tank. To micronize the ACL (ACL240), the liquid was added through the inlet and beaten continuously for 240 minutes.

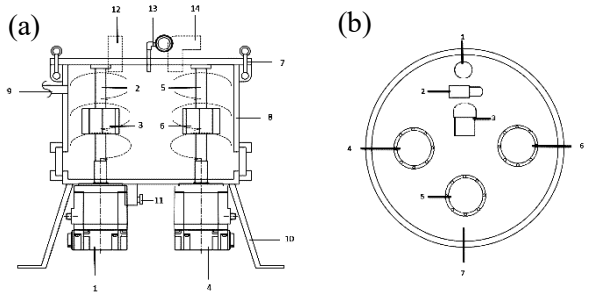

Figure 1. (a) schematic of the nanometerization machine (1, 4: high-speed motors; 2, 5: shafts; 3, 6: stirring blades; 7: lid; 8: tank body; 9: thermometer; 10: stands; 11: outlet; 12: inlet; 13: pressure valve; 14: exhaust vent. (b) plan view of the nanometerization machine (1: inlet; 2: pressure valve; 3: exhaust vent; 4, 5, 6: observation holes; 7: lid.

2.3. Dispersants

The three tested dispersants (Yihe Co., Ltd) were microcrystalline cellulose, silicon dioxide, and polyethylene glycol. The ACL240 was divided into nine bottles, 20mL each. The nine bottles were then divided into three groups, with each group having three different concentrations of dispersants (0.5%, 0.75%, and 1.0%). The samples containing microcrystalline cellulose were named A1, A2, and A3. The samples containing silicon dioxide were called B1, B2, and B3. The samples containing polyethylene glycol were named C1, C2, and C3.

2.4. Particle size detection

The equipment—Malvern Zetasizer Nano ZS (Malvern Panalytical, USA)—used to measure the particle size in this experiment is based on the principle of dynamic light scattering. When suspended particles undergo Brownian motion, they produce scattered stripes that change with time. The detector converts the scattered light signal into an electric current and obtains the diffusion coefficient of the particles in the solution through digital gas correlation. The size of the particle size can then be obtained using the Stock-Einstein equation. The equipment's particle size range falls within 0.6 nm to 6000 nm, while the concentration range is 0.1 mg/ml to 40 wt%, the temperature range is between 2°C to 90°C, and the LASER specification is 4 Mw He-Ne, 633 nm. The nine samples were tested and recorded for particle size distribution each week.

3.Results and discussions

3.1. Particle size distribution

After 56 days of sampling and analysis, the average particle sizes of the different samples were summarized (Table 1). On the first day, the particle sizes were similar, but eventually they began to differ according to the effectiveness of the dispersants. The average particle sizes of the

A1, A2 A3, B1, B2, B3, C1, C2, and C3 samples on the first day were 108.6 nm, 159.4 nm, 108.1 nm, 123.7nm, 116.2 nm, 176.3 nm, 131.1 nm, 116.5 nm, and 102.1 nm, respectively. By the 56th day, the average particle sizes had changed to 667.1 nm, 709.4 nm, 234.4 nm, 558.3 nm, 126.2 nm, 210.9 nm, 344.8 nm, 118.5 nm, and 139.5 nm, respectively. In the case of the A1 sample, the average particle size did not change much from the 1st day to the 28th day, but after the 42nd day, the aggregation degree began rising and eventually sky-rocketed to 667.1 nm by the 56th day. In the case of the A2 sample, the average particle size declined slightly in the first few weeks but then jumped to 709.4 nm. In the B1 sample, the average particle size remained pretty stable in the first five weeks, but—as recorded on the 49th day—the particles started to re-aggregate soon thereafter. In the C1 sample, the average particle size increased quickly in the first week and was the only sample in which the average particle size was larger than 200. However, the average particle size of the A3, B2, B3, C2, and C3 samples was relatively stable over the 56 days, with the changes being negligible. After comparing each sample, it was found that A3, B2, and C2 had the best dispersant effect, whereas A2, B1 and C1 could not slow down the re-aggregation process of the mixture. As can be seen in Fig. 2, in the A1 sample, the proportion of particles above 1000 nm rocketed from 15% to 38% over the 56 days. Nevertheless, particles below 100 nm still accounted for 42% of the total, while particles in the range of 100 nm to 1000 nm dropped to 22% on the 56th day. The proportion of particles in the 200 nm to 1000 nm range in the B1 sample increased to 42.32% over the 56 days. At the same time, the proportion of particles with particle size below 100 nm dropped to 21.64%, and particles below 50 nm disappeared completely. In the C1 sample, the proportion of particles in the 200 nm to 1000 nm range increased to 16.41% over the 56 days, with a sudden growth of particles above 500 nm. In contrast, three samples—A3, B2, and C2—had the smallest average particle size and the most stable particle distribution compared with each other sample of the same group (Table 1), and thus the best dispersant effect. The proportion of particles in the 200 nm to 1000 nm range in the case of A3, B2, and C2 on the first day was 12.09%, 13.78%, and 9.09%, respectively. By the end of the eighth week, the numbers were 36,28%, 25.29%, and 13.73%, respectively (see Fig. 3).

Table 1. Average particle size of the different samples over 56 days

Day	**1**	**7**	**28**	**35**	**42**	**49**	**56**
Sample	**Average particle size (nm)**						
A1	108.6	101.0	103.7	124.6	280.7	294.6	667.1
A2	159.4	112.5	104.8	212.5	377.5	571.5	709.4
A3	108.1	138.4	207.4	155.2	340.2	223.7	234.4
B1	123.7	128.1	233.0	134.9	235.0	450.5	558.3
B2	116.2	152.5	176.3	107.4	105.8	174.9	126.2
B3	176.3	196.5	111.9	210.6	199.3	268.1	210.9
C1	131.1	292.8	325.8	215.5	303.5	284.6	344.8
C2	116.5	92.7	102.5	94.5	94.05	214.7	118.5
C3	102.1	188.8	93.64	110.5	102.9	149.3	139.5

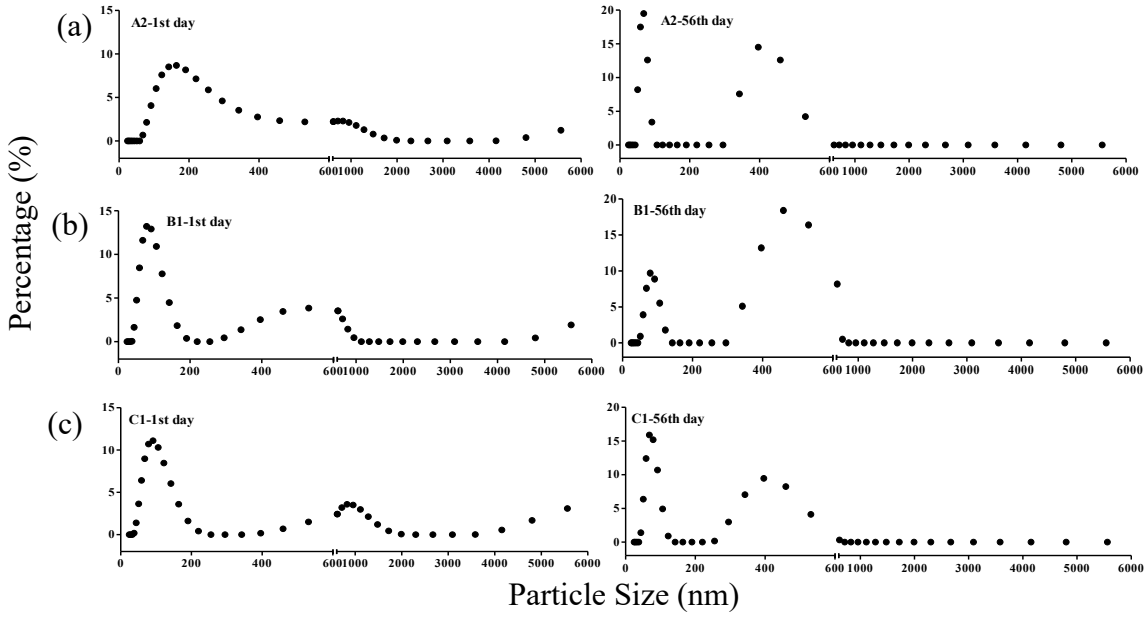

Figure 2. Particle size distribution diagram of the (a) A2 sample, (b) B1 sample, and (c) C1 sample on the 1st day and the 56th day

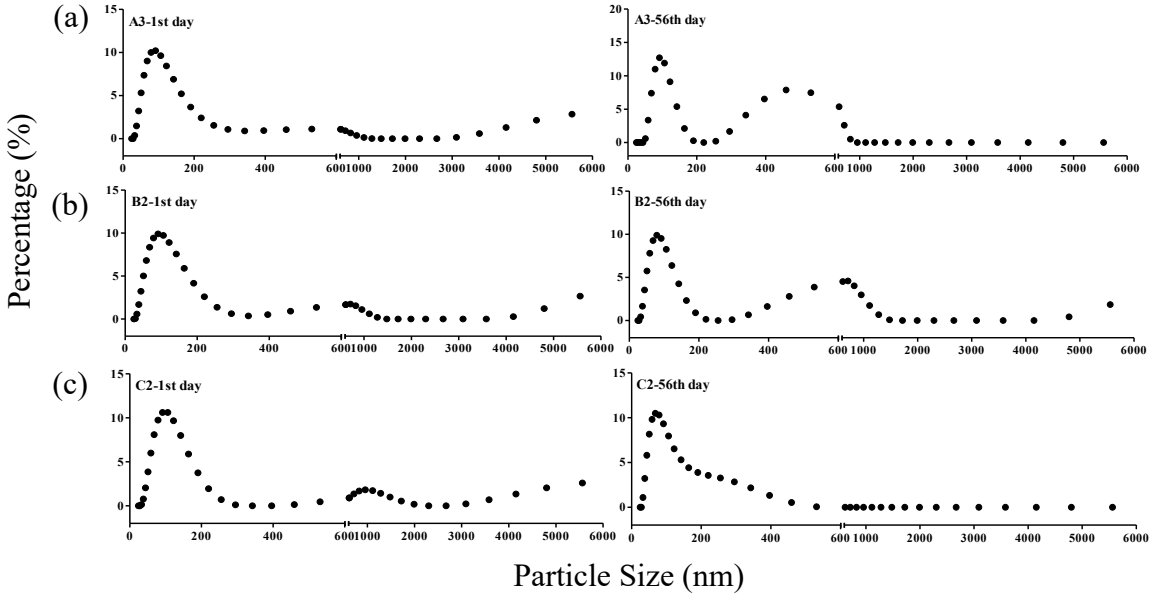

Figure 3. Particle size distribution diagram of the (a) A3 sample, (b) B2 sample, and (c) C2 sample on the 1st day and the 56th day

In order to evaluate the effectiveness of the chemical dispersants, in each sample, the particles whose sum demonstrated a positive growth over time were chosen. In the particles of the A1 sample [Fig. 4. (a)], the 531 nm and 1280 nm particles are negatively correlated. When the proportion of 531 nm particles decreases with time, the proportion of 1280 nm particles increases. Within eight weeks, the total proportion of 531 nm and 1280 nm particles increased to 13.51%. After eight weeks, the 531 nm particles disappeared completely. In the A2 sample [Fig. 4. (b)], the 255 nm and 396 nm particles were chosen. The total proportion of the two particle sizes increased to 9.52%, with the 255 nm particles disappearing totally by the

eighth week. Among the particles in the A3 sample [Fig. 4. (c)], those that had a correlation were the 396 nm and 459 nm particles. Their total proportion rocketed from 1.99% to 14.41%, with the proportion between the two particle sizes remaining stable during the eight weeks. In the B1 sample [Fig. 4. (d)], three particles sizes—396 nm, 459 nm, and 531 nm—had a positive rising trend. The total proportion of the three particles sizes increased to 48% by the eighth week. Among the particles in the B2 sample [Fig. 4. (e)], the 295 nm particles and the 825 nm particles had a negative correlation, with the total proportion of the two particle sizes increasing to 1.98%. In the B3 sample [Fig. 4. (f)], a total of four particle sizes were reported to have had a positive growth over time. The sum of the 220 nm particles, the 255 nm particles, the 531 nm particles, and the 615 nm particles grew to 4.96% over eight weeks. Among the particles in the C1 sample [Fig. 4. (g)], the 342 nm particles and the 459 nm particles had a negative correlation, and over the eight weeks their total proportion increased to 14.41%. In the C2 sample [Fig. 4. (h)], the proportion of the 220 nm particles and the 255 nm particles had a negative correlation. The total proportion of the two particle sizes rose to 4.18% after eight weeks. Among the particles in the C3 sample [Fig. 4. (i)], those that had a correlation were the 190 nm and the 531 nm particles. Their total proportion increased to 3.26% over the eight weeks.

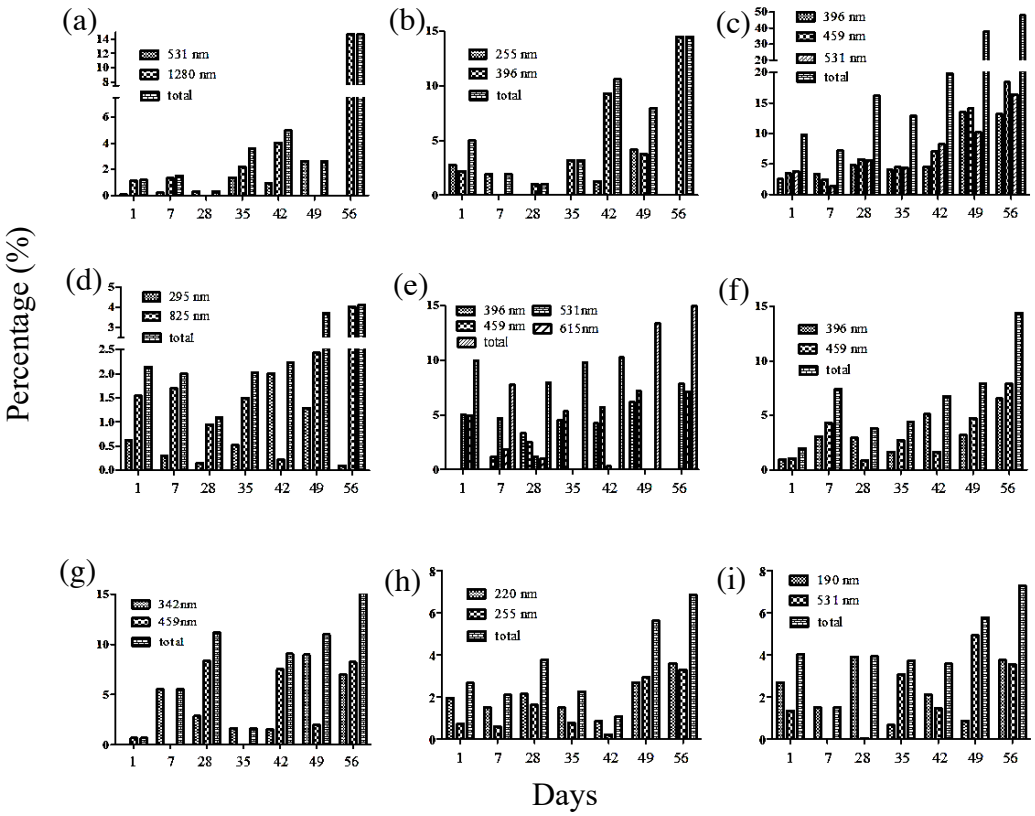

Figure 4. Histogram of the (a) 532 nm particles and 1280 nm particles in the A1 sample, (b) 255 nm particles and 396 nm particles in the A2 sample, (c) 396 nm particles and 459 nm particles in the A3 sample, (d) 396 nm particles, 459 nm particles, and 531 nm particles in the B1 sample, (e) 295 nm particles and 825 nm particles in the B2 sample, (f) 396 nm particles, 459 nm particles, 531 nm particles, and the 615 nm particles in the B3 sample, (g) 342 nm particles and 459 nm particles in the C1 sample, (h) 220 nm particles and 255 nm particles in the C2 sample, and (i) 190 nm particles and 531 nm particles in the B2 sample

3.2. Comparison of the particle sizes of the nine samples

In the A1, A2 and A3 samples, the degree of aggregation at different concentrations varies with particles, aggregation and time. The stability of a sample was indicated by the value of the slope. When the slope value (or S) was large, the degree of aggregation was more obvious. In contrast, a value represented by 1/S meant that the larger the value, the more obvious the dispersion effect. The comparisons of the particle distribution slopes of each dispersant type were recorded as follows (Fig. 5). In the A1 sample, the total percentage of the 531 nm particles and the 1280 nm particles showed a positive correlation between aggregation and time. The sum of the 531 nm particles and the 1280 nm particles had a growing pattern. The degree of aggregation of the A1 sample was represented by the sum of the 531 nm particles and the 1280 nm particles, and the 1/S value was 6.32. In the A2 sample, the total percentage of the 255 nm particles and the 396 nm particles exhibited a positive correlation between aggregation and time. The total percentage changed and posted a growing trend over time. The degree of aggregation of the A2 sample was represented by the sum of the 255 nm particles and the 396 nm particles, and the 1/S value was 5.97. In the A3 sample, the total percentage of the 396 nm particles and the 459 nm particles indicated a positive correlation between aggregation and time. The sum of the 396 nm particles and the 459 nm particles posted a rising trend. Represented by the sum of the 396 nm particles and the 459 nm particles, the 1/S value of the A3 sample was 7.59. In conclusion, the 1/S values of the A1, A2, and A3 samples were 6.32, 5.97, and 7.59, respectively. In the B1 sample, the degree of aggregation of the 396 nm, 459 nm, and 531 nm particles in total was the most obvious, and was therefore regarded as the representative. The 1/S value of the sum of the 396 nm, 459 nm, and 531 nm particles in the B1 sample was 1.59. In the B2 sample, the total percentage of the 295 nm particles and the 825 nm particles showed a positive correlation between aggregation and time. The sum of the 295 nm particles and the 825 nm particles posted a growing pattern. The degree of aggregation of the B2 sample was represented by the sum of the 295 nm particles and the 825 nm particles, and the 1/S value was 6.32. The B3 sample was represented by the sum of the 220 nm and the 255 nm particles, together with the 531 nm and the 615 nm particles, and the 1/S value was 10.47. In summary, the 1/S values of the B1, B2, and B3 samples were 1.59, 30.39, and 10.47, respectively. In the C1 sample, the total percentage of the 342 nm and the 459 nm particles showed a positive correlation between aggregation and time.

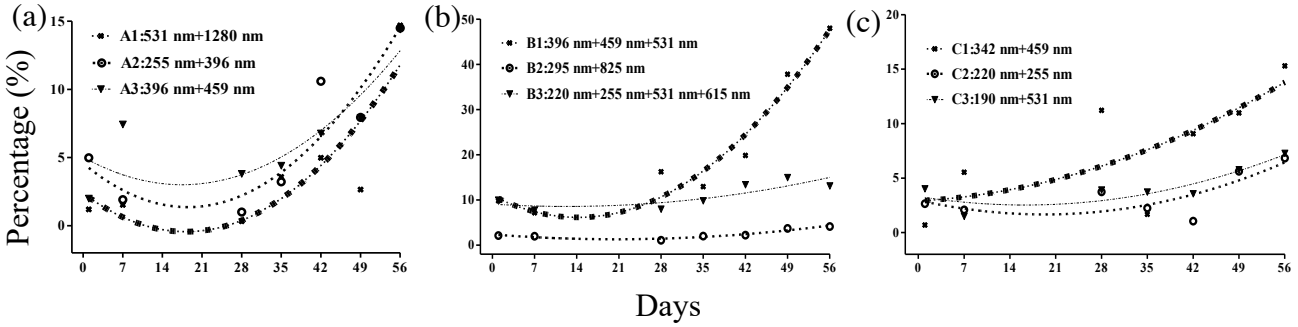

Figure 5. Comparison of the particle distribution slopes of (a) A1, A2, and A3; (b) B1, B2, and B3; (c) C1, C2, and C3

The sum of the 342 nm and the 459 nm particles exhibited a growing pattern, and the 1/S value was 5.23. In the C2 sample, the total percentage of the 220 nm particles and the 255 nm particles showed a positive effect on the relationship between aggregation and time. The sum of the 220 nm particles and the 255 nm particles posted a growing pattern. The C2 sample was represented by the sum of the 220 nm particles and the 255 nm particles, and the 1/S value was 17.32. In the C3 sample, the total percentage of the 190 nm and the 531 nm

particles posted a positive correlation between aggregation and time. The sum of the 190 nm and the 531 nm particles showed a growing pattern. The C3 sample was represented by the sum of the 190 nm and the 531 nm particles, and the 1/S value was 15.36. The 1/S values of C1, C2, and C3 were 5.23, 17.32, and 15.36, respectively.

4. Conclusions

Nanoparticles are affected by the Van der Waals force regardless of what dispersant is used, because as the particle size decreases, the surface area increases. As time passes, the particles are easily re-aggregated into macromolecules. Therefore, dispersant addition is necessary in order to maintain the stability of the micronized extract. After monitoring and testing for the particle size distribution over eight weeks, the results showed that the degree of aggregation did not decrease when the amount of dispersant was increased, but rather the optimal amount worked better.

4.1. Particle size distribution

All the micro-particles displayed an aggregation trend into bigger particles. Among the nine samples, the A1 and A2 samples had the most obvious degree of aggregation. The particles ranging from 100 nm to 1000 nm in the A1 sample aggregated into particles above 1000 nm. In the A2 samples, most of the particles ranged in size from 200 nm to 1000 nm, and by the 56th day, particles below 50 nm no longer existed. In contrast, three samples performed the best in terms of slowing the aggregation process, namely A3, B2, and C2 (Fig. 6). In the A3 sample, the particles ranging in size from 200 nm to 1000 nm had a slight change over time, but the degree of aggregation in this sample was lower than in the other samples of the same group. The particle size in the B2 sample was stable during the 56 days of the test, and no remarkable changes were recorded. Moreover, the degree of aggregation even slowed down in the last week. In the C2 sample, the 200 nm – 1000 nm range particles increased stably over the first six weeks, but accelerated quickly in the seventh week and then dropped.

4.2. Comparison of the particle distribution slopes of the three dispersant representatives

The degree of aggregation differed by particle, aggregation and time in each sample with different concentrations.

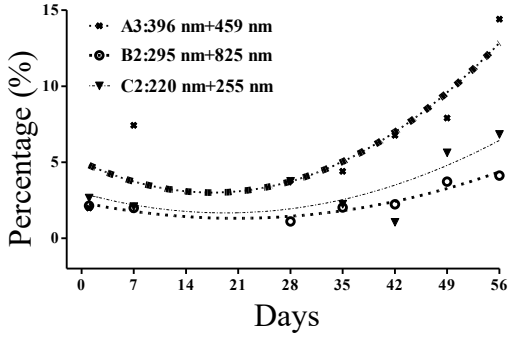

Figure 6. Comparison of the particle distribution slopes of A3, B2, and C2

The slope value (a.k.a. S) represented the degree of aggregation of the samples, with the larger the 1/S, the more obvious the dispersion effect. Each sample had a corresponding set of particles, which represented a positive effect on the relationship between aggregation and time, and thus a growing pattern. The different concentrations of each dispersant were compared and chosen for the best, and each representative was then compared for the best dispersant out of the three. The 1/S values of A1, A2, and A3 were 6.32, 5.97, and 7.59, respectively, meaning that microcrystalline cellulose 1.0% had the best dispersion effect. The 1/S values of B1, B2, and B3 were 1.59, 30.39, and 10.47, respectively, meaning that silicon dioxide 0.75% had the best dispersion effect. The 1/S values of C1, C2, and C3 were 5.23, 17.32, and 15.36, respectively, meaning that polyethylene glycol had the best dispersion effect at a concentration of 0.75%. Figure 6 compares the three particle distribution slopes and indicates the best one out of the three. The A3 particles aggregated faster than the B2 particles, and the aggregation speed of the C2 particles was also faster than that of the B2 particles. Hence, a silicon dioxide concentration of 0.75% gives the best dispersion effect.

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CHARACTERIZATION OF INDIGENOUS YEASTS SPECIES ISOLATED FROM FRUITS FOR PINEAPPLE WINE PRODUCTION

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ABSTRACT

This study was designed to characterize yeast species isolated from fruits and use as starter cultures in pineapple wine production. Forty yeast isolates were obtained from fermenting pineapple, watermelon and cashew juices using culture-dependent method and screened for pathogenicity properties. Eleven of the yeast isolates were non-pathogenic and were investigated for their abilities to produce invertase, tolerate ethanol, sugars, grow at different temperatures and pH by spectrophotometric method. Identification of the yeast isolates was carried out using API (ID 32C) kit. The result obtained showed that Isolate PIN32 *(Saccharomyces cerevisiae* 4) had the highest invertase activity of 40.04±0.5 Umol/min followed by 30.17±0.1 Umol/min produced by WAM8 (*Saccharomyces cerevisiae* 1). The highest tolerance to ethanol was demonstrated by isolate PIN32 (*Saccharomyces cerevisiae* 4) and WAM8 (*Saccharomyces cerevisiae1*) with a growth of 1.31±0.3 and 1.26±0.2 respectively. Optimum glucose tolerance was observed in WAM8 (*Saccharomyces cerevisiae1*), while PIN32 (*Saccharomyces cerevisiae* 4) demonstrated the highest growth in 20% sucrose. Similarly PIN32 (*Saccharomyces cerevisiae* 4) and WAM8 (*Saccharomyces cerevisiae 1*) recorded the highest growth of 1.55 at pH 6. All isolates exhibited optimum growths at 30^0 C with PIN32 recording the highest growth. The isolates were identified as *Saccharomyces cerevisiae, Pichia farinosa, Saccharomyces kluyveri, Kloeckera japonica, Pichia ohmeri, Debaromyces polymorphus, Candida kefyr.* The result showed that PIN32 and WAM 8 could be selected as potential starter cultures for pineapple wine production based on the empirical findings in this work.

1. Introduction

Yeasts are eukaryotic microorganisms and they are widely encountered in ecological niches such as fruits, grains, soil and fermented food but they are mostly isolated from citrus juices and sugar cane (Tamang,2016) They are important in many complex ecosystems, as frequent early colonizers of nutrient rich substrates (Djelal et al.2017). *Saccharomyces cerevisiae* (baker's and brewer's yeast) is the most studied species because it utilizes hexose sugar to produce CO_2, ethanol, and variety of secondary metabolites such as esters, aldehyde and amino acids that contribute to the development of flavour and aroma of fermented foods (Pretotoritus, 2000). Unlike bacteria, yeasts are osmophilic and can grow in media of low water activities and acid pH. (Deak, 2006).) These biotechnological properties exhibited by saccharomyces cerevisiae have made them suitable in the production of wine of good quality and consumers acceptability(Turker, 2014).However Yeast metabolism and physiology are thus strongly dependent on sugar and oxygen Unlike bacteria, yeasts are osmophilic and can grow in media of low water activities and acid pH.(Dickinson and Kruckeberg, 2006)..Its enhanced applications in

alcoholic fermentation, bread-making, single cell protein, vitamin production, synthesis of recombinant proteins, and biological control is well documented (Akabanda et al.,. 2013).Its high tolerance to sugar ,ethanol , high temperature are the biotechnological properties leading to its selection for alcoholic fermentation (Islam et.al.,2015)

These biotechnological properties exhibited by saccharomyces cerevisiae have made them suitable in the production of wine of good quality and consumers acceptability (Turker, 2014).

Wine is a fermented beverage prepared from fresh fruit juices by normal alcoholic fermentation (Okafor,2007). In wine production ,yeast ferments the hexose sugar present in the substrate a process named alcoholic fermentation to ethanol, carbon dioxide and other secondary metabolites.(Robinson,2006) The quality of wine depends largely on the yeast strain used (Idise, 2012).Wines produced from grape (Vitis species) are called the true wines while wines from other fruits are referred to as fruit wines and they are named using the name of the fruit used for their production e.g., orange wine, banana wine, cherry wine and pineapple wine (Lea et al., 2003). It is reported that wine and other alcoholic drinks are used in entertaining guests during conferences, rallies, marriage, christening, and burial ceremonies.. This has made it an integral part of the Nigerian society and many people believe strongly in its ability to prevent cardiovascular disease because of its high content of polyphenol such resveratrol, anthocyanin and catechins (Snopek et al.,2018). Most of the common fruits being utilized for wine and juice making are cashew, pineapple, mango and orange. Fruits supplement the quality of our diet by providing essential ingredients like vitamins, minerals, carbohydrates. They are seasonal products and are available in large quantity during their harvesting period but a higher percentage is wasted due to lack of available storage facilities and underutilization. This wastage can be arrested by processing and conversion of these fruits into other useful valued products which will make them available all year round. Such possible products are wines, concentrate and confectionaries (Bolarin et al., 2016).

Pineapple (Ananas comosus)belongs to the Bromeliaceae family and is planted in different regions of Nigeria either for export or for the local consumption. It can be eaten fresh or process into fruit juices or concentrates for future use. Nutritionally it contains 81.2-86.2% moisture, 13-19% total solids of which sucrose, glucose, and fructose are the main components with 2-3% fiber, a high level of vitamin C and calcium (Sun et al.,2016). It is also reported to possess proteolytic activity due to the presence of bromelin couple with low amount of lipids and nitrogenous compounds (Shetty et al.,2019)The pineapple varieties commonly grown in Africa have sufficient sugar levels and favorable pH (4.5-6.5) for fermentation to occur (Idise, 2012). Thus, through fermentation the highly perishable pineapple fruit could be converted into a highly nutritious wine which can be made available all year round (Keller, 2010). In Nigeria, 50% of the pineapple harvested annually is wasted due to lack of adequate storage facility. It is therefore suggested that government and private investors should look inwards as to developing wineries that will exploit optimally the utilization of this fruit, considering the fact that a huge amount of foreign exchange and employment will be derived from the exportation of wine (Akubo, et al., 2003).

In this present work, attempt was made to characterize indigenous yeast species isolated from fruits to select potential starters for pineapple wine production with the aim of developing wine with long shelf life, desirable sensory attributes coupled with improved nutritional quality and consumer's acceptability.

2. Materials and methods

2.1. Materials

2.1.1. Samples

Samples of pineapple, water melon and cashew nut were collected from Military Cantonment Oshodi in Lagos and Bodija market in Ibadan, Nigeria in sterile ethylene bags and

transported to the Food and Applied Microbiology laboratory, Department of Microbiology University of Ibadan, Nigeria.

2.2. Methods

2.2.1. Treatment of sample and isolation procedure

The barks of the pineapple, water melon and cashew fruits were removed and cut into pieces using a sharp knife. They were allowed to ferment naturally for three days and their juices were extracted using a juice extractor machine. The juices were serially diluted with one ml from dilutions 10^{-4} and 10^{-5} was inoculated into sterile Petri dishes containing malt extract agar and incubated at 30°C for three days. The Petri dishes were observed for microbial growth and representative colonies sub-cultured repeatedly to obtain pure cultures which were maintained on malt extract agar slant at 4°C.

2.2.2. Pathogenicity Test

*2.2.2.1.Gelatin Liquefaction***:**

The method described by dele-Cruz and Torres (2012) was used. One ml from a 24h old culture of the yeast cells suspension was inoculated into sterile gelatin medium in 50mL Erlenmeyer flasks containing 10% malt extract and incubated at 30^0C for 7 days. The temperature was reduced to observe liquefaction. At lower temperature, liquefaction of gelatin indicates positive reaction while un-liquefied gelatin indicates negative reaction.

2.2.2.2. Hemolysis test

This was carried out by streaking a colony from a 24h old culture of the yeast cells on blood agar plates and incubated for 24-72h at 30^0C.The plates were observed for alpha, beta or gamma hemolytic reaction (Akinjogunla *et.al.* 2014).

2.2.2.3. DNase test

The method described by Akinjogunla *et. al.*. (2014). was adopted by picking a colony from a 24h old culture of yeast cells and streaked on DNase agar plates containing methyl green indicator and incubated at 37^0C for 72h. The plate was observed for green color fades surrounded by a colorless zone.

2.3. Identification procedure

Identification of the isolates was carried out by considering their macroscopic and microscopic characteristics (Tika *et al*.(2017) and by employing the API (ID 32C) test kit.

2.4. Determination of invertase production.

Determination of invertase production was carried out by inoculating 1 ml from a 24h old culture of yeast cells suspension into sucrose (4% w/v, 2 ml) in 10ml solution of acetate buffer(pH 5) and incubated for 5 min at 30°C. The amount of reducing sugar released was determined by dinitro-salicylic acid method Tika *et al*.(2017).The amount of enzyme which liberate 1 µ mole reducing sugar per minute was defined as one unit of Invertase activity.

2.5. Determination of ethanol tolerance

This was carried out by inoculating 0.1 ml from a 24h old culture of yeast cells suspension into 10ml sterile malt extract broth containing different concentrations of ethanol (0, 5, 10, 15 and 20%) The tubes were incubated at 30°C for 48 h. and the initial optical densities were read from an UV spectrophotometer (Pharamacia LK13Ultraspec II) set at 560nm. The tubes were transferred into a gyratory shaker incubator set at 150rpm at 30^0C for 24 h and the final optical density was read.

2.6. Determination of sugar tolerance

The modified method described by Shankar *et. al*.(2013) was used by inoculating 0.1 ml from a 24h old culture of the yeast cells suspension into sterile malt extract broth in several 100ml Erlenmeyer flasks containing 10%, 20, 3% and 40%) glucose concentrations and incubation was carried outat 30^0C for 72h. Growth was determined using an UV visible spectrophotometer (Pharamacia LK13Ultraspec II) set at 560nm.The experiment was repeated by replacing glucose with sucrose.

2.7. Determination of growth at different pH

1 ml from a 24h old culture of the yeast cells suspension was into several sterile malt extract broth in 100ml Erlenmeyer flasks with pH

adjusted to 2, 4, 6 and 8 and incubated at 30^0C for 72h h and growth was determined using an UV visible spectrophotometer (Pharamacia LK13Ultraspec II) set at 560nm.

2.8. Determination of growth at different temperatures

One ml from a 24h old culture of the yeast cells suspension was inoculated into sterile malt extract broth in several 100ml Erlenmeyer flasks with pH adjusted to 6. Incubation was carried out at different temperatures (20, 25, 30, 35, 40 and 45) for 72 h and growth was determined by using an UV visible spectrophotometer (Pharamacia LK13Ultraspec II) set at 560nm.

All experiments reported in this study were carried out in triplicates

Statistical analysis

Descriptive statistical method in statistical package for social science (SPSS version17) was used for data analysis to determine means and standard errors.

3. Results and discussions

The screening for pathogenic properties of the yeast isolates is shown in Table 1. It was observed that the eleven isolates tested were negative to gelatin liquefaction, heamolysis and DNase production.

Table 1. Pathogenicity test of yeast isolates

Isolate Code	Gelatin Liquefaction	Haemolytic Test	Dnase Test
WAM 8	_	_	_
WAM 11	_	_	_
WAM 20	_	_	_
WAM 25	_	_	_
PIN 10	_	_	_
PIN 40	_	_	_
PIN 32	_	_	_
PIN 12	_	_	_
CAS 15	_	_	_
CAS 36	_	_	_
CAS 3	_	_	_

Key - WAM-8:Saccharomyces cerevisiae 1,WAM-11:Pichia farinosa,WAM-20: Saccharomyces cerevisiae2,WAM-25:Kloeckera japonica; PIN-10:Saccharomyces kluyveri, PIN–40: Saccharomyces cerevisiae3,PIN-32: Saccharomyces cerevisiae 4, PIN-12:Saccharomyces cerevisiae 5 CAS-15: Pichia ohmeri;CAS-36:Debaryomyces polymorphus;CAS-3: Candida kefyr.

The result of Invertase production by the yeast isolates is presented in Table 2.

All the yeast isolates produced varying amounts of invertase with Isolate PIN32(*Saccharomyces cerevisiae* 4) showing the highest invertase activity of 40.04±0.5Umol/min followed by an activity of 30.17±0.1Umol/min produced by WAM8 (*Saccharomyces cerevisiae* 1) and the least activity of 13.87±0.2Umol/min was recorded in CAS36 (*Debaromyces polymorphus*).

The observed result in the screening of yeast for pathogenicity properties is in conformity with the findings of Eze *et al.*(2011). He previously reported that yeast isolates are not gelatinase producers and the absence of gelatinase, haemolysis and dnase production by these micro-organisms accord them acceptability in the food industry as starter cultures.

Table 2. Invertase production by the yeast isolates

Isolate number	Invertase activity Umol/min
WAM 8	30.17±0.1
WAM 11	18.85±0.1
WAM 20	25.64±0.2
WAM 25	20.51±0.4
PIN 10	28.82±0.3
PIN 40	26.28±0.
PIN 32	40.04±0.5
PIN12	14.66±0.9
CAS 15	20.03±0.7
CAS36	13.87±0.2
CAS3	18.27±0.2

Production of invertase by a wide range of microorganisms such as *Saccharomyces cerevisiae* and *S.carlsbergensis* had been earlier reported (Sivakumar et al.; 2013) Mahesh et al.(2012) reported that *S. cerevisiae* possesses a great ability to secrete invertase while Talekar *et al.* (2010) confirmed that this enzyme is highly significant in the cleavage of α-1,4 glycosidic linkage between α-D-glucose and β-D-fructose molecules of sucrose by hydrolysis and releases monosaccharide. In addition, Guimaraes *et al*. (2007) described the general use of invertase in the production of confectionary with liquid or soft center invert syrup, calf feed preparation, pharmaceutical, food and fermentation of cane molasses into ethanol. The tolerance of the yeasts isolates to different concentration of ethanol is shown in Table 3. The result showed that all the yeasts isolates tolerated the different concentrations (5% -15%) of ethanol used producing different levels of growth with the highest recorded at lower concentration of ethanol. The highest tolerance to ethanol was demonstrated by Isolate PIN32 (*Saccharomyces cerevisiae* 4) by producing a growth of 1.31±0.3 (optical density) followed by a growth of 1.26±0.2 recorded by WAM8 (*Saccharomyces cerevisiae1*) while the least growth of 0.72±0.3 was seen in CAS3 (*Candida kefyr*) at 15% ethanol.

Table 3. Tolerance of yeast isolates to different ethanol concentrations

Isolate code	5%v/v	10%v/v	15%v/v	20%v/v
WAM8	1.74±0.5	1.54±0.5	1.26±0.2	0.00±0.0
WAM11	1.37±0.9	1.15±0.2	1.00±0.3	0.00±0.0
WAM20	1.61±0.1	1.37±0.0	1.21±0.6	0.00±0.0
WAM25	1.44±0.0	1.18±0.9	1.02±0.4	0.00±0.0
PIN10	1.25±0.3	1.14±0.8	0.90±0.0	0.00±0.0
PIN40	1.54±0.3	1.37±0.2	1.11±0.1	0.00±0.0
PIN32	1.83±0.1	1.58±0.3	1.31±0.3	0.00±0.0
PIN12	1.47±0.8	1.04±0.7	0.76±0.0	0.00±0.0
CAS15	1.44 ±0.2	1.05±0.6	0.80±0.0	0.00±0.0
CAS36	1.33±0.6	1.06±0.4	0.81±0.1	0.00±0.0
CAS3	1.37±0.0	1.05±0.1	0.72±0.2	0.00±0.0

The ethanol tolerance of yeast isolates seen in this present work is observed to be species dependent. The yeast species were able to grow in different concentrations of ethanol due to the

ability of their cell wall to withstand osmotic stress (plasmolysis) (Gomar-Alba et al.2015). This observation had earlier been reported by Dash *et al*. (2015); Alloysius *et al*. (2015). However, Osho (2005) reported that the alcoholic tolerance for most brewing yeast strains was within 9-10% ethanol concentration. Studies have shown that ethanol tolerant yeasts are also sugar-tolerant (Balakmar and Arasatnam,, 2012;Techaparin *et al.,*.2017) and the combination of the two properties are important in the selection of yeast species for industrial application especially in fermentative production of ethanol, wine, alcoholic beverages and baking products (Moneke *et al*., 2008;Patruscus *et al*.,2009). Ethanol tolerance is a crucial characteristic of microorganisms involved in the production of alcohol because the process will be inhibited if the fermenting microorganism could not tolerate the alcohol produced (Thammasttirong *et al.,*2013). (Thammasttirong *et al*.,.2013) Albergaria and Arneborg (2016);Alonso del-real *et al*.(2017) explained that the ability of yeast spp. to survive in high alcoholic wine is an indication of their high ethanol tolerance and this characteristics is used in the selection of yeast spp. for industrial ethanol production (Chandasena *et al*., 2006; Patruscus *et al.,*.2009).

Table 4 shows the result of tolerance of yeast isolates to different glucose concentration. All the isolates had maximum growth at 20% glucose but at higher concentration growth decreased. In addition, the highest glucose tolerance was observed in WAM8 (*Saccharomyces cerevisiae1*) recording a growth of 2.46±0.1 followed by PIN32 (*Saccharomyces cerevisiae 4*) with a growth of 2.34±0.1 and the least was observed in WAM25 (*Kloeckera japonica*) with a growth of 2.04±03 in 20% glucose.

Table 4. Tolerance of yeasts isolates to different glucose concentration

Isolates	10%	20%	30%	40%
WAM8	2.11±0.8	2.46±0.1	2.25±0.5	2.16±0.1
WAM11	2.07±0.6	2.20±0.0	2.10±0.6	2.05±0.0
WAM20	2.07±0.6	2.06±0.3	2.00±0.0	1.90±0.5
WAM25	2.01±0.6	2.04±0.3	1.99±0.1	1.83±0.9
PIN10	2.03±0.0	2.06±0.2	1.91±0.2	1.80±0.3
PIN40	2.01±0.1	2.09±0.5	1.81±0.1	1.72±0.4
PIN32	2.09±0.1	2.34±0.1	2..20±0.8	2.10±0.4
PIN12	2.01±0.2	2.18±0.7	2.10±0.0	1.88±0.2
CAS15	2.07±0.5	2.20±0.0	2.00±0.9	1.90±0.6
CAS36	2.06±0.7	2.18±0.6	2.01±0.3	1.70±0.7
CAS3	2.02±0.3	2.07±0.8	1.99±0.1	1.72±0.0

The result of tolerance of the yeast isolates to different sucrose concentration is presented in Table 5. It was observed that PIN32 (*Saccharomyces cerevisiae 4*) recorded the highest growth of 2.39±0.1 followed by a growth of 2.30±0.3 produced by WAM8 (*Saccharomyces cerevisiae1*) while the least growth of 2.10±0.0recorded by WAM11 (*Pichia farinosa)* in 20% sucrose.

Table 5. Tolerance of yeasts isolates to different sucrose concentration

Isolate code	10%	20%	30%	40%
WAM8	2.20±0.3	2.30±0.3	1.83±0.1	1.01±0.7
WAM11	2.02±0.6	2.10±0.2	1.43±0.0	0.89±0.5
WAM20	2.07±0.2	2.14±0.0	1.50±0.6	0..66±0.7

WAM25	2..08±0.7	2.16±0.9	1.53±0.2	0.64±0.6
PIN10	2.10±0.0	2.16±0.4	1.50±0.5	0.59±0.1
PIN40	2.05±0.1	2.11±0.0	1.45±0.1	0.50±0.4
PIN32	217±0.8	2.39±0.1	1.89±0.0	1.18±0.5
PIN12	2.11±0.6	2.28±0.7	1.80±0.2	1.00±0.8
CAS15	2.06±0.6	2.18±0.5	1.60±0.1	0.85±0.3
CAS36	2.08±0.2	2.20±0.1	1.64±0.9	0.93±0.7
CAS3	2.10±0.1	2.16±0.0	1.55±0.3	0.79±0.1

All previously reported observations on sugar tolerance are in agreement with the findings in this work. The ability of the yeast isolate to tolerate sugar concentration is due to the possession of osmotic stress resistant cells caused by dehydration (Homann,2003; Gomar-Alba *et al.*,.2015) They are frequently isolated from high-sugary substrates such as fruits, honey, and jam. (Patruscus *et al.*,.2009; Alakeji *et al.* (2015). Ogunremi *et al.* (2015) and Sulieman *et al*. (2015) confirmed their ability to cause food spoilage. Examples of some sugar tolerant yeast spp are *Candida bombicola; Tolulaspora delbrueckii; Zygosaccharomyces bailli; Zygosaccharomyces rouxii* and it has been reported that they grow in medium containing 40-70% (w/w) sugar .Sugar tolerant yeasts are osmophillic in nature, desirable and are good candidates for production of poly-alcohols thus showing potential for industrial application (Deak, 2006; Turker, 2014) Their sugar-tolerant attribute is exploited in the processing of functional foods (health drinks, enzyme drinks, and fermented vegetable extract) which possess various health-regulating functions. Their immense contribution to the unique functions of these novel foods is partly due to the possession of antibacterial activity (Boirivant and Stober,2007)

The result of growth of yeast isolates at different pH is shown in Table 6. It was observed that all the yeast species grew within the pH range of 2 to 8 showing different levels of growth with optimum growth at pH 6 after which no growth was observed. PIN32 (*Saccharomyces cerevisiae 4*) and WAM8 (*Saccharomyces cerevisiae 1*) recorded the highest growth of 1.55 followed by WAM 20 (*Saccharomyces cerevisiae* 2) producing a growth of 1.47±0.3 and least (0.94±0.1) was recorded by CAS36 (*Debaromyces polymorphus*). Minimum growths were recorded at extreme pH (2 and 8) and all the yeast isolates showed no growth at pH 8. The growth pattern demonstrated by yeast spp. with pH variation had previously been reported by Narendramata and Power (2005) and Deak,(2006). Alakeji *et al.*(2015) reported that mould and yeasts could tolerate range pH between 2-8 and their ability to tolerate low pH (acidic pH) serves as a strategy to eliminate spoilage microbes and create a conducive environment for growth of desirable microorganisms (Boirivant and Stober,2007).

Table 6. Tolerance of yeasts isolate to different pH

Isolate code	**2**	**4**	**6**	**8**
WAM8	0.36±0.2	1.12±0.1	1.55±0.5	0.00±0.0
WAM11	0.34±0.1	1.27±0..6	1.29±0.1	0.00±0.0
WAM20	0.47±0,1	1.23±0.2	1.47±0.3	0.00±0.0
WAM25	0.18±0.0	1.01±0.0	1.18±0.2	0.03±0.0
PIN10	0.34±0.0	1.11±0.3	1.32±0.0	0.00±0.0
PIN40	0.22±0.1	0.94±0.0	1.25±0.7	0.00±0.0
PIN32	0.21±0.0	1.27±0.1	1.55±0.1	0.00±0.0
PIN12	0.26±0.3	1.09±0.5	1.28±0.6	0.00±0.0

CAS15	0.21±0.2	0.71±0.2	0.94±0.4	0.00±0.0
CAS36	0.19±0.1	1.04±0.3	1.31±0.0	0.09±0.0
CAS3	0.17±0.0	1.13±0.7	1.40±0.2	0.00±0.0

Table 7 represents the growth of the yeast isolates. at different temperatures The optimum temperature for growth of all the yeast isolates was 30^{o}C but a sharp decline in growth was observed as the temperature reached 40^{o}C and no growth was recorded at 45^{0}C.The isolates grew optimally within a temperature range of 30-35^{0}C with PIN32 (*Saccharomyces cerevisiae Saccharomyces*4) producing the highest growth of 2.50±0.6 followed by WAM8 (*Saccharomyces cerevisiae* 1) with a growth of 2.45±0.1 and the least 2.15±0.3 was recorded by CAS 36 (*Debaromyces polymorphus*).The growth pattern of yeast isolates obtained at different temperatures in this study is in consonance with the finding of Caspeta *et.al.*(2016) and Taluhder *et al*.(2016) that reported the activity of yeasts within a temperature range of 20-30^{0}C The inability of the yeast species to grow at extreme temperature of 45^{0}C is due to the high stress associated with this temperature (Sathees *et al.*, 2011;Choudhary,2016). The growth of yeast spp. within a temperature range of 20^{0}C-40^{0}C confirmed the vast difference in their thermo stability and maximum growth at 30^{0}C makes the yeast species suitable in fermentation process as this temperature corresponds with the temperature of fermentation which usually takes place within a temperature range of 20-30^{o}C (Keller, 2010).

Table 7. Tolerance of yeasts isolate to different temperatures

Isolate code	**20^{0}C**	**25^{0}C**	**30^{0}C**	**35^{0}C**	**40^{0}C**	**45^{0}C**
WAM 8	1.05±0.4	1.60±0.6	2.45±0.1	2.34±0.2	0.50±0.1	0.00±0.0
WAM 11	1.17±0.9	1.55±0.3	2.29±0.6	2.19±0.3	0.41±0.2	0.00±0.0
WAM 20	1.21±0.5	1.59±0.5	2.21±0.8	2.13±0.2	0.33±0.0	0.00±0.0
WAM 25	1.12±0.4	1.64±0.1	2.34±0.0	2.20±0.1	0.36±0.1	0.00±0.0
PIN 10	1.38±0.2	1.73±0.3	2.16±0.1	2.08±0.0	0.46±0.1	0.00±0.0
PIN 40	1.24±0.2	1.47±0.0	2.18±0.2	2.05±0.5	0.46±0.3	0.00±0.0
PIN 32	1.21±0.5	1.66±0.2	2.50±0.6	2.32±0.2	0.78±0.2	0.00±0.0
PIN 12	1.16±0.1	1.59±0.5	2.11±0.1	2.04±0.3	0.14±0.1	0.00±0.0
CAS 15	0.79±0.4	1.46±0.7	2.16±0.2	2.11±0.3	0.06±0.2	0.00±0.0
CAS 36	1.14±0.0	1.58±0.2	2.15±0.3	2.10±0.8	0.09±0.0	0.00±0.0
CAS 3	1.29±0.3	1.62±0.0	2.19±0.1	2..08±0.2	0.09±0.1	0.00±0.0

Table 8 shows the result of the identification process of the eleven yeast isolates. They were identified as *Saccharomyces cerevesiae* (5)*, Saccharomyces kluyveri* (1)*,* Pichia *farinose* (1)*, Kloeckera japonica*(1)*, Pichia ohmeri* (1) *Debaryomyces polymorphus*(1), *Candida tropicalis* (1)and *Candida kefyr.*(1).

Table 8. Identification result of the Isolates

Isolate code	Source	Number of isolate	Identity
WAM 8	Water melon	1	*Saccharomyces cerevisiae* 1
WAM 11	Water melon	1	*Pichia farinose*
WAM 20	Water melon	1	*Saccharomyces cerevisiae* 2
WAM 25	Water melon	1	*Kloeckera japonica*
PIN 10	Pineapple juice	1	*Saccharomyces kluyverii*
PIN 40	Pineapple juice	1	*Saccharomyces cerevisiae* 3
PIN 32	Pineapple juice	1	*Saccharomyces cerevisiae* 4
PIN 12	Pineapple juice	1	*Pichia ohmeri*
CAS 15	Cashew juice	1	*Saccharomyces cerevisiae* 5
CAS 36	Cashew juice	1	*Debaromyces polymorphus*
CAS 3	Cashew juice	1	*Candida kefyr*

Isolation and identification of yeast from fruits, fermented foods and beverages had earlier been reported by Maragatham and Panneerselvam (2011), Somdatta et. al. (2011) and Zerihun (2016). The involvement of yeasts in different types of indigenous fermented foods and fruits has been documented (Ogunremi et al.,2015). Their existence in fermented foods and fruits is due to their sugar loving characteristics and their fermentative ability (Djelal et al.,2017)

4. Conclusions

Considering the result obtained in this study isolates PIN32 (Saccharomyces cerevisiae 4) and WAM 8 (Saccharomyces cerevisiae 1) may be selected as potential starter cultures for the production of pineapple wine.

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EFFECT OF SOIL NUTRIENT MANAGEMENT AND COOKING METHODS ON NUTRIENT AND PHYTOCHEMICAL COMPOSITION OF *IPOMEABATATAS* (UMUSPO 3 VARIETY)

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ABSTRACT

Findings from the investigations on the effects of different soil nutrient managements and cooking methods on nutrient and phytochemical composition of orange-fleshed sweet potato (Umudike Sweet Potato Variety 3) (UMUSPO 3 Ipomea batatas) are reported in this article. UMUSPO 3 potato vines (main plot) were planted on eight sub- plots treated with different soil nutrients (Poultry manure, agrolyser and NPK) combinations (VB1 – VB8) and a control (VB9) at the National Root Crops Research Institute experimental farm, Umudike, Abia State, Nigeria. Potato tubers were harvested after 4 months of planting and both cooked and raw samples were subjected to chemical and sensory analysis. Carbohydrate and dry matter contents were highest and respectively 16.4% and 29.79% in potatoes harvested from the control soil (VB9). Both control and treated soils yielded potatoes that were high in calcium (86.94 – 96.47 mg/100g), magnesium (73.62 - 86.87 mg/100g), phosphorus (151.26 - 195.97 mg/100g), potassium (790.30 – 901.54 mg/100g) and sodium (74.50 – 81.84 mg/100g). Only soil treatments with NPK 15:15:15 at 400 kg/ha (VB5) improved protein contents of raw potatoes from 5.26% to 6.13% while other soil treatments decreased the protein content. Potato harvested from the control and various soil treatments (VB1 –VB8) were rich in vitamin C (53.34 – 95.37 mg/100 g) and carotene (28.52 – 29.73 mg/100 g) but relatively low in the B vitamins. Soil treatments VB1 to VB8 did not improve vitamin contents of potatoes beyond that of the control for vitamin B1. Cooking methods and cooking time affected carotenoid retention in UMUSPO 3 potato. Carotenoid retention was higher in oven-dried samples than in roasted and boiled samples. Oven-drying for 24h decreased total carotenoid retention to 76.4%, and for 48h to 36.3%. Boiling for 10 min decreased carotenoid content to 56.4%, and for 30 min to 17.1%. Roasting for 10 min reduced it to 50.6%, and for 30 min to 30.9%.

1.INTRODUCTION

Sweet potatoes (*Ipomeabatata*(L.) LAM.),arefamine crops cultivated throughout the tropics and warm temperate regions of the world for their starchy roots (Mitra 2012). Potatoes are good sources of carbohydrates, lipids, proteins, vitamins, and minerals. The intensity of the orange-fleshed colour is directly correlated to the beta-carotene content of orange-fleshed sweet potato (OFSP).

Sweet potato is grown to a lesser extent in West Africa where it is priced by the region's poor farmers. In West Africa, it is valued as a reliable, low input, food security crop, for its commercial potential. Sweet potato requires moderate temperature (21 – 26^{O}C) and a well distributed rainfall of 75 – 150 cm. The crop

requires a lot of sunshine, tolerates drought to some extent but cannot withstand water logging (Nedunchezhiyan*et al*., 2007). Sandy loam with clay subsoil is the ideal. Heavier, clayey soils restrict development of the storage roots as a result of compacted lumps of soil (Nedunchezhiyan*et al*., 2012). Sandy soil encourages the development of long cylindrical pencil-like roots (Nedunchezhiyan*et al*., 2007). Thus, soil types, rainfall distribution and soil management practices influence yield and composition of sweet potatoes. Also, processing and cooking methods influence final composition and sensory properties of foods including sweet potatoes (Yang and Gradi, 2008; Chukwu*et al*, 2010). Potato can be boiled, fried or grilled, steamed, braised, baked and roasted (Abidin, 2004). Two most common ways of consuming potato in Nigeria are boiling in water and frying in refined vegetable oil or palm oil.This study investigated the effect of different soil nutrient management and cooking methods on the nutrient composition, phytochemical content and retention of carotenoids in an orange-fleshed sweet potato (UMUSPO 3 *Ipomeabatatas*).Results for detailed carotenoid profile and constituents of other OFSP varieties have been reported in literature (Ojimelukwe and Okpanku, 2020). Figure 1 shows the UMUSPO3 Orange-fleshed sweet potato variety.

Figure 1. UMUSPO3 Orange-fleshed sweet potato variety

2.MATERIALS AND METHODS

2.1.Raw Materials

The raw materials were poultry manure obtained from the poultry farm of Michael Okpara University of Agriculture, Umudike, Nigeria, artificial nitrogen fertilizer (NPK) obtained from the Abia State Agricultural Development Programme, and orange fleshed sweet potato (Umudike sweet potato variety 3 (UMUSPO 3***Ipomeabatatas*variety**) vine cuttings obtained from a students' research farm at the National Root Crops Research Institute, Umudike, Abia State, Nigeria.

2.2.Field Work Experimental Design and Treatments

The UMUSPO 3 sweet potato variety was planted at the National Root Crops Research Institute experimental farm, Umudike, Abia State, Nigeria situated between latitude 05°29N; longitude 07°33 E and an altitude of 122m. The sandy loam soil used for planting contained 78.8% sand, 6.8% silt, 14.4% clay, pH 4.7, 1.59% organic matter, 0.08% Nitrogen (N), 32.1 mg/kg Phosphorus (P) and 0.35 mg/kg, Potassium (K). (Ukom et al., 2011). The treatment wasasplit plot in randomized complete block design with three replications. Main plot was UMUSPO 3 Orange-Fleshed Sweet Potato (OFSP) while sub-plots were eight nutrient combinations and a control, viz: VB1 = poultry manure at 5.0 x 10^3 kg/ha; VB2 = Poultry manure at 2.5 x 10^3kg/ha + NPK 15:15:15 at 200 kg/ha; VB3 = Agrolyser 2.7 kg/ha + NPK at 200 kg/ha; VB4 = Poultry manure at 5.0 x 10^3kg/ha + NPK at 200 kg/ha; VB5 = NPK 15:15:15 at 400 kg/ha VB6 = Agrolyzer at 5.3 kg/ha; VB7 = Poultry manure at 10.0 x 10^3 kg/ha; VB8 = Poultry manure at 2.5 x 10^3kg/ha + NPK at 200 kg/ha + Agrolyser at 2.7 kg/ha; VB9 = Control (untreated soil).

(Agrolyser contains: Ca – 20.4%; Na – 1.04%; Zn – 0.11%, S – 2.72%; Fe, Mn, Mo trace).The poultry manure had pH 7.06, N 2.17%, P 1.06% and K 0.62%, and was applied into appropriate plots after ridging while NPK were applied by band placement 4 weeks after planting. Each of the sub-plots measured 3 m x 2 m (6 m^2). Sweet potato vine cuttings of 20 cm length with at least 4 nodes were planted along the crest of the ridges. Replacement of vacant stands was done at 2 weeks after planting. Potato roots were harvested after 4 months of planting. The raw samples were subjected to chemical analysis while the processed (boiled, oven-dried and roasted) samples were subjected to carotenoid analysis only.

2.3.Methods

2.3.1.Determination of Proximate composition of UMUSPO 3 sweet potato roots

Moisture, crude protein (% N x6.25), fat, fibre and ash contents of the potato samples were determined in triplicates using standard procedures of the Association of Official Analytical Chemist (AOAC, 2000).

2.3.2.Mineral Content Determination

Mineral contents were determined using the method of AOAC (2000). Milled dry potato samples (2.0 g) of 0.5 mm size using a milling machine (Superintermentmilling machine, Model No: 242, Japan) was mixed with 5 ml of a digestion mixture (selenium + H_2SO_4 + salicylic acid solution) in 150-ml conical flask, allowed to stand overnight (12 h) and then digested at 30°C for 2 h. Concentrated H_2SO_4 (5 ml) was added, and the mixture heated more vigorously at higher temperature until completely digested, releasing profuse fumes. The digest was cooled, transferred into a 50 ml volumetric flask and made up to the mark with distilled water. This was used for the determinations. Calcium (Ca) and magnesium (Mg) contents were determined by the EDTA versanatecomplexometric titration method; potassium (K) and sodium (Na) contents by the flame photometric method while phosphorus (P) was determined by the Vanado-molybdate reagent method using spectrophotometer.

2.3.3.Vitamin Determination

2.3.3.1.Determination of pro-vitamin A

Pro-vitamin A (β-carotene) content was determined using method of the Association of Vitamin Chemist as described by Kirk and Sawyer (1998). Milled dried potato (5 g) was mixed with 30 ml of absolute ethanol and 3 ml of 50% KOH solution; boiled under reflux for 30 minutes, cooled rapidly, and 30 ml of distilled water added. The mixture was carefully washed thrice, each with 50 ml of ether into a separating funnel after which the upper aqueous layer was discarded. The lower oily layer was again washed thrice, each with 50 ml distilled water but with caution to avoid formation of emulsion. The washed extract (Vitamin A) was evaporated to near dryness over a steam bath and then allowed to dry completely at room temperature (25˚C - 27˚C) in a stream of air. It was dissolved in 10 ml of isopropyl alcohol and its absorbance read in a spectrophotometer (72 series VIS/UV, England) at 325 nm with a reagent blank set at zero. Also, a standard vitamin A (retinol) was dissolved in isopropyl alcohol and its absorbance read at the same wavelength as the sample. Vitamin A content was calculated as follows:

$$\textbf{Vitamin A}\left(\frac{\textbf{mg}}{\textbf{100}}\textbf{g}\right) = \frac{(\textbf{100}\ x\ Au\ x\ C)}{W\ x\ As}, \quad (1)$$

Where W = Weight of sample, Au = Absorbance of sample, As = Absorbance of standard, C = Concentration.

2.3.3.2.Determination of vitamin C (ascorbic Acid)

Ascorbic acid contentwas determined using Basket titration method described by Okwu and Ndu (2006). A ground quantity (5 g) of the sample was thoroughly mixed with 50 ml of distilled water, allowed to stand for 30 min at room temperature (25˚C - 27˚C), shaken and then filtered through What man No. 42 filter paper. The filtrate (extract) was treated with 20 ml of 10% potassium iodide solution followed by titration with Copper sulphate ($CuSO_4$) solution. Vitamin C content was calculated as follow: Vitamin C (mg/100) =

$$\textbf{Vitamin C (mg per 100g)} = \frac{\textbf{100}}{\textbf{W}}\ \textbf{x 0.88mg x Titre}, \quad (2)$$

Where W = Weight of sample analyzed and 1 ml of 0.01 m $CuSO_4$ solution = 0.88 mg vitamin C.

2.3.3.3.Determination of vitamin B_1 (Thiamine)

Thiamine was determined according to method of Jorg Augustin as slightly modified by Okwu and Ndu, (2006). Five (5) g of sample was homogenized in normal ethanolic Sodium hydroxide solution, filtered through What man No. 42 filter paper, and an aliquot (10 ml) of the filtrate mixed with 10 ml of 0.1 N potassium dichromate solution. Also, 10 ml of standard thiamine solution was treated with 10 ml of 0.1N Potassium dichromate solution as well as with a

reagent blank. The absorbance was read spectrophotometrically at 36 nm (72 series VIS/UV, England), and thiamine content calculated thus:

$$\text{Thiamine mg/100 g} = \frac{100}{W} x \frac{Au}{As} x\ C\ x \frac{Vf}{Va},, \quad (3)$$

Where: W = Weight of sample analyzed, Au = Absorbance of sample, As = Absorbance of standard , C = Concentration 1µ/ml.

2.3.3.4.Determination of vitamin B_2 (riboflavin)

Riboflavin was determined calorimetrically using the method of Jorg Augustin as described by Okwu and Ndu (2006). Five grams (5 g) of ground dry Potato sample (5) was extracted with 50 ml of 50% ethanol with intermittent shaking for 1 hour. This was filtered through what man No. 42 filter paper, and 10 ml of the filtrate mixed with 10 ml each of 5% $KMnO_4$ solution and 30% Hydrogen peroxide solution. The mixture was maintained on a hot water bath for 30 minutes, and thereafter, 20 ml of 40% Sodium sulphate solution was added, and the mixture made up to 50 ml with distilled water. The absorbance was measured spectrophotometrically at 520 nm. A standard riboflavin solution was prepared similarly, the absorbance measured similarly, and vitamin B_2 content calculated thus:

$$\text{Riboflavin mg/100 g} = \frac{100}{W} \text{ x Au/As x C x Vf/Va}, \quad (4)$$

Where w = weight of sample analyzed, Au = Absorbance of sample, As = Absorbance of standard, C = Concentration of standard (mg/1ml), Vf = Total volume of extract, and Va = Volume of extract analyzed.

2.3.3.5.Determination of vitamin B_3 (niacin)

Niacin content was determined by the Skater colorimetric method as described by Okwu (2004). Five grams (5 g) of ground dried potato was mixed with 30 ml of normal H_2SO_4 solution, shaken for 30 minutes and filtered through Whatman No. 42 filter paper. The extract was made alkaline by adding 3 drops of conc. NH_4OH, and 10 ml of extract treated with 5 ml of normal Potassium ferrocynanide solution, followed by 5 ml of 0.02 NH_2SO_4 solution. This was allowed to stand for 5 min at room temperature (25˚C - 27˚C) after which absorbance was read at 470 nm. Niacin content was calculated thus:

$$\text{Niacin (mg/100 g)} = \frac{100}{W} \text{ x Au/As x C x Vf/Va}, \quad (5)$$

Where: W = Weight of sample analyzed, Au = Absorbance of sample, As = Absorbance of standard, C = Concentration 1µ/ml, Vf = Total volume of extract, and Va = Volume of extract analysed.

2.4.Determination of Phytochemical Constituents

2.4.1.Determination of tannin content

The vanillin–HClmethod described by Niseteo *et al.*(2012) was used. Ground dry potato sample (1 g) was mixed vigorously with 3ml of methanol at room temperature (28± 2ºC) and rested for 1 min to extract tannin. This was filtered, and 3 ml of the extract reacted with 3 ml each of 0.1mol NH_4Cl and 0.08 mol $k_3Fe(CN)_6$for 2 min in a test tube. Absorbance was read at 720nm, and tannin content extrapolated from a standard curve previously prepared with catechin as tannin standard. The process was repeated in triplicates and the average value expressed in mg/100g tannin content

2.4.2.Alkaloid Determination

Five grams of the sample were weighed into a 250 ml beaker and 200 ml of 20% acetic acid in ethanol was added and covered to stand for 4 hours. This was filtered and the extract was concentrated using a water-bath to one quarter of the original volume. Concentrated Ammonium hydroxide was added drop-wise to the extract until precipitation was complete. The whole solution was allowed to settle and the precipitate was collected by filtration, dried and weighed (Harborne, 1973).

2.4.3.Determination of total flavonoid Content

The Iron (iii) Chloride ($AlCl_3$) method of AOAC(2000) was adopted.Ten gram of potato

powder (10 g) was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature and filtered through what man filter paper No 42 (125 mm). Aliquot(1.5 ml) of filtrate was mixed with 1.5 ml of a 2% $AlCl_3.6H_20$ (2g in 100ml methanol) solution, shaken and incubated for 10 min at room temperature. Absorbance was read at 367 nm after incubating for 10 min at room temperature in the dark. A standard curve for flavonoid was prepared from 0.0, 0.02, 0.05, 0.10, 0.50, 1.50 and 2.0 mg/ml garlic acid concentrations at367 nm and flavonoid content (expressed as garlic acid equivalents (GAE/100g) extrapolated from the curve.

2.4.4.Determination of oxalate content:

Oxalate content was determined as described by Oke (1966). One gram of potato powder (W1) was mixed with 190 ml of de-ionized water and 10 ml of 6M HCl; and then digested at 90 ^{O}C for 4 h. This was centrifuged at 2000 rpm for 5 min; the supernatant diluted with distilled water to 250 ml and titrated with concentrated Ammonium hydroxide solution to faint yellow colour end point, using methyl orange indicator.The resultant mixture was heated (90 ^{O}C) for 20 min and 10ml of 5 % Calcium chloride ($CaCl_2$) solution added to precipitate Calcium oxalate. This was rested overnight, centrifuged, decanted and the residue oven-dried at 60 ^{O}C for 48 h. This was cooled and weighed (W2). This was repeated three times and the mean weight expressed as:

$$\% \, Oxalate \; content = \frac{W2}{W1} x \frac{100}{1} \quad (5)$$

2.5.Data Analysis

Data obtained were subjected to descriptive test and one-way analysis of variance used to determine level of significance, using the SPSS software version 17. Means where significantly different at $p<0.05$ were separated using Duncan Multiple Range Test (DMRT).

3.RESULTS AND DISCUSSION

3.1.Proximate Composition of Orange Fleshed Potato (UMUSPO 3 *Ipomea Batatas* Variety) Grown under Different Soil Nutrient Management treatments

Table 1 shows the proximate composition of *Ipomea batatas*(variety: UMUSPO 3) cultivated on soils with different nutrient treatments. The moisture content ranged from 70.21 g/100 g in potatoes planted onVB9 (untreated plot) andon VB8(treated with poultry manure at 2.5 x 10^3 kg/ha plus NPK at 200 kg/ha plus Agrolyser at 2.7 kg/ha)to 76.08 g/100 g in potatoes planted on VB2(treated with poultry manure at 2.5 x 10^3 kg/ha plus NPK at 200 kg/ha).Moisture content increased with soil nutrient treatments, implying that the crops will be more prone to post-harvest biochemical activities and may have shorter shelf life on storage.

It was only soil nutrient treatment VB5(NPK 15:15:15 at 400 kg/ha) that improved protein content from 5.26% to 6.13% in OFSP potatoes. Treatments VB3 and VB6 did not significantly ($p> 0.05$) affect protein content of potatoes. Other treatments decreased this nutrient from 5.26% to 4.37% through 3.39% protein contents. The 3.49 g/100 g – 6.13 g/100 g protein contents in this work was comparable with the values reported by Ukom et al (2009), but higher than the 1.2% to2.3% reported by Onuh*et al*. (2004) on harvested potatoes. Carbohydrate content increased from 16.40% in the control to 19.04% in VB8 (potatoes grown on Poultry manure at 2.5 x 10^3kg/ha + NPK at 200 kg/ha + Agrolyser at 2.7 kg/ha). Also, dry matter content (29.79%) was also decreased by all the soil nutrient treatments, except in treatment VB8where it was not ($p >0.05$) significantly affected. Crude fibre contents of the control (VB9) and VB3, were not significantly different ($p>0.05$) from one another. Soil nutrient treatments VB4 to VB6; VB7 and VB8 decreased crude fibre content of potatoes which ranged from 3.03% to 5.05% (but the values were not significantly different ($p>0.05$). The dry matter content was relatively lower for other treatments, except for VB6,(1.71%). Crude fat decreased from 1.68% in potatoes fromVB9, (the control), to as low as 1.51% in potatoes from treated soils. Soil

nutrient management treatments decreased the fat content of sweet potatoes (in comparison with the control) except for those grown on Agrolyzer at 5.3kg/ha (VB6).

Table 1. Effect of different soil nutrient management on the proximate composition (g/100 g) of UMUSPO 3 *Ipomeabatatas*Variety

Treatments	Moisture	Ash	Crude Fat	Crude Fibre	Protein	Carbohydrate	Dry Matter
VB1	73.10^{d}±0.14	2.07^{a}±0.04	1.56^{d}±0.01	3.09^{c}±0.01	4.37^{c}±0.01	15.82^{c}±0.23	26.90^{d}±0.14
VB2	76.08^{a}±0.03	1.2±0.02	1.64^{c}±0.00	3.06^{c}±0.02	4.37^{c} ±0.01	13.65^{g}±0.02	23.92^{b}±0.03
VB3	72.09^{e}±0.01	0.99^{e}±0.00	1.66^{bc} ±0.01	5.09^{a} ±0.02	5.25^{b} ±0.01	14.93^{e} ±0.03	27.91^{c} ±0.01
VB4	74.78^{b}±0.03	0.99^{e}±0.01	1.52^{e} ±0.00	4.06^{b}±0.02	3.49^{d}±0.02	15.18^{d}±0.05	25.22^{g}±0.03
VB5	71.09^{f}±0.02	1.66^{b}±0.00	1.55^{d}±0.01	3.03^{c}±0.01	6.13^{a}±0.01	16.55^{b}±0.01	28.92^{b}±0.02
VB6	73.08^{d}±0.01	0.81^{f}±0.01	1.71^{a}±0.00	4.05^{b}±0.07	5.26^{b}±0.00	14.37^{f}±0.06	26.19^{e}±0.01
VB7	74.41^{c}±0.01	0.80^{f} ±0.00	1.55^{d} ±0.01	3.98^{b} ±0.00	4.38^{c} ±0.00	14.89^{e} ±0.00	25.59^{f}±0.01
VB8	70.21^{g}±0.01	0.81^{f}±0.01	1.51^{e}±0.01	4.07^{b}±0.04	4.37^{c}±0.00	19.04^{a}±0.06	29.79^{a}±0.01
VB9	70.21^{g} ±0.01	1.41^{c} ±0.01	1.68^{ab} ±0.01	5.05^{a} ±0.04	5.26^{b} ±0.01	16.40^{b} ±0.04	29.79^{a} ±0.01

Values are means ± SD. means with different superscripts in the same column are significantly different ($p<0.05$), VB1 = poultry manure at 5.0 x 10^3 kg/ha; VB2 = Poultry manure at 2.5 x 10^3kg/ha + NPK 15:15:15 at 200 kg/ha; VB3 = Agrolyser 2.7 kg/ha + NPK at 200 kg/ha; VB4 = Poultry manure at 5.0 x 10^3kg/ha + NPK at 200 kg/ha; VB5 = NPK 15:15:15 at 400 kg/ha; VB6 = Agrolyzer at 5.3 kg/ha; VB7 = Poultry manure at 10.0 x 10^3 kg/ha; VB8 = Poultry manure at 2.5 x 10^3kg/ha + NPK at 200 kg/ha + Agrolyser at 2.7 kg/ha; VB9 = Control

3.2.Effect of Different Soil Nutrient Management Treatments on Mineral Content of UMUSPO 3 *IpomeaBatatas*Variety (FW) (Mg/100 G)

Table 2 shows mineral composition potatoes samples (UMUSPO 3*Ipomeabatatas*) cultivated on soils given different nutrient managements. All the potato samples, including those harvested from the control, untreated soil (VB9) were rich in calcium (86.94 – 96.47 mg/100g), magnesium (73.62 - 86.87 mg/100g), phosphorus (151.26 - 195.97 mg/100g), potassium (790.30 – 901.54 mg/100g) and sodium (74.50 – 81.84 mg/100g). Soil nutrient treatments significantly ($p <0.05$) improved phosphorus contents of potatoes; this ranged from 151.26 mg/100g in the control potatoes to 152.87 mg/100g through 159.97 mg/100g in potatoes from the treated soils. Magnesium and sodium contents (mg/100g) significantly ($p < 0.05$) decreased with soil nutrient management. Treatment VB7 produced potatoes with more balanced minerals for human health. The approximate 11: 1 ratio of potassium to sodium in this UMUSPO 3*Ipomeabatatas,*is geometrically higher than the minimum 2: 1 recommended for hypertensive-free diets supports its use by hypertensive patients.

Calcium contributes in normal bone and teeth development, blood clotting and enzymic function (Gropper *et al.*, 2005; Ojimelukwe*et al.*, 2005). Magnesium is needed for heart and muscles functioning, maintenance of blood pressure and myocardial contraction (Umesh, 2009). Phosphorus helps bone and teeth development, sugar phosphate is involved in carbohydrate and fat metabolism; and helps in cell activity and maintenance of pH in the body (Gropper *et al.*, 2005; Ojimelukwe*et al.*, 2005). VB3 gave the best improvement of the mineral content amongst all the treatments.

Table 2. Effect of different soil nutrient management treatments on mineral content of UMUSPO 3*Ipomeabatatas*variety (mg/100 g)

Treatment	Calcium	Magnesium	Phosphorus	Potassium	Sodium
VB1	94.95^{abc}±0.03	73.62^{b}±1.90	152.87^{ef}±2.28	901.54^{a}±1.54	74.50^{d}±1.04
VB2	93.46^{bc}±0.30	75.43^{b}±0.27	159.05^{ab}±0.62	863.61^{b}±1.49	80.93^{ab}±0.00
VB3	96.47^{a} ±1.12	75.03^{b}±0.10	156.63^{bcd} ±1.00	797.34^{c} ±1.57	77.81^{bcd} ±2.34
VB4	95.43^{ab} ±1.82	76.52^{b}±0.28	159.57^{a} ±0.23	867.06^{b} ±1.90	81.98^{a} ±2.66
VB5	93.10^{c}±0.85	73.49^{b}±0.34	155.22^{cde}±1.29	785.72^{d}±0.62	77.28^{cd}±1.16
VB6	94.85^{abc}±0.47	74.73^{b}±0.89	157.46^{abc}±0.23	790.30^{cd}±1.54	79.78^{abc}±0.13
VB7	93.77^{bc} ±0.74	85.18^{a} ±1.45	159.97^{a} ±0.24	896.31^{a} ±1.65	75.63^{cd} ±0.72
VB8	86.94^{d}±1.03	86.87^{a}±3.69	154.61^{de}±0.30	791.25^{cd}±7.35	77.35^{cd}±1.08
VB9	93.91^{bc} ±0.16	85.22^{a} ±0.41	151.26^{f} ±1.25	798.64^{c} ±0.27	80.03^{abc} ±0.29

Values are means ± SD. means with different superscripts in the same column are significantly different ($p<0.05$), VB1 = poultry manure at 5.0 x 10^3 kg/ha; VB2 = Poultry manure at 2.5 x 10^3kg/ha + NPK 15:15:15 at 200 kg/ha; VB3 = Agrolyser 2.7 kg/ha + NPK at 200 kg/ha; VB4 = Poultry manure at 5.0 x 10^3kg/ha + NPK at 200 kg/ha; VB5 = NPK 15:15:15 at 400 kg/ha; VB6 = Agrolyzer at 5.3 kg/ha; VB7 = Poultry manure at 10.0 x 10^3 kg/ha; VB8 = Poultry manure at 2.5 x 10^3kg/ha + NPK at 200 kg/ha + Agrolyser at 2.7 kg/ha; VB9 = Control

3.3.Vitamin Content of UMUSPO 3 *Ipomeabatatas*Variety Cultivated under Different Soil Nutrient Management Treatments

Table 3 show vitamin composition of nine (9) potato (*Ipomeabatatas*UMUSPO 3) samples grown on untreated soil and eight soils samples with different soil nutrient treatments.

Soil nutrient management did not significantly ($p > 0.05$) improve vitamin B_1(Thiamine) and pro-vitamin A contents. Vitamin B_1 was 0.29 mg/100g in both the control, VB9 and the treated samples, VB2 – VB6 and there were no significant differences between these samples and values for other samples. However, values of thiamine content reported in this study are higher than 0.08 mg/100 g reported by USDA (2001) National Database on sweet potato. Soil nutrient management, improved vitamin B_3(niacin) contents of the potatoes. Pro-vitamin A (Beta-carotene) ranged from 28.52-29.53 mg/100g in the potato samples. On the other hand, vitamin B_3 content increased from 1.01mg/100g in potatoes from the control soil, VB9, to 1.42 mg/100g through 1.94 mg/100g in potatoes from treated soils. Also, vitamin B_2(Riboflavin) was poor in all the potato samples harvested from soils with different nutrient treatment. It remained0.09 mg/100g in potatoes harvested from VB9 (the control), VB1 and VB2 but improved to 0.10 mg/100g in potatoes harvested from VB8 (treated with Poultry manure at 2.5 x 10^3kg/ha + NPK at 200 kg/ha + Agrolyser at 2.7 kg/ha) and 0.11mg/100g in VB3 (soil, treated with Agrolyser 2.7 kg/ha + NPK at 200 kg/ha). Also, only nutrient treatment VB4 (Poultry manure at 5.0 x 10^3 kg/ha + NPK at 200 kg/ha) significantly ($p < 0.05$) improved vitamin C (ascorbic acid) content of potatoes from 84.55 mg/100g in the control to 95.37 mg/100g. Other treatments either did not or decreased its content in the potatoes. UMUSPO 3 *Ipomeabatatas* is an excellent source of vitamin C. Vitamin C boosts immune systems, improves iron absorption, neutralizes blood toxins, and helps in maintaining epithelial cells and tissues. It promotes healthy gums, healing of wound and acts as natural antioxidant (Ojimelukwe*et al.*, 2005; Umesh, 2009). UMUSPO 3 *Ipomeabatatas* is also a rich source of vitamin B_3, pro-vitamin A and vitamin C but poor in vitamins B_1 and B_2. Niacin (vitamin B_3) is necessary for glycolysis, tissue respiration and fat synthesis (Gropper *et al.*, 2005). Beta-carotene is essential for good vision, differentiation of epithelial issues, proper immune functions, healthy skin; hair and nails

(Gropper *et al.*, 2005; Ojimelukwe*et al.*, 2005; Umesh, 2009). The soil nutrient treatments did not improve the pro-vitamin A content of the samples.

Table 3. Effect of different nutrient management treatments on vitamin Content mg/100 g of UMUSPO 3 *Ipomeabatatas*variety

Treatment	Vitamin B_1	Vitamin B_2	Vitamin B_3	Pro-vitamin A	Vitamin C
VB1	0.24^{a}±0.02	0.09^{bc}±0.00	1.94^{a}±0.00	28.52^{c}±0.12	73.79^{c}±0.18
VB2	0.29^{a}±0.00	0.09^{bc}±0.01	1.42^{e}±0.02	29.45^{ab}±0.26	84.53^{b}±0.16
VB3	0.28^{a}±0.01	0.11^{a}±0.01	1.47^{e}±0.01	29.53^{a}±0.11	63.34^{d}±0.09
VB4	0.28^{a}±0.00	0.06^{d}±0.00	1.93^{a}±0.00	29.33^{a}±0.16	95.37^{a} ±0.01
VB5	0.28^{a}±0.00	0.08^{cd}±0.00	1.54^{d}±0.00	29.35^{b}±0.13	84.53^{h}±0.16
VB6	0.29^{a}±0.00	0.08^{cd}±0.00	1.63^{c}±0.00	29.43^{ab}±0.42	58.33^{d}±7.16
VB7	0.28^{a}±0.00	0.07^{d}±0.00	1.72^{b} ±0.00	29.44^{ab} ±0.04	84.52^{b} ±0.17
VB8	0.28^{a}±0.02	0.10^{a}±0.00	1.73^{b}±0.12	29.73^{a}±0.4	84.45^{b}±0.04
VB9	0.29^{a}±0.05	0.09^{bc} ±0.00	1.01^{f} ±1.42	29.53^{a}± 0.12	84.55^{b} ±0.10

Values are means ± SD. Means with different superscripts in the same column are significantly different ($p<0.05$),; VB1 = poultry manure at 5.0 x 10^3 kg/ha; VB2 = Poultry manure at 2.5 x 10^3kg/ha + NPK 15:15:15 at 200 kg/ha; VB3 = Agrolyser 2.7 kg/ha + NPK at 200 kg/ha; VB4 = Poultry manure at 5.0 x 10^3 kg/ha + NPK at 200 kg/ha;VB5 = NPK 15:15:15 at 400 kg/ha; VB6 = Agrolyzer at 5.3 kg/ha; VB7 = Poultry manure at 10.0 x 10^3kg/ha ;VB8 = Poultry manure at 2.5 x 10^3kg/ha + NPK at 200 kg/ha + Agrolyser at 2.7 kg/ha; VB9 = Control

3.4.Phytochemical composition of UMUSPO 3 *Ipomeabatatas*variety cultivated under different soil nutrient management treatments

Table 4 shows phytochemical (alkaloid, flavonoid, tannin and oxalate) contents of*Ipomeabatatas*(variety: UMUSPO 3) cultivated under different soil nutrient management treatments. There was no significant differences ($p>0.05$) in oxalate contents of UMUSPO 3 OFSP given different soil management treatments. The alkaloid content was highest for VB8 (Poultry manure at 2.5 x 10^3kg/ha + NPK at 200 kg/ha + Agrolyser at 2.7 kg/ha), but its tannin content was one of the lowest and only higher than VB1 (poultry manure at 5.0 x 10^3 kg/ha) Flavonoid, tannin and oxalate were very low in all the potato sample, and ranged from 0.81 mg/100g to 0.04 mg/100g in the potato samples. However, soil nutrient management significantly reduced oxalate content of potatoes from 1.22mg/100g in the control sample to as low as 0.60 in samples harvested from soil treated with Poultry manure at 2.5 x 10^3kg/ha + NPK 15:15:15 at 200 kg/ha (VB2). Flavonoids are secondary metabolites with health benefits and potentials for the prevention of carcinogenesis, cardiovascular diseases, diabetes, inflammation, arteriosclerosis and aging. They act as free radical scavengers (Slimetad and Verhel, 2009; Ukom*et al.*, 2014).Shekhar et al. (2015) as well as Anthoney and Owenga (2014) observed variations in the phytochemical contents of sweet potatoes.

Table 4. Effect of different nutrient management treatments on phytochemicalcomposition mg/100 g of UMUSPO 3 *Ipomeabatatas*variety

Treatment	Alkaloid	Flavonoid	Tannin	Oxalate
VB1	0.63^{e}±0.04	0.22^{c}±0.28	0.24^{c}±0.01	0.05^{a}±0.04
VB2	0.60^{e} ±0.01	0.42^{b}±0.03	0.43^{a}±0.03	0.05a ±0.01
VB3	0.84^{d} ±0.05	0.21^{c} ±0.01	0.43^{a}±0.01	0.04^{a}±0.02
VB4	1.10^{bc} ±0.01	0.44^{b} ±0.06	0.42^{a}±0.03	0.05^{a}±0.01
VB5	0.62^{e}±0.03	0.24^{c}±0.57	0.45^{a}±0.00	0.04^{a}±0.03
VB6	1.00^{c}±0.00	0.81^{a}±0.01	0.43^{a}±0.04	0.04^{a}±0.03
VB9	1.22^{b} ±0.03	0.40^{b} ±0.00	0.42^{a} ±0.00	0.04^{a} ±0.00
VB7	1.23^{b} ±0.04	0.43^{b} ±0.00	0.43^{a} ±0.00	0.06^{a} ±0.02
VB8	1.46^{a}±0.09	0.43^{b}±0.04	0.32^{b}±0.00	0.08^{a}±0.04
VB9	1.22^{b} ±0.03	0.40^{b} ±0.00	0.42^{a} ±0.00	0.04^{a} ±0.03

Values are mean ± SD. Mean values with different superscripts in the column are significantly different ($p<0.05$)., VB1 = poultry manure at 5.0 x 10^3 kg/ha; VB2 = Poultry manure at 2.5 x 10^3kg/ha + NPK 15:15:15 at 200 kg/ha; VB3 = Agrolyser 2.7 kg/ha + NPK at 200 kg/ha; VB4 = Poultry manure at 5.0 x 10^3kg/ha + NPK at 200 kg/ha; VB5 = NPK 15:15:15 at 400 kg/ha; VB6 = Agrolyzer at 5.3 kg/ha; VB7 = Poultry manure at 10.0 x 10^3kg/ha; VB8 = Poultry manure at 2.5 x 10^3kg/ha + NPK at 200 kg/ha + Agrolyser at 2.7 kg/ha; VB9 = Control

3.5.Effect of cooking methods on beta-carotene (µg/g) retention in UMUSPO 3 *Ipomeabatatas*variety cultivated under different soil nutrient management treatments

Table 5 shows the effects of cooking methods (oven-drying, roasting and boiling) on total carotenoid retention of *Ipomeabatatas* (UMUSPO 3). Oven-drying, roasting and boiling significantly ($p < 0.05$) affected total carotenoid retention. Carotenoid loss increased with increasing cooking time. Oven-drying for 24 h reduced carotenoid content to76.4%, and for 48 h reduced the content to 36.9%. Roasting for 10 min reduced total carotenoid content to 50.6%, and for 30 min to 20% retention. On the other hand, boiling for 10 min reduced total carotenoid content to 56.4% while boiling for 30 min reduced total carotenoid content to 17.1%. The longer time used in oven-drying was as a result of the low temperature regiment of ≤ 70^{O}C generally employed in drying agricultural products to retain its functional properties. Effects of various traditional processing methods on carotenoids content of sweet potato have also been reported by Ukom and Ojimelukwe (2016);Bengtson*et al*. (2008), among others. The difference in carotenoid retention in boiled sweet potato (85 – 90%) (Vimala*et al*, 2011) and highest values obtained in this work 56.4% (UMUSPO 3) may be attributed to differences in cooking conditions, time and temperature (Timalsina et al., 2019). Roasting has been considered a good carotenoid retention method because roasting reduces moisture and concentrates the carotenoid per weight of the food (Ukom*et al.*, 2011).Pre-drying, frying temperature and frying time affect the colour, crispiness moisture content and hardness of sweet potatoes (Tamalsina et al., 2019).

Table 5. Effect of cooking methods on total carotenoid (µg/g) retention of UMUSPO 3 *Ipomeabatatas*variety

Processing Method	**Cooking Time**	**Carotenoid Retention**	**%Total Carotenoid retention**
Oven-drying	0 h	41.70^{a}±1.50	
	24 h	31.69^{b}±0.00	76.4%
	36 h	27.48^{c}±0.00	65.9%
	48 h	15.14^{d}±0.00	36.3%
Roasting	0 min	41.70^{a} ±1.50	
	10 min	21.10^{b} ± 0.14	50.6%
	20 min	12.20^{c} ± 0.00	29.2%
	30 min	8.70^{d} ± 0.00	20.9%
Boiling	0 min	41.70^{a}±1.50	
	10 min	23.50^{b}±0.57	56.4%
	20 min	11.00^{c}±0.00	26.4%
	30 min	7.15^{d}±0.71	17.1%

Values are means ± SD. Means with different superscripts in the same column are significantly different ($p<0.05$).

The average loss of carotenoid was more in boiling than in roasting but least in oven-drying (see Figure 2). The figure indicates that oven-drying is a superior method of processing for carotenoid retention when compared with moist heat treatment and roasting.During heat treatment, there could be enzymatic or non-enzymatic oxidation as well as isomerization of cis to trans isomers of β-carotene, which alters their biological activity and denatures the food.There is the need to adapt the processing methods that will reduce β-carotene degradation (Rodriguez-Amaya, 1999 and Vimala*et al.*, 2011).

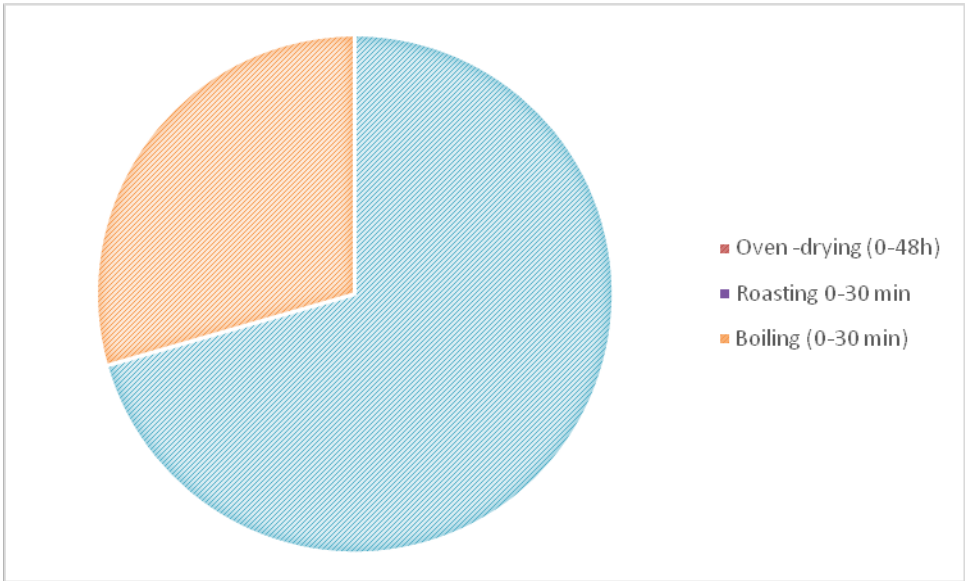

Figure 2. Average carotenoid retention after processing.

4.Conclusions

Use of NPK fertilizer improves the protein content, while the use of poultry manure improves the ash content off orange-fleshed sweet potatoes. Application of poultry manure improves the niacin content of the potatoes. Vitamin B1 and pro-vitamin A contents are not improved by soil nutrient management treatments. Carotenoid retention during processing is highly dependent on the processing methods as carotenoids undergo some qualitative and quantitative changes during processing.

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FRUIT PEELS: A STRONG NATURAL SOURCE OF ANTIOXIDANT AND PREBIOTICS

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ABSTRACT

The aim of the present study was to evaluate the nutritional, anti-nutritional, antioxidant, antimicrobial and prebiotic activity in peels of four selected fruits (apple, sweet lime, papaya and banana) commonly consumed in India. The nutritional and anti-nutritional constituents along with antioxidant activities were determined. Anti-bacterial activity and prebiotic potential of selected fruit peels was also estimated. All the selected fruit peels exhibit good nutritional value along with acceptable level of anti-nutritional factors. The highest values of antioxidant activity, total phenolics and flavonoids were recorded in peels of banana and apple. The results indicate that peels of different fruits can be regarded as natural plant source of antioxidants with high prebiotic potential.

1.Introduction

Historically fruits have been recommended as a significant necessity of human diet and are extensively recommended for their health-promoting benefits. Worldwide, India is the second largest consumer and producer of fruits and generates million tons of fruit waste per year affecting as a solid waste (Patel and Goyal, 2012; Pathak et al, 2017). The organized sectors concerned with fruit processing, packaging, distribution and consumption in Philippines, China, United States of America and India generates approximately 55 million tones of fruit wastes which are dumped in landfills causing environmental hazards (Wadhwa and Bakshi, 2013).

These fruit peels can be utilized for many purposes as they are loaded with nutrients instead of disposing them and causing environmental hazards. The outer protective layer of a fruit commonly known as peel, rind or skin provides mechanical support to fruits. In botanical terms the outermost layer of the fruit is epicarp or exocarp which forms the outer tough skin of the fruit mainly composed of cellulosic material along with other components like pigments (flavonoids,
carotenoids, chlorophylls), enzymes, fattyacids, essential oils, triterpenoids, steroids, bitter principles (limonin) and waxes. Different fruit peels contains significant amount of polyphenols, dietary fiber, polyunsaturated fatty acids, proteins, essential amino acids and potassium.

The peel of the fruits also exhibits good prebiotic potential due to presence of non-digestable fiber compounds and stimulate the growth of beneficial bacteria of large bowel system which decrease the infectious episodes. The most superior form of prebiotics is Galactooligosaccharides (GOS) belonging to a group of particular nutrient fibers. Consumption of fruits either raw or processed provides considerable proportion of the whole antioxidant in the diet. According to researches, fruit peels are the foundation of many other biological functions along with the prevention of many chronic diseases such as anti-cancer, anti-diabetic, anti-hypertensive, anti-aging and anti-inflammatory (Ke et al, 2015). Preliminary research has established other potential effects of prebiotic consumption such as reducing abdominal discomfort and bloating, boosting immune system, regulating weight management and maintaining bone mineral density along with improving brain health (Patel and Goyal, 2012).

Fruit peels are usually considered as a waste and represent an environmental problem by most of the industries as their disposal becomes a serious problem. On the other hand many studies have been carried out about the potential utilization of several fruit peels originated by-products for their inclusion in the daily diet of humans as this can reduce industrial costs and provide correct solution for the pollution problem by justifying the new investments in equipments connected with broadest applications in pharmaceuticals, alcoholic beverages, food processing and textile industries (Kumar et al, 2018). Peel waste contains a biomass-rich material such as lignocelluloses, which stimulate new pathways for the production of low cost, sustainable and renewable adsorbents for water treatment applications. (Bhatnagar *et al*., 2015)

Peels of some widely consumed fruits like apple, sweet lime, papaya and banana represents a great therapeutic potential as they are highly rich in vitamins, minerals and antioxidants. They can be easily utilized to produce variety of value added products such as organic acids, prebiotics, polysaccharides, enzymes, ethanol, single cell protein, bioactive compounds, natural fertilizers and pollution repellents. Overall, fruit peels possess numerous pharmacokinetic and pharmacodynamics properties and its constituents are widely utilized for many clinical and therapeutic applications.

Apple peel (*Malus pumila*) are dense source of nutrients and it's a powerhouse for health, due to presence of high antioxidant content, pectins, phytochemicals (phenol and flavonoids), fiber, vitamins A and C, minerals like potassium, calcium, phosphorus, iron and folate.

Sweet lime (*Citrus limetta*) peels are rich in active phytochemicals that can protect health. It provides ample amount of pectin, folic acid, potassium and vitamin C. Citrus species contributes in preventing life threatening diseases (Gorinstein et al., 2004).

Papaya peel (*Carica* papaya L.) exhibits high antioxidant activity by scavenging free radicals and prevents life threatening chronic diseases like diabetes, cancer cardiac diseases (John and Shahidi, 2010). It is a good source of phenolic compounds, minerals and fibre.

Banana peel (*Musa Acuminata*) contains significant amount of lignin, protein, fiber, starch, vitamin C, pyridoxine, potassium and magnesium. It is equally dense in antioxidant luetin, which enhance and protects eye and cardiac health.

The objective of the present investigation was to evaluate the nutritional composition, antioxidant capacity, prebiotic potential and anti-microbial property of four different fruit peels (apple, sweet-lime, papaya and banana) by using *in-vitro* methods.

2.Material and Methods

2.1. Procurement of fruits

The required fruits of apple, sweet lime, papaya and banana were collected from a local fruit market and were stored in cold conditions until the moment of analysis. Other raw materials were purchased from the licensed and authorized shops.

2.2. Samples preparation

Healthy mature fruits were selected free from bacterial infection and mechanical damages. The fruits were peeled, cleaned and dried at 60 ± 5ºC for 12 h in hot air oven, ground and stored in airtight polythene bags for further analysis

2.3. Chemicals

Standards of gallic acid and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2'-diphenyl-1-picrylhydrazil (DPPH) and Folin-Ciocalteau's reagent were obtained from Fluka (United Kingdom). All the other reagents were of analytical grade.

2.4. Gross chemical composition

Moisture, ash, crude protein, crude fat, fibre and total carbohydrate content of fruit peels were estimated according to the methods of AOAC (2000).

2.5. Determination of minerals

In 1% hydrochloric acid, the ash samples were dissolved and the solutions were used for the determination of the following minerals: Iron, calcium, potassium and phosphorus according to the methods of AOAC (2000).

2.6. Determination of anti-nutritional factors

The phytic acid content was determined by the colorimetric method reported by Wheeler and Ferrel (1971) and the optical density is read at 480nm, by using a spectrophotometer. Phytic acid solution was used to obtain a standard curve.

Tannins were determined by the method given by Marigo (1973) and the optical densities were measured at 700nm. The formula used to estimate the tannic acid content is given below:

$$\text{Tannic acid \%} = \frac{\text{mg of Tannic acid} \times \text{dilution} \times 100}{\text{mg of sample taken of color development} \times \text{Weight of sample} \times 1000} \quad (1)$$

Oxalic acid were estimated by volumetric analysis (titration method) given by Adeiyi et al., 2009. The following formula was used to obtain the oxalate %.

$$\text{\% Oxalate} = \frac{\text{titre value} \times \text{M of KMnO4} \times \text{vol. makeup} \times \text{molecular wt. of oxalate} \times 100}{\text{aliquot taken for titration} \times \text{wt. of sample} \times 1000} \quad (2)$$

2.7. Extract Preparation

150 g of different fruit peels powder was dissolved in 300ml of organic solvent (ethanol) in conical flask and incubated at room temperature at 150 rpm (rounds per minute) for two days. Filtered with Whatman No.1 filter paper and concentrated to dryness at 40 – 60 °C on hot water bath to get the semi solid crude extracts which were stored at 4 °C in airtight bottles till further use.

2.8. Determination of percentage yield

The percentage yield was evaluated by comparing the weight of fresh whole fruit and

dried powder of peel obtained from the same amount of fruit.

Yield (%) = Weight of dried fruit peel powder x 100

Weight of fresh fruit

(3)

2.9. Determination of Total Phenol Content

The total phenolic content was estimated by the Folin–Ciocalteu method using standard phenolic compound gallic acid as described by (Singleton and Rossi, 1965) with some modification. 1ml of aliquot (extract or standard solution of gallic acid) (20, 40, 60, 80 and 100mg/L) was taken in a test tube was diluted with 9ml of nanopure water. 1ml of Folin–Ciocalteu reagent was combined to the mixture and vortexed. It was allowed to react for 5min then 10 ml of 7% Na_2CO_3 solution was added with constant mixing. Immediately the solution was diluted to volume 25ml with nanopure water and was thoroughly mixed and incubated for 90min in darkness at 23ºC. The absorbance verses prepared blank was measured at 750nm and the obtained results of total phenols were expressed as mg gallic acid equivalents (GAE; mg/100 g fresh mass).

2.10. Determination of Total flavonoid content

The total flavonoids content was estimated by the method described by Sakanaka et al, (2004). The standard solution was prepared by dissolving 10mg of quercetin in 10ml of methanol to obtain 1000 µg/ml solution. Different concentration of aliquots (6.25, 12.5, 25, 50, 80 and 100 µg/ml) was prepared from stock solution in 6 different test tubes. Each sample was mixed with 1.5ml of methanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate solution followed with 2.8ml of distilled water and the reaction mixture was mixed well and left at room temperature for 30 min. The absorbance of the resulting solution was measured immediately at 415 nm against the sample blank which was prepared in similar way by replacing aluminium chloride with distilled water. The results of samples were expressed as mg of quercetin equivalents of total dried fractions. All the fractions were run in triplicate. For the preparation of test solution 10mg of extract was dissolved in 10ml of methanol to get 1mg/ml solution. The required volume of the above solution was transferred into the test tubes and the color development was carried out as for the standard. The absorbance of the test solution was measured at 415 nm against blank in a double beam spectrophotometer (Perkin Elmer). In the extract the total flavonoid content was expressed as µg/ml. In the test sample the concentration of total flavonoid content was calculated from the calibration curve by plotting absorbance verses concentration.

The calibration curve was prepared by using different concentrations of quercetin which is expressed in mg/gm dry weight.

2.11. Determination of Antioxidant activity

The free radical scavenging activity was measured by using the 1,1- diphenyl-2-picrylhydrazyl (DPPH) assay (Brand-Williams et al., 1995) with some modifications. The preparation of stock solution was done by dissolving 2.15mg of DPPH in 1.65ml methanol. The test tube was covered with aluminum foil to protect it from light and stored at 20ºC until required. For control reading 150µl DPPH was added to 3000µl methanol and immediately absorbance was taken at 517nm. Different concentrations (10 - 50µg ml^{-1}) of the extract as well as standard compound (ascorbic acid) were taken and the volume was made uniformly to 150 µl with methanol. Each sample was further diluted with methanol upto 3ml and to each 150 µl DPPH was added. The reaction mixture was allowed to stand in dark for 15 min at room temperature, methanol was used as blank and the absorbance was recorded at 517nm spectrophotometrically. The concentration of sample required to scavenge 50% of DPPH radical (IC_{50} value) was noted by linear regression analysis of dose response curve plotting between percentage inhibition and concentration.

The percentage of scavenging activity was calculated using the following formula:

% Scavenging = [(Absorbance control – Absorbance sample)/Absorbance control] × 100

(4)

2.12. Determination of anti-bacterial property

The agar cup-plate diffusion method was used to analyze the antibacterial activity of the prepared methanolic extracts of apple, sweet-lime, papaya and banana peels. The crude extracts were diluted in 100% Dimethyl Sulphoxide (DMSO) at a concentration of 25, 50, 70 and 100 mg/mL respectively. 20ml of sterile nutrient agar medium was poured into sterile petri-dishes and was allowed to solidify. The prepared medium was seeded by pour plate method with the micro-organisms using 4ml of sterile top agar containing 1 ml culture. On the agar plate, wells (6 mm) were made by using sterile cork borer No. 4. The different concentrations of (25 mg/ml, 50 mg/ml, 70 mg/ml, 100 mg/ml) peel extracts were loaded into the wells and all the plates were incubated overnight at a temperature of 37°C for 24 hours with appropriate positive and negative controls. The drug tetracycline was used as a positive control in a concentration of 10 µg/mL and 100 % (DMSO) was used as negative control. The

antibacterial activity of the peel extract was analyzed by measuring the diameter of the inhibition zone in mm with a transparent scale. The antibacterial assay for each of the extracts against all microorganisms tested was performed in triplicates.

Analysis of relative percentage (%) zone of inhibition

The relative percentage zone of inhibition of two extracts of apple, sweet-lime, papaya and banana peels against four bacterial strains was calculated by formula:

Percentage relative zone of inhibition in mm =

$$\frac{Zone\ of\ inhibition\ of\ sample\ (Plant\ extract) - Zone\ of\ inhibition\ of\ negative\ control}{Zone\ of\ inhibition\ of\ positive\ control (antibiotic\ standard\ drug)} \times 100 \quad (5)$$

2.13. Determination of Prebiotic Potential

The prebiotic potential of different fruit peels were analysed according to the method given by Hussein *et al.,* 2015. Three strains viz. *L. casei, L. rhamnosus* and *L. plantarum* were used to determine the prebiotic activities of the apple peel extract (500mg/ml) and sweet lime peel extract (500mg/ml) produced by crude extract method. The prebiotic activity was expressed as the 'prebiotic index'. This index relates the growth intensities of the probiotics with that of the pathogen *E. coli*.

Prebiotic Index

$$= \frac{\text{Optical Density of the growth of probiotic culture}}{\text{optical density of growth of E coli}} \quad (6)$$

Control =

$$\frac{Optical\ Density\ of\ the\ probiotics\ without\ extract}{Optical\ Density\ of\ E.Coli\ without\ extract} \quad (7)$$

2.14. Statistical analysis

The experimental data collected was tabulated and analyzed statistically with the help of approved statistical techniques (Imran and Coover, 1983). Percentage, mean scores, standard deviation (SD) of three replicates was analysed using the application available for Microsoft Excel (XLSTAT 2016). Significant differences among the samples were calculated using one-way ANOVA followed by Duncan's multiple range test at the 5% level (p B 0.05).

3.Results and discussions

Nutritional profiling of different fruit peels (apple. sweet-lime, papaya and banana) were studied additionally studying the antioxidant activities, anti-microbial property and prebiotic potential of these peel extracts.

3.1 Nutritional profiling

Nutritional profile of the four different peels were analysed by determining its moisture, ash, protein, fat, carbohydrate, energy, fibre and minerals. In Table 1, data summarizes the proximate composition and mineral content of the different fruit peels. Papaya peel had the highest moisture content 88.62% followed by banana peel 87.94%, apple peel 82.60% and sweet lime peel with a value of 73.60%. The high moisture level in all the investigated samples suggests that the fruit peels cannot be stored for long duration and it can be easily spoiled. Higher water activity in fruit peels will increase the microbial action and bring about food spoilage. Jenson (1978) reported that around 85% of moisture content is present in fruits and vegetables. The banana peel had maximum (12.33 ± 0.15 g/100g) ash content whereas apple peel had minimum (1.48 ± 0.02 g/100g). The high ash content indicates the amount of inorganic elements (potassium, calcium, copper, zinc and magnesium) present in fruit peels (Igile et al., 2013). The apple peel had the maximum amount of fat content (8.73±0.11 g/100g) followed by sweet-lime (7.76±0.11 g/100g), banana (6.43 ± 0.07 g/100g) and papaya (6.23 ± 0.01 g/100g). The fruit peels are low in fat content; therefore it can be used in product formulation especially for weight reduction diets. The protein content for the fruit peels varied different species, falling within the extremes of higher value (8.7 ± 0.17 g/100g) in sweet-lime and lower value (2.74 ± 0.05 g/100g) in apple. Parni and Verma (2014) reported that crude protein content in the peel of *Carica papaya* is 5.03 mg/g. The carbohydrate and energy content was high in sweet-lime peel (59.56±0.75 g/100g and 342.97 ± 4.30 Kcal/100g) while papaya peel had minimum carbohydrate and energy content (23.92±0.39 g/100g and 164.9 ± 16.34 Kcal/100g). The presence of complex carbohydrates especially natural sugars, fibre and starches in fruit peels. Anhwange et al. (2009) and Osarumwense et al. (2013) estimated that significant amount of carbohydrate was present in Banana and citrus peels.

The crude fibre content (29.52 ± 0.02 g/100g) was high in banana peel, which aids in better digestion and softens the stools resulting in prevention of constipation {Ayoola and Adeyeye, 2009).

The mean concentration of iron, calcium and phosphorus in the different fruit peels ranged 18.8±0.26 to 33.4±0.2 mg/100g, 14.45±0.50 to 169.3±3.60 mg/100g and 10.13±0.30 to 83.0±0.52 mg/100g with banana peel having the highest concentration of iron (33.4±0.2 mg/100g) and sweet-lime peel having the highest concentration of calcium (169.3±3.60 mg/100g) and phosphorus (83.0±0.52 mg/100g). All these metals are usually related with plants

or plant products. It was indicated from the results that fruit peels can supplement the body with some important macro and micro nutrients required by the body.

Table 1. Chemical composition and minerals content of different fruit peels

Chemical Composition (g/100g)	**Apple (*Malus pumila*)**	**Sweet-lime (*Citrus limetta*)**	**Banana (Musa acuminata)**	**Papaya (*Carica papaya*)**
Moisture (%)	82.60±0.73	73.6±0.42	87.94±5.62	88.62±0.77
Ash (g)	1.48±0.02	4.6±0.1	12.33±0.15	11.5±0.3
Fat (g)	8.73±0.11	7.76±0.11	6.43±0.07	6.23±0.01
Protein (g)	2.74±0.05	8.7±0.17	8.68±0.07	7.27±0.05
Carbohydrate (g)	53.01±0.34	59.56±0.75	45.67±5.87	23.92±0.39
Energy (Kcal)	301.6±56	342.97±4.30	274.33±25.02	164.9±26.34
Fibre (g)	12.3±0.05	15.16±0.05	29.52±0.02	10.5±0.25
Mineral content (g/100mg)				
Iron (mg)	23.93±0.23	18.8±0.26	33.4±0.2	23.83±0.35
Calcium (mg)	14.45±0.50	169.3±3.60	17.73±1.15	15.56±0.30
Phosphorus (mg)	75.4±0.1	83.0±0.52	10.13±0.30	20.46±0.25

3.2. Anti -nutritional Profiling

The most abundant anti-nutritional factors present in selected fruit peels (viz. oxalate, phytate and tannin) are shown in Table 2. The highest oxalate level (247.00±5.89mg/100g) was observed in banana peel followed by apple peel (85.36±0.05 mg/100g) and sweet lime peel (55.6±0.1mg/100g). Papaya peel had the lowest oxalate level (43.42±0.66mg/100g). Oxalate can bind to different minerals present in food thus rendering them inaccessible for normal biochemical and physiological role such as blood coagulation, nerve impulse transmission, cofactor in enzymatic reaction, maintenance of teeth and bone Ladeji (2004). The obtained values of different fruit peels were below the established toxic level.

The phytate content in different fruit peels range from 5.5±0.2mg/100g in papaya peel to 251.66±7.63mg/100g in sweet lime peel. Phytate forms an indigestible complex with different mineral elements (iron, calcium, magnesium, phosphorus, zinc and managanese), thus decreasing the absorption and bioavailability of these minerals (Umaru et al., 2007).

The maximum tannin content was observed in apple peel (75.34±0.05%) while lowest values were observed in banana peel (42.3±0.2%). The astringent taste in fruit peels was imposed by tannins which reduce the food intake and affects the palatability. Tannins also bind together enzymes of the digestive tract along with endogenous and exogenous proteins, therefore affecting the protein utilization (Sotelu et al., 1995). All the above mentioned fruit peels contains lower amounts of anti-nutrients, consequently they are recommended for consumption.

Table 2. Concentration of anti-nutritional factors in selected fruit peels

Anti-nutritional Factor	**Apple (*Malus pumila*)**	**Sweet-lime (*Citrus limetta*)**	**Banana (Musa acuminata)**	**Papaya (*Carica papaya*)**
Oxalate (mg/100g)	85.36±0.05	55.6±0.1	247.00±5.89	43.42±0.66
Phytate (mg/100g)	146.66±11.54	251.66±7.63	7.27±0.24	5.5±0.2
Tannin %	75.34±0.05	67.28±0.32	42.3±0.2	54.33±0.15

3.3 Antioxidant Activity

The total phenolic and flavonoid content in the different fruit peels was quantified in Table 3.

The total phenolic contents exhibited in different fruit peels range from 3.04±0.02 in papaya peel to 25.1±0.1mg/g GAE in banana peel. Thus, results show that fruit peel of banana had a highest TPC content. The presence of phenolic in fruit peels explains the potential of antioxidant activity. These phenolic compounds react with phosphomolybdate and

phosphotungstic acid contained in the Folin–Ciocalteu reagent undergoes complex oxidation-reduction reactions (Kaur and Kapoor, 2011). The total phenolic content was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW).

According to Sir Elkhatim (2018) fruit peels are the readily available inexpensive source of bioactive compounds (natural antioxidant) and it contains high phenolic compounds than those in pulps. The total flavonoid content varied from 9.03±0.01 in banana peel to 26.43±0.01 mg quercetin equivalent/g of extract powder in apple peel. Apple peel showed the highest level of total flavonoid content, followed by sweet-lime peel and papaya peel, while banana peel presented the lowest. Research findings suggest that fruit peels are good source of flavonoids and it possess excellent radical scavenging activity Sir Elkhatim (2018). According to Wanpeng et al., 2017 highest phenolic and flavonoid content was found in fruit peels followed by whole fruit and seeds whereas juice lowest phenolic content was seen in juices.

Table 3. Total phenol and flavonoid content in different parts of fruit peels

Phytochemicals	**Fruit peels**			
	Apple (*Malus pumila*)	**Sweet-lime (*Citrus limetta*)**	**Banana (*Musa acuminata*)**	**Papaya (*Carica papaya*)**
Total Phenol (mg/g)	11.60±0.04	14.42±0.08	25.1±0.1	3.04±0.02
Flavonoid (mg/g)	26.43±0.01	19.26±0.20	9.03±0.01	15.01±0.01

Total phenolic and total flavonoid were expressed as gallic acid equivalents (GAE) and rutin equivalents (RE), respectively

In this study *in vitro* antioxidant assay was performed to evaluate the antioxidant potential of different fruit peels. The most widely used method for assessing the antioxidant activity is scavenging model of DPPH radical. Table 4 displayed the antioxidant activity of different fruit peels. The DPPH values ranged from 10.71±0.03 to 77.5±0.80 mg/g. The highest value was found for banana peel, whereas the lowest was found for sweet-lime peel. The higher antioxidant activity in different fruit peels is due to the higher content of polyphenols and flavonoids. Sir Elkhatim (2018) reported that in three types of citrus fruit peels the antioxidant activity is significantly ($p < .05$) higher than that in pulp and seeds.

The antioxidant capacity can also be studied by a simple method known as FRAP assay (Pulido et al. 2000). The FRAP values in different fruit peels varied from 15.17 ± 0.14 to 60.65 ± 0.32 mg/g. Apple peel had the highest FRAP value, whereas sweet-lime peel had the lowest FRAP value. According to Wanpeng et al., (2017) fruit peels had the higher FRAP values than other tissues like seeds, whole fruit and juice.

Table 4. Antioxidant activity of different fruit peels

Antioxidant	**Apple (*Malus pumila*)**	**Sweet-lime (*Citrus limetta*)**	**Banana (Musa acuminata)**	**Papaya (*Carica papaya*)**
DPPH (mg/g)	75.31±0.08	10.71±0.03	77.5±0.80	57.3±0.02
FRAP (mg/g)	60.65 ± 0.32	15.17±0.14	20.5±0.2	26.13±0.97

3.4 Antibacterial activity

Antibacterial potential of the peels were checked against gram positive and gram negative pathogenic bacteria by using different concentration of ethanolic extracts for all the fruit peels.

Table 5. Antibacterial activity of apple , banana, Papaya and sweet-lime peel extracts indicated by Zone of inhibition (250mg dry wt/ml,mm)

Peel	**Extract**	***Escherichia. Coli***	***Pseudomonas***	***Shigella***	***Staphylococcus. Aureus***
	Aqueous	$24.33±1.15^{d}$	$16.93±0.11^{d}$	$15.03±0.15^{a}$	$26.8±0.26^{c}$
Apple	50% ethanol	$23.7±0.17^{d}$	$18.16±0.15^{e}$	$16.33±0.57^{b}$	$26.96±0.057^{c}$
	70% ethanol	$30.93±0.11^{f}$	$19.66±0.57^{f}$	$19.9±0.1^{d}$	$30.33±0.57^{d}$
	Aqueous	$20.16±0.15^{c}$	$15.06±0.11^{c}$	$17.13±0.15^{c}$	$40.66±0.57^{c}$
Sweet lime	50%	$21.16±0.28^{c}$	$10.23±0.20^{a}$	$20.1±0.17^{d}$	$43.3±0.26^{d}$

	ethanol				
	70% ethanol	26.3±0.26^{e}	14.06±0.11^{b}	23.1±1.74^{e}	45.93±0.11^{e}
Banana	Aqueous	20.2+0.25^{c}	19.3±0.47^{f}	19.7±0.35^{d}	21.3±0.50^{b}
	50% ethanol	20.2±0.11^{c}	20.3±0.20^{f}	20.1±0.05^{d}	21.5±0.30^{b}
	70% ethanol	22.3+0.25^{c}	21.3±0.47^{g}	21.6±0.35^{d}	22.6±0.50^{b}
Papaya	Aqueous	15.5±0.87^{a}	10.1±0.05^{a}	18.3±0.05^{c}	18.3±0.25^{a}
	50% ethanol	16.2±0.36^{a}	10.5±0.37^{a}	19.2±0.11^{d}	18.6±0.11^{a}
	70% ethanol	18.3±0.^{b}	11.3±0.05^{a}	20.3±0.05^{d}	20.3±0.25^{a}

Each value represents the mean and standard deviation from three lots;
Means with different superscripts for each sample are significantly different ($p<0.05$)

It is evident from the results (Table 5) that highest zone of inhibition was observed for 70% ethanolic extract of sweet-lime peel (45.93mm) and 70% ethanolic extract of apple peel (30.33mm) against_*S.aureus* . Substantial zone of inhibition was observed for 70% ethanolic extract of sweet-lime peel against *Shigella* (23.1mm) and *E.coli* (26.3 mm). The overall data clearly states that 70% ethanolic extract of all the peels manifests good anti-bacterial potential. All the extracts showed inhibition in following sequence *S.aureus* > *Ecoli*> *S.flexneri*> *Pseudomonas.* According to Ghai et al (2018) sweet lime has shown maximum zone of inhibition for *Bacillus cereus* (9mm) > *Escherichia coli* > (7.67mm) *Enterobacter aerogenes* (8.33mm)> *Pseudomonas aeruginosa* (4.67mm). The citrus fruit peel extract using *Citrus sinensis* (orange), *Citrus limon* (lemon), and *Citrus limetta* (sweet lime) also showed high antimicrobial activity against the common gastrointestinal pathogens (Nisha et al., 2013) (fig 1).

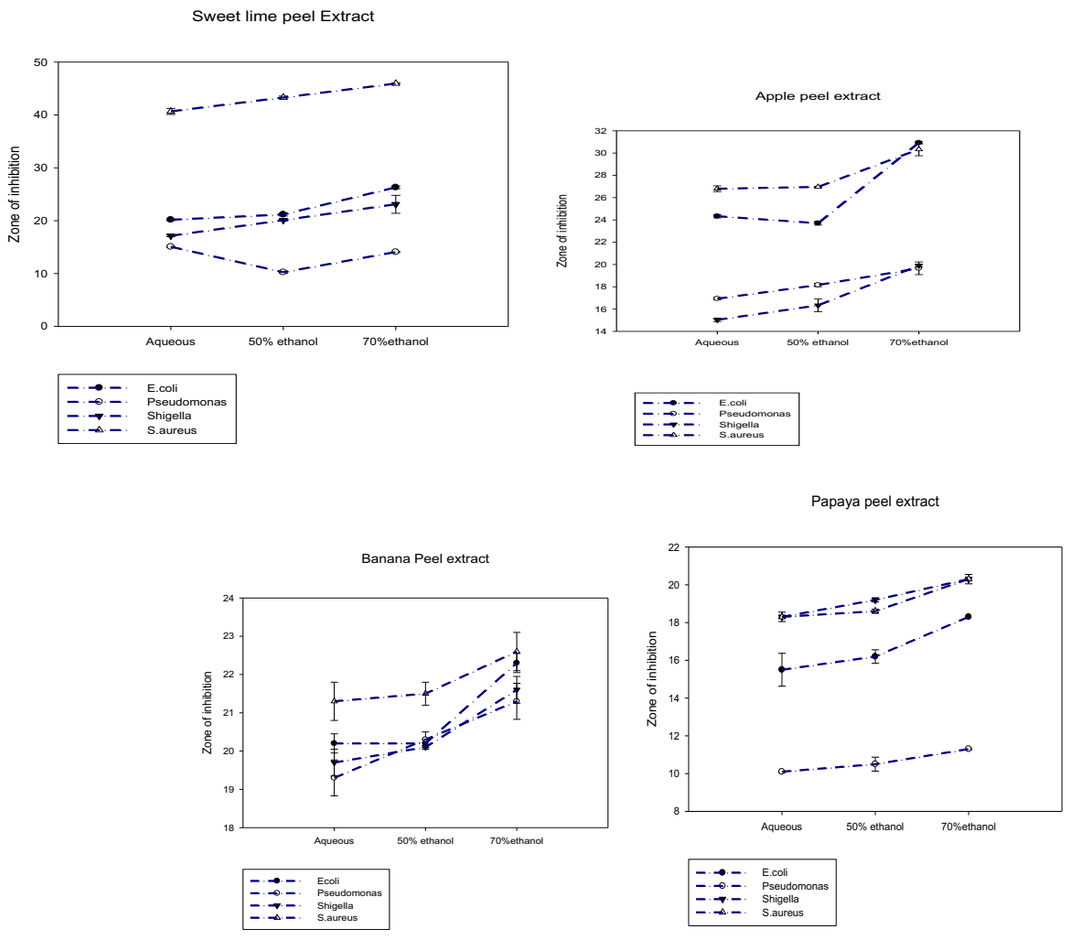

Figure 1. Antibacterial activity of fruit peels extracts indicated by Zone of inhibition

The above data indicates the least concentration at which clear zone of inhibition against bacterial strains was observed. All active extracts characterized by MIC values as equal to or higher than 50 mg/ml. Aqueous extracts of both the peels have shown the higher values of MIC as 250 mg /ml and 175 mg /ml for apple and sweet lime respectively. 70% ethanolic extract of apple and sweet-lime peel extract shows the clear zone of inhibition at lowest concentration.

Table 6. MIC of apple , banana, Papaya and sweet-lime peel extracts

Peels	Extracts	***Escherichia. coli***	***Pseudomonas***	***Shigella***	***Staphylococcus. Aureus***
Apple	Aqueous	250mg/ml	250mg/ml	250mg/ml	175mg/ml
	50% ethanol	175mg/ml	175mg/ml	175mg/ml	50mg/ml
	70% ethanol	150mg/ml	150 mg/ml	150mg/ml	50mg/ml
Sweet Lime	Aqueous	175mg/ml	175mg/ml	250mg/ml	175mg/ml
	50% ethanol	50mg/ml	50mg/ml	175mg/ml	50mg/ml
	70% ethanol	20mg/ml	50mg/ml	50mg/ml	20mg/ml
Banana	**Aqueous**	250mg/ml	500mg/ml	500mg/ml	250mg/ml
	50% methanol	250mg/ml	500mg/ml	500mg/ml	250mg/ml
	70% methanol	175mg/ml	250mg/ml	250mg/ml	175mg/ml
Papaya	**Aqueous**	500mg/ml	750mg/ml	750mg/ml	250mg/ml
	50% methanol	500mg/ml	500mg/ml	500mg/ml	175mg/ml
	70% methanol	250mg/ml	500mg/ml	500mg/ml	125mg/ml

3.5. Prebiotic Activity

Three strains viz. *L .casei, L. rhamnosus* and *L. plantarum* were used to determine the prebiotic activities of the apple peel extract (500mg/ml) and sweet lime peel extract (500mg/ml) produced by crude extract method. The prebiotic activity was expressed as the 'prebiotic index'. This index relates the growth intensities of the probiotics with that of the pathogen *E. coli*. The results recorded in Table 7 indicate that both the extracts enhanced the growth of probiotic culture. The prebiotic index for *Lactobacillus plantarum* was greater for apple peel as compared to sweet-lime peel. The prebiotic index for *Lactobacillus rhamnosus* was greater for sweet-lime peel as compared to apple peel. The prebiotic index for *Lactobacillus casei* was almost equal for apple peel and sweet-lime peel.

Table 7. Prebiotic Index of apple and sweet-lime peels

Extracts	Prebiotic Indices using various Probiotics		
	L.plantarum	***L.rhamnosus***	***L. casei***
Apple Peel	25.55	16.88	33.55
Sweet Lime Peel	20.46	21.11	33.48
Papaya Peel	2.91	1.17	0.976
Banana Peel	4.32	1.19	1.29
Control	0.80	0.98	1.04

4.Conclusions

The obtained results revealed that peels of different fruits are dense in medicinally important health-promoting nutrients and it is an excellent source of natural antioxidants which enhance the bioavailability of bioactive compounds. The methanolic extract of different fruit peel extracts shows greater resistance against the number of pathogenic microorganisms. Fruit peels are the natural source of prebiotics with its potential applications as functional food ingredients or as natural preservatives. The extracts from these fruit peels could be a good source in designing

the useful drugs or pharmaceutical products for human health.

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EVALUATION OF TOXIC POTENTIALS OF *Cola millenii* K. Schum SEED AND PULP FLOUR IN FOOD FORMULATIONS

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ABSTRACT

Different parts of *Cola millenii* have been used in traditional medicine and available information in the literature has highlighted some nutritional potentials of C*ola millenii* seed and pulp. Previous studies on the toxicity of the seed were conducted on the extracts and conflicting evidences were obtained. Therefore, this study assesses the health risk of consuming *Cola millenii* seed and pulp flour in Wistar Albino rats.

The result obtained showed insignificant ($p>0.05$) difference in the organ weight of rats across the groups. Feeding of animals with the whole seed, pulp, and defatted seed flour showed no toxic effects on the food and water intake. Significant ($p<0.05$) increase was observed in the AST activity of group fed with whole seed and defatted seed flour while no significant ($p>0.05$) difference was observed in the ALT and GGT activity across the groups. Creatinine concentration decreased significantly ($p<0.05$) in the group fed with the defatted seed flour while urea concentration decreased significantly ($p<0.05$) in the group fed with the whole seed flour. Degenerative changes indicated by inflammation, necrosis and fibrosis were observed in the liver of group fed with the whole seed flour as well as in the kidney of groups fed with whole seed, defatted seed, and pulp of *C. millenii*. The result of this study showed that *C. millenii* seed might be slightly toxic. Therefore, there is a need for caution in the use of *C. millenii* as food.

1.Introduction

Plants remain a widely accepted component of Western diets and several health benefits are attributed to the consumption of plant foods (Deanna, 2019). Due to the increasing demand on valuable foods with sufficient nutrient, efforts are being made to boost food production with interest focused on the possibility of exploring the less familiar plants of the wild (Anhwange *et al.,* 2004). The tropical Africa sub-region is home to many potentially valuable fruit species of which many are yet to be fully exploited. Therefore, there is need to promote crop and food diversifications to include neglected and underutilized species that are readily affordable and accessible to the rural and urban dwellers and most times can withstand the stresses linked to climate change (Bello et al., 2008). Although some of these plants that grow in the wild are gaining attention among

researchers. One of these is *Cola millenii* K. Schum, commonly known as monkey kola. The fruit is bright red in a stellate cluster and its seed is covered with a fibrous coat with an edible kernel. The pulp has a varying characteristics sweetness which makes it useful as snack while seed is mostly appreciated for its therapeutic uses (Orisakeye and Ojo, 2013; Borokini *et al*., 2014).

The nutritional potentials of *C. millenii* showed that the seed contain sufficient amount of carbohydrate, protein and fibre as well as essential minerals required for general wellbeing (Ibironke *et al.* (2013). In addition, phytochemical studies on the seed and pulp showed that it has medicinal potentials as it contains important phytochemicals such as saponins, terpenoids, tannins and alkaloids (Giwa *et al.,* 2012). Furthermore, the seed and pulp possess strong antimicrobial and antioxidant activity (Adeniyi *et al*., 2004; Orisakeye *et al*., 2013; Borokini *et al*., 2014). Therefore, *C. milleni* is an important medicinal plant with valuable nutritional usefulness.

Although, plant foods contain essential nutritional constituents, there are increasing concerns because of their safety when consumed (Suzanne *et al.,* 2011). Currently, most toxicity studies conducted on *C. milleni* were performed on the seed and leaf extracts and a range of potential contraindications such as hepatic and renal toxicity have been identified (Oyemitan *et al.,* 2016; Itoandon *et al.,* 2016). Ubon *et al.* (2017) on the contrary reported that the ethanolic extract of the seed is non-cytotoxic, non-hepatotoxic and non-cardiotoxic. Therefore, this conflicting evidences need to be investigated. Hence, the study assessed the health risk of consumption of *C. millenii* seed and pulp flour in Wistar Albino rats.

2. Methods

2.1. Sample collection

Ripe monkey cola fruits were collected in February 2019 from (Omo Odo Agba Area, Near Baba Kekere, Osogbo) Latitude 7.762216, longtitude (4.579217) Oke-Baale area, Osogbo local government in Osun State, Nigeria.

2.2. Sample preparation

The ripe fruits were washed and left to dry off at room temperature. The fruit's endocarp was cut open with the use of a knife and then the clustered seeds were removed. The pulp (mesocarp) was removed from the seed itself and was left to air-dry separately at room temperature. After drying, the seeds and pulp were ground separately using a using a blender (Power Deluxe CB 8231 N model) until a fine homogenous sample was obtained and the ground sample was kept an air-tight container. A portion of the ground seed sample was defatted by weighing into a filter paper and inserted into the thimble of a Soxhlet apparatus. 200 ml of n-hexane was measured into the round bottom flask of the soxhlet setup. The condenser was coupled and the setup was heated to reflux using a heating mantle. The oil sample was extracted continuously for 4 hours. The cake was then placed in oven at less than 60^{o}C for 2 hours until uniform weight was obtained. The deffated seed (seed cake) was then kept in an air-tight container for animal feeding.

Commercial stock rat diet was obtained from Glory veterinary, Oritanaira Area, Ogbomoso, Oyo State. Diets were prepared by mixing 95 % of the commercial diet with 5 % of pulp, cake and seed of *C. milleni* separately every day (Datta *et al.,* 2011).

2.3. Experimental Design

Adult Swiss male Albino rats (120-180g) used in this study were divided into 4 groups of 5 animals. Group A received the normal commercial diet, Group B received feed mixed with 5 % pulp flour, Group C received diet mixed with 5 % defatted seed flour while Group D received diet mixed with 5 % seed flour. The animals were maintained in Basket Cages with wood shave as beddings and kept under standard condition of relative humidity and temperature. Standard protocol on animal handling and care were followed throughout the study whereas approval and permission for animal use were obtained prior to the commencement of the study.

2.4. Acute Toxicity Study

After grouping of animals, they were fed with diet containing 5 % of *C. milleni* seed, defatted seed, and pulp respectively for 21 days. Daily food and water intake and weekly body weights were monitored (Datta *et al.,* 2011). At the end of the 21 days of experiment, animals were fasted overnight and sacrificed by cervical dislocation. The blood was collected via heart puncture into plane sterile bottles while the liver and kidney were harvested and fixed in formalin buffer for histopathological analyses. Blood sample was allowed to clot and then centrifuge at 4000 rpm for 10 minutes. The serum was then separated and used for further biochemical studies.

2.4.1. Liver and renal function Analysis

All biochemical analysis Aspartate Amino Transferase (AST), Alanine Amino Transferase (ALT), Gamma Glutamyl Transferase (GGT), Urea and Creatinine)were performed with Fortress Assay Kit, Fortress Diagnostics Limited, Unit 2C Antrim Technology Park, Antrim, BT41 1QS (United Kingdom) following the standard procedure in the manufacturer guide.

2.4.1.1 Estimation of AST, ALT and GGT activities

The AST and ALT activity was determined by the method described by Tietz, (1995). The AST activity was estimated using the equation, AST activity = ΔA / min × 1908.

While the ALT activity was estimated using the equation, ALT activity = ΔA / min × 1746. GGT activity on the other hand was determined by the method of Szasz, (1976). Its activity was estimated using the equation,

GGT (U/L) = 1158 × ΔA 405nm/min where ΔA 405nm/min is the change in absorbance

2.4.1.2 Determination of Serum creatinine and Urea concentration

The serum creatinine concentration was determined by the method of Tietz, (1995). While the serum urea concentration was determined by the method described by Fawcett and Scott, (1962). The serum creatinine concentration was estimated using the equation,

$$\text{Serum creatinine concentration} = \frac{\Delta\text{abs sample}}{\Delta\text{abs standard}} \times 177 \quad (1)$$

Urea concentration was estimated using the equation,

$$\text{Serum urea concentration} = \frac{\Delta\text{abs sample}}{\Delta\text{abs standard}} \times \text{concentration of standard} \quad (2)$$

2.4.2. Histological Analysis

The histo-pathological analysis was performed by the hematoxylin and eosin staining (Pearse, 1980). Firstly, the samples were dehydrated through ascending grades of alcohol, cleared in xylene, and then impregnated in paraffin wax of melting point between 55^0C-56^0C for infiltration. Tissue sects were mounted on slides and then stained with hematoxylin and eosin (H & E). The stained tissues were then observed under microscope.

2.5. Statistical Analysis

Data were expressed as mean ± SEM and analyzed by One-way Analysis of Variance (ANOVA) using Statistical Package for Social Science (SPSS) 21.0. The level of significance was determined at 95 % confident level and separation between mean was determined by Duncan multiple test.

3. Results and Discussions

3.1. Results

3.1.1. Acute toxicity of *Cola milleni* Seed, Pulp, and Cake on Albino Rats

Table 1 showed the weekly food and water intake of rats fed with the seed, pulp, defatted seed, and commercial diet. Incorporation of 5 % of seed sample showed no effects on the food intake of the treatment groups. The animals showed comparable food and water intake with the control (commercial diet). Figure 1 showed the body weight gain of rats during the period of study. The result showed total body weight increase across the groups. Feeding of the animals with *C. milleni* had no negative impact on the growth and body weight. In table 2, the

effects of feeding the experimental animals with *C. milleni* on the weight of organs were recorded. No significant variation was observed in the organ to body weight ratio of the animals fed with seed, defatted seed, and pulp. Only in the spleen to body-weight ratio was lower ratio obtained in the treated groups compared with the control.

Table 1. Weekly food and water intake of Albino Rats fed with *C. milleni*

Groups	Weekly Food and Water Intake of Rats					
	Week 1		Week 2		Week 3	
	Food(g)	Water (ml)	Food(g)	Water (ml)	Food (g)	Water (ml)
Control	86.33±6.33	75.33±17.76	92.57±3.39	84.29±7.75	77.57±1.56	88.57±8.84
Pulp	96.67±0.61	71.17±10.56	98.29±0.64	85.71±4.68	93.43±1.29	98.57±19.33
Defatted seed	87.50±2.59	98.33±16.10	93.71±1.36	88.00±10.83	89.86±3.14	89.25±6.21
Seed	87.33±3.00	79.00±17.05	90.14±2.41	94.43±8.55	93.71±1.23	112.86±10.17

Values were expressed as mean±SEM

Table 2. Organ to weight ratio of Albino Rats fed with seed and pulp of *C. milleni*

Groups	Organ to weight ratio			
	Liver	Kidney	Heart	Spleen
Control	0.04	0.006	0.004	0.007
Pulp	0.03	0.004	0.003	0.004
Defatted seed	0.03	0.007	0.004	0.004
Seed	0.03	0.005	0.003	0.004

Organ weight/Weight of animals at week 3 (Sample size, 5)

Table 3. Liver function enzymes in Albino Rats fed with *C. milleni*

Groups	Liver Function Markers (U/L)		
	AST	ALT	GGT
Control	43.52 ± 5.69^{a}	28.52 ± 3.51^{a}	2.53 ± 0.43^{a}
Pulp	56.78 ± 2.46^{ab}	32.26 ± 2.16^{a}	1.89 ± 0.25^{a}
Defatted seed	74.89 ± 3.95^{b}	26.15 ± 1.61^{a}	1.56 ± 0.29^{a}
Seed	65.83 ± 7.73^{b}	27.54 ± 2.79^{a}	1.66 ± 0.17^{a}

Values were expressed as mean±SEM and considered significant at $p<0.05$. Values with different superscript along the same row are significantly different (Sample size, 5)

3.1.2. Effects of *C. milleni* on the live function of Rats

The liver function markers of rats fed with *C. milleni* seed and pulp are showed in Table 3. Aspartate Amino Transferase (AST) activity increased significantly ($p<0.05$) in the groups fed with the defatted seed and seed, while no significant difference ($p>0.05$) was observed between the control and group fed with pulp. No significant difference was observed in the Alanine Amino Transferase (ALT) and Gamma Glutamyl Transferase (GGT) activities across the groups.

3.1.3. Effects of *C. milleni* on the Renal function of Rats

Figure 2a and b represented the creatinine and urea concentration respectively of rats fed with *C. milleni* and the commercial diet. Highest creatinine concentration was observed in the control group. Comparison between the control

and groups fed with pulp and seed of *C. milleni* showed no significant difference ($p<0.05$). However, significant decrease was observed in the creatinine concentration of the group fed with the defatted seed. Urea on the other hand decreased significantly ($p<0.05$) in the group fed with seed of *C. milleni* while no significant difference ($p>0.05$) was observed across the other groups.

3.1.4. Effects of *C. milleni* on the histology of liver and kidney of rats

3.1.4.1. Effects of C. milleni on the liver histology of rats

Plate 3a and b represent the photomicrograph of haematoxylin and eosin (H and E) stained liver section of rats in the control and treatment groups at 100um and 50um magnifications respectively. The representative photomicrograph showed the portal triad (PT), hepatic vein (HV), hepatic artery (HA), hepatic duct (HD), bile duct (BD) and the well distributed hepatocytes (H). Observed in the photomicrograph included milddegenerative changes that can be termed as necrosis, some mild fibrosis and hemorrhage characterized by the presences of infiltrated red hemorrhagic and inflammatory cells (Group B-D). Also observed is a distortion (well defined dilation in the portal triad) in the walls of the blood vessels (red arrow), pyknotic hepatocytes and signs of inflammation (red arrow) is seen more especially in the group fed with *C. milleni* seed powder (group D). Group A on the other hand showed normal morphological presentation with an array of well outlined hepatocytes as well as the portal triad system.

3.1.4.2 Effects of C. milleni on the kidney histology of rats

The representative photomicrograph of H and E stained section of the kidney of rats across the treatment and the control groups are presented in plate 4a and b. Demonstrated across the study groups is the Renal Corpuscles (RC), Renal glomeruli (G), Macula densa (MD), Distal and Proximal (DCT & PCT) convoluted tubules (renal tubules RT) and the bowman's capsule (BC). The red arrows indicate areas with marked pathomorphological changes.

The collagen (type IV) of the basement membrane outlines the glomerular capillaries. The collagen of the parietal layer (PL) of Bowman's capsule (BC) and the basal membrane (BM) of a distal tubule are observable from the photomicrographs. Marked degenerative changes characterized with fibrosis and hemorrhage (red arrows) showing varying degrees of renal injury evidenced by focal sclerosis of the glomerulus, widening of the Bowman's space and hyper-cellularity and complete collapse of the glomerulus. There is hyaline arteriolosclerosis, interstitial fibrosis, interstitial inflammation, as well as acute tubular necrosis are all observed in a mild to severe degree in treated group B-D relative to group A that appears normal. Features are consistent with chronic glomerulonephritis and or glomerulosclerosis. (Red arrows).

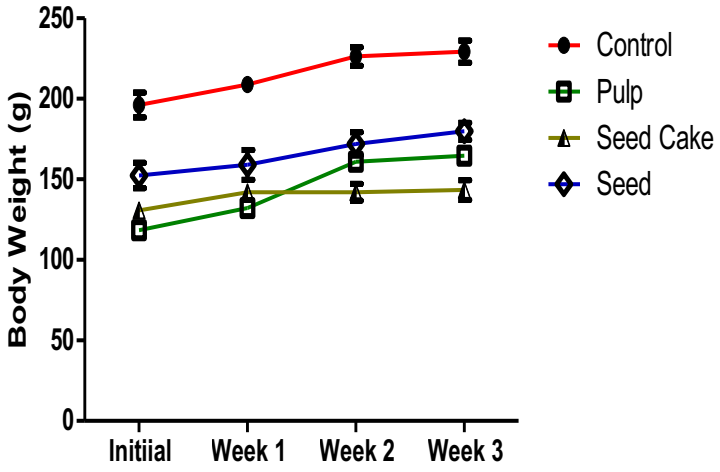

Figure 1. Weekly total body weight of Rats fed with *C. milleni*
Values were expressed as mean±SEM

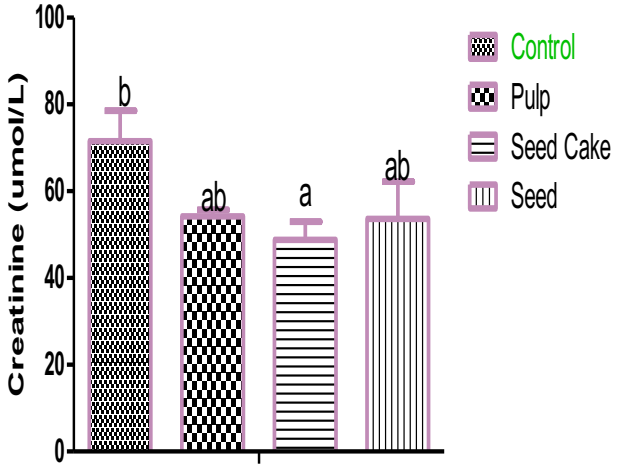

Figure 2 a, Serum creatinine concentration in Rats fed with *C. milleni*
Values were expressed as mean±SEM and considered significant at $p<0.05$. Values with different superscript are significantly different

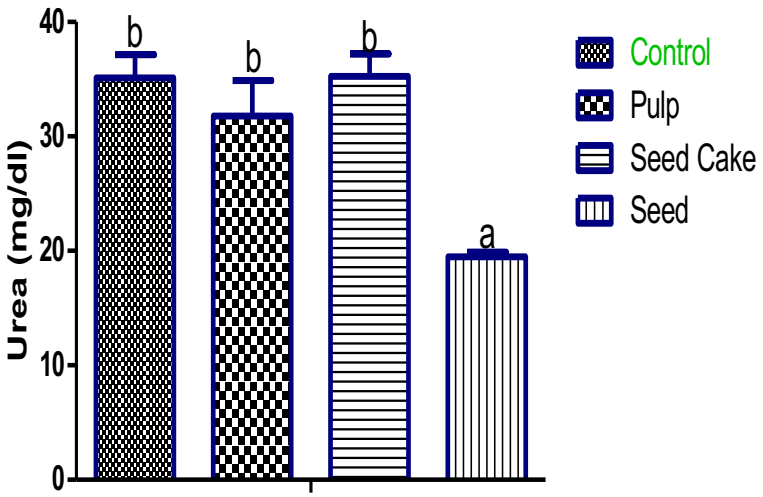

Figure 2b, Serum Urea concentration in Rats fed with *C. milleni*
Values were expressed as mean±SEM and considered significant at p<0.05. Values with different superscript are significantly different

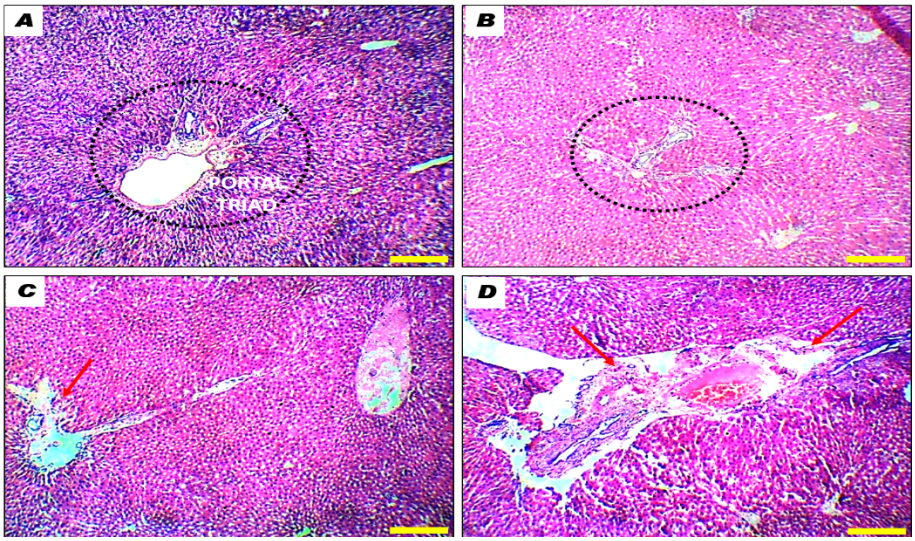

Plate 3a, Photomicrographs of the panoramic views of liver general micromorphological presentations in Adult Wistar rats across the study groups. Hematoxylin and Eosin stain (scale bar 100um).

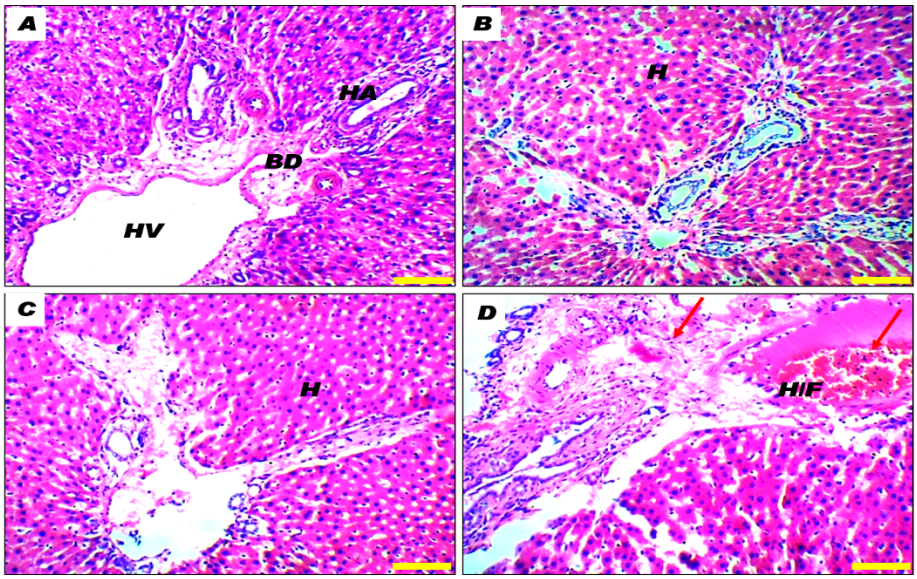

Plate 3b, Photomicrographs of liver general micromorphological presentations in Adult Wistar rats across the study groups. Hematoxylin and Eosin stain (scale bar 50um).

Key
A, Control group
B, Group fed with the pulp of *C. milleni*
C, Group fed with the defatted seed of *C. milleni*
D, Group fed with the seed of *C. milleni*

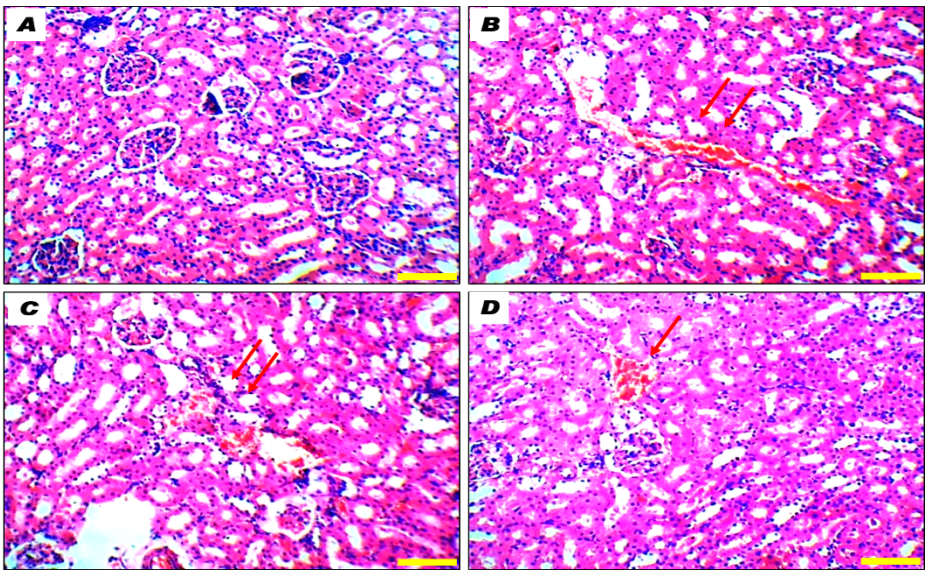

Plate 4a, Photomicrographs of the renal cortex showing panoramic views of Kidney general micromorphological presentations in Adult Wistar rats across the study groups. Hematoxylin and Eosin stain (scale bar 100um).

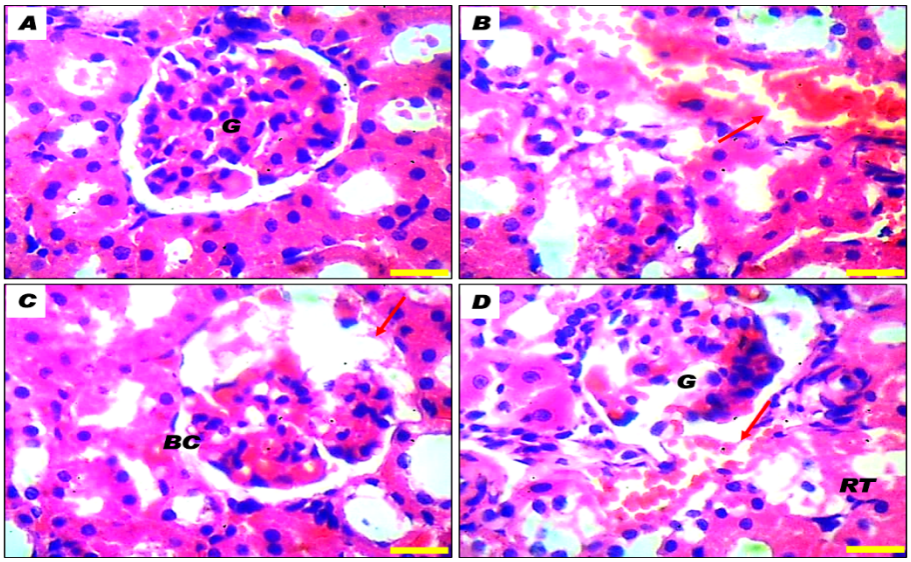

Plate 4b, Photomicrographs of the renal cortex showing magnified views of Kidney general micromorphological presentations in Adult Wistar rats across the study groups. Hematoxylin and Eosin stain (scale bar 50um).

Key
A, Control group
B, Group fed with the pulp of *C. milleni*
C, Group fed with the defatted seed of *C. milleni*
D, Group fed with the seed of *C. milleni*

3.2. Discussions

Cola millenii is an important plant of the transitional forest of Southern Nigeria (Ubon *et al*., 2008). The current interest of various experiment on the plant is owned to its many nutritional potentials (Borokini *et al.,* 2014; Ibironke *et al.,* 2013; Odugbemi, 2006). Data in the literature presents conflicting evidences as regards the nutritional risk of consuming *C. millenii* as food. Therefore, this present study assessed the health risk of consuming *C. m*illenii seed and pulp in Albino rats. In this study, no death was observed among the experimental groups and the animals gained weight normally throughout the period of study. Therefore, feeding of the animals with *C. millenii* had no lethal effects as well as negative impact on the growth, food and water intake of the experimental animals.

Liver, which is the organ responsible for metabolism of most xenobiotics, is susceptible to chemical injury (Hodgson *et al.,* 1988). Kidney on the other hand functions in getting rid of the waste materials, which are either ingested or produced by detoxification process of the liver (Olorunnisola *et al.,* 2019). Therefore, accumulation of these toxic metabolites or chemicals predisposes them to damage (Arthur and John, 2000). In this study, the toxic effect of *C. milleni* seed and pulp was assessed in the liver and kidney of rats.

Hence, the biochemical parameters monitored are useful indices for the assessment of liver and renal toxicity. It included the Asparatate Amino Transferase (AST), Alanine Amino Transferase (ALT), Gamma Glutamyl Transferase (GGT), creatinine, and urea.

AST, ALT and GGT enzymes are mainly localized in the liver and are released into circulation upon damage to the hepatic cells. Therefore, increase in their serum activity is usually an indication of damage to the liver cells and their concentration could reflect the extent of hepatotoxicity (Olorunnisola *et al.,* 2019). Observation in this study i.e. increased observed in the AST activity of the group fed with the

defatted seed and whole seed might reflect the toxic nature of *C. millenii* seed.

The histopathological studies on the liver further substantiate the result of the biochemical studies. The mild degenerative changes observed in the group fed with the seed of *C. millenii* are a reflection that the oil of *C. mileni* is toxic to the liver. Ogbeh *et al.* (2018) previously recorded the high acidity of *C. millenii* oil. According to the study, *C. millenii* had acid value far above the recommended for cooking oil and the value established by the Ministry of Public Health for various types of edible fats and oils. The results in this study also support the report of Itoandon *et al.* (2016) who reported potential toxicity of leaf extracts of *C. milleni.*

In the monitoring of renal function, urea and creatinine concentration can give insight to the effects of the plant sample on the tubular and glomerular functions of the kidney (Ukwuani *et al*., 2012). Therefore, high serum urea and creatinine level may be a pointer of renal failure (Hodgson *et al.*, 1988). Furthermore, removal of creatinine from plasma is achieved during glomerular filtration process and excreted in the urine. However, when the plasma level increases above normal, creatinine could also be excreted through the tubules. The implication is that the serum creatinine level in renal disease generally does not increase until the renal function is substantially impaired (Faulkner*et al.,*, 1982).

Observations in this study substantiate the claim as no distortion was observed in the renal excretion of creatinine and urea whereas, histological studies revealed mild damage in the renal architecture.

Anti-nutritional factors in plants have been reported to cause modification in biochemical processes of the body. For instance, tannin was reported to reduce protein digestibility consequently reduce amino acid bioavailability (Aletor, 1993; Carnovale *et al.,* 1991; Chai and Liebman, 2004). In this study, decreased serum urea concentration, which is an excretory product of protein digestion observed in the group fed with the seed, relative to the control group might be a reflection of decreased amino acid bioavailability rather than renal protection.

4. Conclusions

The result of this study showed that *C. millenii* seed might be slightly toxic to the liver and kidney. The study also revealed that the oil of the seed might have contributed to this toxicity in rats.

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EFFECT OF PRETREATMENTS AND DRYING METHODS ON PHYSICOCHEMICAL PROPERTIES OF UNRIPE PLANTAIN FLOUR AND SENSORY ACCEPTABILITY OF ITS COOKED DOUGH (*AMALA*)

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ABSTRACT

Effect of pretreatments (sulphiting and blanching) and drying methods (sun, oven and freeze) on physicochemical properties and acceptability of unripe plantain flour was investigated. Proximate compositions, selected mineral contents and functional properties of the flour, as well as the sensory attributes of their cooked dough (*amala*) were determined using standard methods. Pretreatments and drying methods had varying individual and interactive effects on the unripe plantain flour. Moisture contents of the flour, except the blanched sun-dried sample, were lower than the 10% recommended by Standard Organisation of Nigeria as the maximum safe-keeping limit for flour. Carbohydrate contents were generally high (80.33-83.06%) but protein (2.17-2.87%), fat (1.30-1.56%) and ash (1.75-2.10%) were most retained in freeze-dried samples and blanching caused about 40-47% reduction in crude fibre. Pretreatments reduced ($p<0.05$) calcium (32.15-30.07 mg/100g) but increased ($p<0.05$) phosphorus (21.51-23.00 mg/100g) contents of sun-dried flour while sodium generally increased with sulphiting. Freeze-dried flour had highest bulk density (0.83-0.84 g/ml), swelling index (4.12-4.17%), water absorption capacity (24.80-27.68%) and dispersibility (60.10-63.00%). Cooked dough (*amala*) prepared from the various unripe plantain flour were all accepted by the panelists but those from freeze-dried flour were the most generally acceptable with the sulphited-freeze-dried unripe plantain cooked dough being rated as the best.

1. Introduction

Plantain (*Musa paradisiaca*) is a perennial tree crop which belongs to the kingdom Plantae and the family *Musaceae*. It is attractive to farmers due to its low requirement of labour and agricultural input for production (Marriott and Lancester, 1983) and has been reported to contribute to subsistence economies in Africa. Production of plantain in Nigeria was reported to have doubled within two decades before 2010 and the fruit was ranked third among

starchy staples (Akinyemi *et al.,* 2010), serving as a source of carbohydrates for millions of people in the country. Plantain is low in fat and protein but rich in essential minerals (Karim *et al*., 2015).

The African landrace, "Agbagba*",* at the green stage, has a moisture content of about 61% (on wet basis), which increases during ripening to about 68% due to carbohydrate hydrolysis (Adeniji *et al.*, 2006). The high moisture content of plantain makes it a perishable fruit. In Nigeria, for instance, quite a fraction of over 80% of plantain harvested during the peak period between September and February goes into a waste (Ogazi, 1996). This limits off-season availability of the fruit and, in particular, its use by urban populations. As noted by Karim (2005) for fruits and vegetables, one of the most difficult aspects of plantain production is the maintenance of its freshness as there is a physiological deterioration during storage resulting to a significant loss of nutritional value and in many cases, loss of the whole fruits. This phenomenon is usually worse with stored ripe fruits. Unripe plantain on the other hand has a relatively longer shelf life and a more stable physical and nutritional quality. In addition, it offers some health benefits such as reduction of blood sugar level owing to its rich content of dietary fibre (8.20%) and resistant starch (16.20%) as well as essential micronutrients (Chinma *et al*., 2012).

A technique to further extend the shelf life and utilization of unripe plantain is the processing of the fruit into flour which can then be stirred in appropriate quantity of boiling water to form gel (*amala*) and eaten with vegetable soup or any other soup of one's choice. Processing into flour adds value to plantain for both local market and export purposes (Zakpaa *et al.,* 2010; Falade and Ogunwolu, 2012) while also helping to curtail glut (Ogundare-Akanmu, *et al.,* 2012). Drying prior to milling into flour significantly extends the shelf life of a food material as moisture content and water activity will have greatly reduced. Different methods of drying including cabinet, solar, sun, oven, microwave oven, foam-mat, fluidized bed, tray and freeze drying have been reported to influence the physical, chemical, functional and nutritional qualities of unripe plantain flour (Emperatríz *et al.,* 2008; Falade and Olugbuyi, 2010; Falade and Oyeyinka, 2015; Yarkwan and Uvir, 2015; Arinola *et al*., 2016; Fadimu *et al*., 2018a; Ndayambaje *et al*., 2019). This is associated with different rate and pattern of heat and mass transfer, due to different structural make-up of the material being dried (Karim, 2010). Although most of these authors employed a single or combined pretreatments among sulphiting, osmotic dehydration and blanching to control the plantain products' qualities, the influence of pretreatments, singly or in combination with different drying methods was not distinctively elucidated.

Enzymatic browning reaction, attributable to the activity of polyphenol oxidase (tyrosinase) (Carbonaro and Mattera, 2002) which often influences the appearance of food materials, is one of the challenges faced during the processing of plantain. This makes pretreatment an essential part of plantain processing operation. Based on this, Fadimu *et al*. (2018b) have researched and reported blanching temperature of 50 ^{0}C, a blanching time of 6.447 min and 1% potassium metabisulphite to be the optimum pre-treatment conditions for unripe plantain based on the functional properties and proximate composition of the unripe plantain flour. However, the effectiveness of pretreatments, singly in arresting enzymatic browning and other undesirable changes and in combination with different drying methods to produce high quality and acceptable plantain products is worth given special consideration. Therefore, this study aimed at investigating the effect of different pretreatments (sulphiting and blanching) and drying methods (sun, oven and freeze) on the physicochemical properties of unripe plantain flour and sensory acceptability of its cooked dough (*amala*).

2. Materials and methods

2.1. Materials

Mature unripe plantains (*Agbagba*) used for this study were obtained from Ganmo market, Ilorin, Kwara State. The plantains were of stage 1 maturity with acceptable quality for consumption.

2.2. Experimental Design

A two-factorial design adopted for this study was from three levels of pretreatments (control, sulphiting and blanching) and three drying methods (sun, oven and freeze-drying). There were 3 controls (no pretreatment), resulting to nine (3 x 3) treatments (Table 1).

2.3. Pretreatments

The unripe plantains were cleaned, peeled, washed and manually sliced (approximately 2 mm thickness). The sliced plantains were divided into three batches. Two batches each containing 5 kg of plantain slices were subjected to different pretreatments. One batch was dipped in 1,500 ppm sodium metabisulphite ($Na_2S_2O_5$) solution for 2 min, while the other was placed in a plastic drainer and then immersed in hot (70 ^{0}C) water for 2 min. Both were adequately drained on wire mesh. The third batch of the sliced plantain was used as the control (without pretreatment).

2.4. Drying of Pretreated Unripe Plantain Flour

2.4.1. Freeze Drying

Each of the batches of pretreated unripe plantain slices and the batch without pretreatment were spread out in layers of about 1 cm thickness in a separate tray-drying accessory at -18 ^{0}C for 24 hrs and then lyophilized using a Freezone 4.5 lyophilizer (Labconco; Missouri, USA) for 5 hrs as described by Emperatríz *et al*. (2008). The resulting dried unripe plantain slices were milled into flour, packed and sealed inside hermetic plastic containers and stored in a cool dry place for further analyses.

2.4.2. Oven Drying

Pretreated unripe plantain slices and the ones without pretreatment were dried in an air draft oven (Gallenkamp 300 plus series, England) at 70^{0}C for 10 hrs (Chinma *et al.,* 2012). These were then milled into flour and subsequently handled as described for freeze-dried samples.

2.4.3. Sun Drying

Another batch of unripe plantain slices (with and without pretreatments) were thinly spread out on trays and placed in the sun between 10:00 am and 4:30 pm (Agoreyo *et al*, 2011) for 3 days at 35$\pm$2 ^{0}C after which they were milled into flour, packaged and stored as described for freeze-dried samples.

2.5. Determination of Chemical Properties of Unripe Plantain Flour

The proximate compositions including moisture, crude protein, crude fat, crude fibre and ash were determined following the methods of AOAC (2005). Carbohydrates were determined by difference. Mineral contents such as calcium, iron and sodium, were determined using Atomic Absorption Spectroscopic method while colorimetric method involving the use of ammonium vanadate reaction was adopted for phosphorus determination (James, 1995).

2.6. Determination of Functional Properties of Unripe Plantain Flour

Bulk density, swelling index and water absorption capacity of the unripe plantain flour were determined following the methods described by Zakpaa *et al.* (2010) while the method described by Ohizua *et al.* (2017) was used to determine dispersibility of the flour in water.

2.7. Preparation of Unripe Plantain Cooked Dough (*Amala*)

Cooked stiff dough (*amala*) was prepared following the procedures described by Karim *et al*. (2013). Briefly, unripe plantain flour was gently and gradually poured into boiling water with continuous stirring using a wooden stirrer until a homogenous paste was formed. The

paste was covered and left on the heat source for about 5 min to cook. It was further stirred before being wrapped in polythene nylon and kept in a food warmer for subsequent sensory analysis.

2.8. Sensory Evaluation of Unripe Plantain Cooked Dough (*Amala*)

Fifty (50) panellists, comprising staff and students who are conversant with the quality attributes of *amala* made from yam flour, were drawn from the Department of Home Economics and Food Science, University of Ilorin. They were requested to assess the colour, aroma, mouldability and overall acceptability of the coded unripe plantain *amala* samples using a 9-point hedonic preference scale ranging from 1 (dislike extremely) to 9 (like extremely).

2.9. Statistical Analysis

Results of the various analyses were subjected to analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS, version 16.0) and means generated were separated using Duncan multiple range test ($p < 0.05$).

3. Results and discussion

3.1. Proximate Composition of Unripe Plantain Flour

Results show that the various drying and pretreatment methods had significant ($p < 0.05$) effects on the moisture content of unripe plantain flour (Table 2). For each of the pretreated and control (without pretreatment) set of samples, freeze-dried plantain flour recorded the lowest moisture contents (7.36-9.29%). The highest rate of moisture removal by freeze-drying obtained in this study is similar to the finding of Emperatríz *et al.* (2008) and Shofian *et al.* (2011) who reported lower moisture contents with freeze-drying than any other methods of drying studied. The range of moisture contents of the various plantain flour, except for the blanched sun-dried sample, was below the level (10%) recommended by Standard Organisation of Nigeria (SON) for safe keeping quality of flour (Chinma *et al.,* 2012). Moisture content is an important indication of product quality stability. Unripe plantain flour without pretreatment were higher (4.07-4.13%) in crude fibre than the sulphited (3.20-3.85%) and blanched (2.13-2.46%) samples. Drying methods brought about no significant difference in crude fibre contents of the unripe plantain flour without pretreatment. Emperatríz *et al.* (2008) and Gwanfogbe *et al*. (1988) observed that dehydration methods had no significant effect on the dietary fibre of flour. However, the effect of drying methods appeared to be enhanced by the pretreatments with blanching exhibiting most effect and reducing the crude fibre content by about 40% in freeze-dried and 47% in oven-dried samples. In their report, Arisa *et al.* (2013) recorded 35% reduction in the crude fibre content of blanched plantain flour. This suggests that alteration of food tissues through pretreatment breaks down some of the components, such as resistant starch, that may have rather been present as fibre constituent. The range of values obtained in this study are however similar to the range of 2.28-4.44% reported by Arisa *et al.* (2013) and 2.56-3.21% reported by Fadimu *et al*. (2018a). Crude fibre, which is a type of dietary fibre remaining after food has been subjected to acid and alkali treatments, has useful biological functions such as aiding of digestion and diabetes management (Anderson *et al*., 2009).

The protein content of the unripe plantain flour without pretreatment was significantly ($p < 0.05$) higher with freeze-drying than sun and oven drying methods. Since the reverse was recorded in moisture contents, the apparent effect of drying methods on protein content may actually be an indirect effect of reduced moisture, bringing about different levels of concentrated proteins in the flour samples. Generally, sulphiting had a more significant increase on the protein proportion of unripe plantain flour than did blanching. Though the range of protein contents recorded in this study

for unripe plantain flour was generally low (2.17-2.87%), it was not unexpected since unripe plantain flour is typically poor in protein supply (Zakpaa *et al.,* 2010; Karim *et al*. 2015; Fadimu *et al*. 2018a). Besides, the cooked dough (*amala*) is commonly eaten alongside soup prepared with meat or any other source of protein such as soycheese (meat analog). Another strategy to compensate for the low protein content is fortification of the flour with a protein rich material such as soyflour.

Pretreatment methods did not show a particularly defined significant effect on the fat contents of the unripe plantain flour but freeze-dried samples generally had the highest range of fat contents (1.45-1.56%) while the oven-dried samples had the lowest (1.35-1.36%). A similar finding was reported by Emperatríz *et al.* (2008) that freeze-dried and oven-dried unripe plantain flour had highest and lowest fat contents, respectively. Freeze-dried unripe plantain flour recorded the highest ash contents (1.97-2.10%), significantly varied with pretreatment methods, while sun-dried samples recorded the lowest (1.75-1.79%) but did not show any significant response to pretreatment differences (Table 2). The ash contents obtained in this study were similar to those (1.98-2.30%) reported by Emperatríz *et al.* (2008). Ash content represents the amount of minerals in food stuff.

The carbohydrate content of the unripe plantain flour ranged from 80.33-83.06% with blanched samples having the highest values while the samples without pretreatment (control) had the lowest. This again is suggestive of the possibility that as pretreatment alters the tissue matrix of food materials, more carbohydrate constituents may have been made more available for chemical analysis, which by extension, could be indicative of a digestive advantage. A similar effect of pretreatment on the carbohydrate content of plantain flour was observed by Arisa *et al.* (2013). Carbohydrate rich foods supply energy to the body more readily than any other energy source, and energy supply is the main contribution of plantain to diet (Zakpaa *et al.,* 2010).

3.2. Mineral Content of Unripe Plantain Flour

Calcium and phosphorus were more predominant in the unripe plantain flour than iron and sodium (Table 3). Sulphiting had a significant ($p < 0.05$) effect, increasing the sodium content of the unripe plantain flour. This could be attributed to sodium up-take from sodium metabisulphite solution used as one of the pretreatments. Blanching and sulphiting had significant ($p < 0.05$) effect on sun-dried unripe plantain flour, both causing decreased in calcium and increased in phosphorus contents. Freeze-dried samples were generally significantly ($p < 0.05$) higher in iron content than their sun-dried counterparts. The sodium contents (2.07-2.39%), though a bit higher than the range of values (0.52-1.05%) reported by Arisa *et al.* (2013) for pretreated plantain flour, were generally low, and this could be recommended for a low-sodium diet (Ojure and Quadri, 2012). It can be used in the management of high blood pressure and heart disease (Dzomeku *et al*., 2007) The findings of Ojure and Quadri (2012) support the relatively higher amount of calcium obtained in this study for unripe plantain flour. The authors reported a value as high as 71.5 mg/100g in the variety of plantain studied.

3.3. Functional Properties of the Unripe Plantain Flour

Generally, the bulk densities of freeze-dried unripe plantain flour (0.83-0.84 g/ml) were higher than those of oven and sun-dried samples (0.76-0.81 g/ml and 0.79-0.82 g/ml, respectively), but drying methods had no individual significant effect (Table 4). Blanching was the only pretreatment that appeared to have singly reduced bulk density significantly ($p < 0.05$) and this was just in oven-dried unripe plantain flour (i.e., between oven-dried blanched and oven-dried unblanched samples). This was similar to the finding of Arisa *et al*. (2013) who reported that

blanched plantain flour had significantly lower bulk density than sulphited plantain flour and control (without pretreatment). Zakpaa *et al.* (2010) reported a slightly lower value (0.755 g/ml) for ripe plantain flour. Bulk density is an important factor for bulk storage and transportation.

Similarly, freeze-dried unripe plantain flour showed the highest swelling indices (4.12-4.17%) while oven-dried samples recorded the lowest range of value of 3.11-3.25%. Drying and pretreatment methods had both individual and interactive significant ($p < 0.05$) effects on the swelling indices of unripe plantain flour, with blanching causing most increase in swelling indices. Freeze-dried plantain flour had better swelling indices probably due to the destructive effect of other drying methods on the starch components of the plantain (Olawuni *et al.,* 2013). Swelling index is an indication of the absorption index of the starch granules during heating.

Methods of drying showed a significant ($p < 0.05$) effect on the water absorption capacity of unripe plantain flour without pretreatment, with freeze-dried and oven-dried controls having 27.68% and 25.31%, respectively. Pretreatments methods had significant ($p < 0.05$) effect on water absorption capacity of the unripe plantain flour, except in oven-dried samples. The unripe plantain flour that were not pretreated showed significant ($p < 0.05$) differences in dispersibility with drying methods. The dispersibility of the flour which ranged between 60.10% and 63.00% was higher than 52.00-56.00% reported for unblanched cooking banana and sweet potato flour, respectively, by Ohizua *et al.* (2017). This means that the unripe plantain flour will be relatively easier to reconstitute in the making of consistent cooked dough (*amala*).

3.4. Sensory Quality of Pretreated Sun, Oven and Freeze-Dried Unripe Plantain Cooked Dough (Amala)

Table 1. Factorial design for the study

Pretreatment	Control (No pretreatment)	Sulphiting	Blanching
Drying method	Oven drying	Sun drying	Freeze drying

Table 2. Proximate composition of pretreated sun, oven and freeze-dried plantain flour

Sample	**Moisture (%)**	**Crude Protein (%)**	**Fat (%)**	**Ash (%)**	**Crude fibre (%)**	**Carbohydrate (%)**
NS_U	9.19 ± 0.09^{bcd}	2.30 ± 0.04^{fg}	1.30 ± 0.01^{ef}	1.75 ± 0.01^{e}	4.10 ± 0.09^{a}	81.36 ± 0.05^{ab}
BS_U	10.11 ± 0.05^{a}	2.36 ± 0.15^{ef}	1.44 ± 0.04^{bcd}	1.77 ± 0.01^{e}	2.42 ± 0.01^{e}	81.90 ± 0.03^{a}
SS_U	9.80 ± 0.02^{bc}	2.61 ± 0.01^{cd}	1.45 ± 0.03^{bc}	1.79 ± 0.01^{e}	3.64 ± 0.04^{bc}	80.71 ± 0.00^{b}
NO_V	8.15 ± 0.02^{de}	2.17 ± 0.03^{g}	1.36 ± 0.03^{de}	1.87 ± 0.02^{d}	4.07 ± 0.05^{a}	82.38 ± 0.14^{a}
BO_V	8.74 ± 0.05^{bcde}	2.65 ± 0.03^{bc}	1.38 ± 0.03^{cde}	2.04 ± 0.03^{b}	2.13 ± 0.02^{f}	83.06 ± 0.06^{a}
SO_V	9.53 ± 0.01^{bcd}	2.63 ± 0.02^{cd}	1.35 ± 0.01^{ef}	2.04 ± 0.02^{b}	3.20 ± 0.01^{d}	81.25 ± 0.08^{ab}
NF_R	7.36 ± 0.03^{e}	2.67 ± 0.04^{bc}	1.45 ± 0.04^{bcd}	1.97 ± 0.01^{c}	4.13 ± 0.08^{a}	82.42 ± 0.08^{a}
BF_R	8.46 ± 0.04^{cde}	2.84 ± 0.01^{ab}	1.53 ± 0.01^{ab}	2.04 ± 0.01^{b}	2.46 ± 0.01^{e}	82.67 ± 0.03^{a}
SF_R	9.29 ± 0.01^{bcd}	2.87 ± 0.0^{a}	1.56 ± 0.01^{a}	2.10 ± 0.01^{a}	3.85 ± 0.01^{b}	80.33 ± 0.07^{b}

Data are reported as mean ± standard deviation of triplicate determinations. Mean scores within the same column with different superscripts are significantly ($p < 0.05$) different.

NS_U = Non-pretreated sun-dried unripe plantain flour

BS_U = Blanched sun-dried unripe plantain flour

SS_U = Sulphited sun-dried unripe plantain flour

NO_V = Non-pretreated oven-dried unripe plantain flour

BO_V = Blanched oven-dried unripe plantain flour
SO_V = Sulphited sun-dried unripe plantain flour
NF_R = Non-pretreated freeze-dried unripe plantain flour
BF_R = Blanched freeze-dried unripe plantain flour
SF_R = Sulphited freeze-dried unripe plantain flour

Table 3. Mineral contents of pretreated sun, oven and freeze-dried unripe plantain flour

Samples	Calcium (mg/100g)	Phosphorus (mg/100g)	Iron (mg/100g)	Sodium (mg/100g)
RP	29.31±0.02^{d}	21.47±0.06^{e}	2.28±0.04^{c}	2.07±0.02^{d}
NS_U	32.15±0.05^{a}	21.51±0.03^{e}	2.30±0.03^{c}	2.13±0.02^{c}
BS_U	30.69±0.03^{bc}	22.63±0.01^{d}	2.33±0.05^{c}	2.19±0.01^{c}
SS_U	30.07±0.01^{c}	23.00±0.04^{c}	2.35±0.01^{c}	2.27±0.04^{bc}
NO_V	31.74±0.05^{a}	23.14±0.05^{c}	2.39±0.01^{bc}	2.39±0.01^{bc}
BO_V	31.39±0.08^{ab}	23.63±0.02^{bc}	2.42±0.02^{b}	2.18±0.07^{c}
SO_V	31.43±0.02^{ab}	24.03±0.02^{ab}	2.44±0.03^{ab}	2.29±0.03^{b}
NF_R	29.97±0.06^{c}	23.92±0.01^{ab}	2.47±0.03^{a}	2.09±0.01^{d}
BF_R	29.72±0.02^{cd}	24.80±0.01^{a}	2.48±0.02^{a}	2.14±0.01^{c}
SF_R	29.84±0.01^{cd}	25.31±0.03^{a}	2.46±0.01^{a}	2.38±0.05^{a}

Data are reported as mean ± standard deviation of triplicate determinations. Mean scores within the same column with different superscripts are significantly ($p < 0.05$) different.
RP = Ripe plantain flour
NS_U = Non-pretreated sun-dried unripe plantain flour
BS_U = Blanched sun-dried unripe plantain flour
SS_U = Sulphited sun-dried unripe plantain flour
NO_V = Non-pretreated oven-dried unripe plantain flour
BO_V = Blanched oven-dried unripe plantain flour
SO_V = Sulphited sun-dried unripe plantain flour
NF_R = Non-pretreated freeze-dried unripe plantain flour
BF_R = Blanched freeze-dried unripe plantain flour
SF_R = Sulphited freeze-dried unripe plantain flour

Table 4. Functional properties of pretreated sun, oven and freeze-dried unripe plantain flour

Samples	**Bulk density (g/ml)**	**Swelling power (%)**	**Water absorption capacity (%)**	**Dispersibility (%)**
NS_U	0.82±0.01^{ab}	3.05±0.01^{h}	26.41±0.01^{b}	62.36±0.04^{c}
BS_U	0.79±0.01^{b}	3.29±0.01^{d}	25.50±0.04^{d}	61.03±0.02^{f}
SS_U	0.81±0.01^{ab}	3.10±0.01^{g}	25.01±0.01^{c}	62.63±0.03^{b}
NO_V	0.79±0.02^{b}	3.11±0.01^{g}	25.31±0.01^{cd}	61.94±0.01^{d}
BO_V	0.76±0.01^{c}	3.25±0.01^{e}	25.00±0.01^{cd}	62.02±0.04^{d}
SO_V	0.81±0.01^{ab}	3.13±0.01^{f}	25.52±0.01^{cd}	61.98±0.01^{d}
NF_R	0.83±0.02^{ab}	4.12±0.01^{c}	27.68±0.02^{a}	60.10±0.14^{g}
BF_R	0.83±0.01^{a}	4.31±0.01^{a}	26.92±0.02^{b}	61.64±0.02^{e}
SF_R	0.84±0.02^{a}	4.17±0.01^{b}	24.80±0.01^{d}	63.00±0.14^{a}

Data are reported as mean ± standard deviation of triplicate determinations. Mean scores within the same column with different superscripts are significantly ($p < 0.05$) different.
NS_U = Non-pretreated sun-dried unripe plantain flour
BS_U = Blanched sun-dried unripe plantain flour

SS_U = Sulphited sun-dried unripe plantain flour
NO_V = Non-pretreated oven-dried unripe plantain flour
BO_V = Blanched oven-dried unripe plantain flour
SO_V = Sulphited sun-dried unripe plantain flour
NF_R = Non-pretreated freeze-dried unripe plantain flour
BF_R = Blanched freeze-dried unripe plantain flour
SF_R = Sulphited freeze-dried unripe plantain flour

Table 5. Sensory attributes of pretreated sun, oven and freeze-dried unripe plantain cooked dough (*amala*)

Samples	Colour	Aroma	Texture	General Acceptability
NS_U	4.80 ± 0.44^{bc}	5.20 ± 0.33^{b}	5.20 ± 0.18^{b}	5.10 ± 0.34^{b}
BS_U	5.80 ± 0.39^{bc}	5.80 ± 0.33^{b}	5.70 ± 0.31^{b}	5.90 ± 0.53^{bc}
SS_U	5.90 ± 0.56^{b}	5.50 ± 0.30^{b}	5.90 ± 0.47^{b}	6.20 ± 0.56^{b}
NO_V	5.60 ± 0.26^{b}	4.90 ± 0.31^{b}	4.60 ± 0.40^{bc}	5.70 ± 0.45^{bc}
BO_V	6.30 ± 0.36^{b}	5.50 ± 0.37^{b}	4.80 ± 0.30^{bc}	6.70 ± 0.51^{bc}
SO_V	6.50 ± 0.58^{d}	5.10 ± 0.55^{b}	6.40 ± 0.53^{c}	6.80 ± 0.58^{c}
NF_R	5.20 ± 0.64^{bc}	6.00 ± 0.49^{b}	7.20 ± 0.41^{b}	6.10 ± 0.68^{bc}
BF_R	7.90 ± 0.31^{a}	7.30 ± 0.30^{a}	7.80 ± 0.32^{a}	7.80 ± 0.32^{a}
SF_R	8.10 ± 0.23^{a}	7.70 ± 0.34^{a}	8.10 ± 0.30^{a}	8.30 ± 0.27^{a}

Mean scores within the same column with different superscripts are significantly ($p < 0.05$) different.
NS_U = Non-pretreated sun-dried unripe plantain flour *amala*
BS_U = Blanched sun-dried unripe plantain flour *amala*
SS_U = Sulphited sun-dried unripe plantain flour *amala*
NO_V = Non-pretreated oven-dried unripe plantain flour *amala*
BO_V = Blanched oven-dried unripe plantain flour *amala*
SO_V = Sulphited sun-dried unripe plantain flour *amala*
NF_R = Non-pretreated freeze-dried unripe plantain flour *amala*
BF_R = Blanched freeze-dried unripe plantain flour *amala*
SF_R = Sulphited freeze-dried unripe plantain flour *amala*

The cooked dough (*amala*) prepared from the various unripe plantain flour (with and without pretreatment) generally had good sensory attributes as indicated by panellists' ratings (Table 5). Similarly, highest rating for colour, aroma, mouldability, consistency, mouthfeel and overall acceptability had been reported for unripe plantain *amala* compared to its moringa leaf powder fortified counterparts (Karim *et al*., 2015). Though both pretreatments (sulphiting and blanching) improved the sensory qualities, including colour, aroma and mouldability of the *amala*, only their interactive effect with freeze-drying was found to be significant ($p < 0.05$). The cooked dough (*amala*) from freeze-dried unripe plantain flour samples had higher general acceptability ratings than those from sun and oven-dried samples. The highest dispersibility of sulphited freeze-dried unripe plantain flour may have contributed to the corresponding highest value of the general acceptability of the *amala* prepared from it.

4. Conclusions

Pretreatments and drying methods had varying individual and interactive effects on the proximate, mineral and functional properties of unripe plantain flour. Sulphiting and blanching enhanced the protein, fat, ash and moisture but reduced the crude fibre of the unripe plantain flour irrespective of drying methods. Moisture contents, except of sun-dried blanched plantain flour, were generally below the maximum level

(10%) recommended for safe keeping quality of flour. Though carbohydrates predominate the proximate compositions of the unripe plantain flour, protein, fat and ash contents were most retained in freeze-dried samples. The unripe plantain flour samples were all rich in calcium and phosphorus but low in sodium, which however, significantly increased with sulphiting. Blanching and sulphiting increased the phosphorus, iron and sodium but reduced the calcium content of the unripe plantain flour. Freeze-drying improved most of the functional properties of the flour and, blanching and sulphiting significantly increased the swelling power and dispersibility of freeze-dried unripe plantain flour. The stiff cooked dough (*amala*) prepared from all the plantain flour samples were favourably rated by the panellists, with sulphiting and blanching improving all sensory attributes. However, the cooked dough (*amala*) prepared from sulphited freeze-dried unripe plantain flour was the most generally accepted.

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A COMBINED QFD-GAHP TECHNIQUE TO TRANSLATE CUSTOMER REQUIREMENTS INTO THE PRODUCTION PROCESS OF MEAT PRODUCTS

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ABSTRACT

Quality Function Deployment (QFD), as one of the quality engineering methods, tries to attract customer's satisfaction and produce a product suitable for the customers' needs. The aim of the present study is to describe the process of producing a meat product (hamburger) based on the customers' need defined on a higher quality level via combining quality function deployment (QFD) and group analytic hierarchy process (GAHP) techniques. At first, the target customers and what they need from a hamburger product were recognized. Then, using GAHP Method the relative weight of each requirement was calculated. The 4-matrix QFD model was used and the house of quality matrix, product design matrix, process design matrix, and process control planning matrix were completed based on technical principles and hygienic standards of meat product producing units. Among recognized customer requirements of hamburger quality, "not being harmful to body" is the most important requirement and "packed in different weights" is the least important one. Research findings indicates that improving the level of technical and engineering characteristics of "frozen meat" would have a significant effect on enhancing the quality of hamburger and consequently on the satisfaction of the customer. Further, it is necessary that in process design matrix, "microbial test of meat" and "microbial test of semi-processed product" be under a precise control.

To our knowledge, the current study investigates the first application of 4-matrix QFD method and its combination with GAHP to identify and develop the technical and qualitative characteristics of the meat products in accordance with the customers' requirements. There is no study in the literature.

1. Introduction

In these times, considering the socio-cultural changes of human societies, fast foods are among items which have a high place in the food baskets. Hamburgers are among these products and are used by many people throughout the world. In Iran, hamburger is a mixture of beef, onion, rusk flour, garlic, and other additives and is produced in different foodstuff industries (Hajimohammadi et al., 2014). With the aim of identifying the type of additives of meat products and their amounts (Jiang and Xiong,

2016) and packing methods of these products (Godziszewska et al., 2017; Hęś and Gramza-Michałowska, 2016; Molina-Besch, 2016) several studies have been made and different countries have enforced standards and rules governing on foodstuff products (Moore et al., 2012; Rowe et al., 2004; Scramlin et al., 2010; Solomakos et al., 2008). However, in development and improvement of these standards and production processes, the demands and expectations of customers have not sufficiently been considered. Taking the customer requirements when drawing up the standards will make foodstuff factories to become more interested and eager to adapt and implement new hygienic rules in production of their products.

In the arena of competitive markets, emphasis has been put on expectations of customers and meeting them in the technical requirements of the product (Paiva and Pinto, 2012). Satisfying customer requirements and expectations and responding properly to the market changes are a prerequisite for companies to compete (Kazemzadeh et al., 2009).

Quality Function Deployment (QFD) is a suitable tool for translating the market requirements into the technical language used by designers and engineers (Kowalska et al., 2015) in different stages of production to answer the voice of customers (Jia and Bai, 2011). The concept of QFD was put forward for the first time in 1966 by Yoji Akao in Japan (Akao and Mazur, 2003). QFD includes three models: 4-Matrix Model, 30-Matrix Model, and 3-Matrix Model (Revelle et al., 1998).

In recent years, QFD underwent different changes and has been combined with many techniques. Among them are: Fuzzy Logic, Kano model, AHP, analytic network process (ANP) and so forth (Sivasamy et al., 2016).

Year 1987 may be considered the beginning year of entering QFD into the world of foodstuff industries. Since then several researches have been published in which the application of QFD in foodstuff industries and its benefits, especially in respect of customer-oriented development, have been explained. In some of these researches, QFD was introduced as a suitable and promising method in facilitating foodstuff development process (Benner et al., 2003). Others, also have introduced QFD as a structured and useful approach to identify customers' needs and expectations from food products (Rudolph, 1995).

The technique has been used in different studies focusing on foodstuff industries. Dalen (1996) studied the capabilities of QFD and possibility of applying it to meat industry. Dalen's findings showed that with the help of QFD, one can find out the important and real demands of the customers in respect of the meat they consume. Accordingly, he took those needs in designing the techno-engineering characteristics of the final product. Waisarayutt and Tutiyapak (2006) studied and evaluated the capability of QFD in process development of a new type of macaroni with the help of cross-cultural consumer characterization (4Cs) and sensory analysis. Mattsson and Helmersson (2007) reviewed customers' opinions about hamburger consumption in order to develop food products and then clustered these opinions by their approach, named text analytical approach. Sayadi et al. (2017) identified the consumers' expectations of olive oil, and reviewed 439 customers' opinions, to formed the House of Quality matrix, in order to incorporate them in the final product design.

QFD has been also used as a tool for increasing the sales amount of a type of probiotic macaroni's in the market (Pinto and Paiva, 2010); as a method for examining the degree of wheat flour customers' satisfaction (Kristianto et al., 2012); as a method for knowing the expectations who use a beef-bearing food (Park et al., 2012); and for examining the production of organic products (de Fátima Cardoso et al., 2015). Also, Expansion of QFD and its combination with Fuzzy numbers has been investigated (Bevilacqua et al., 2012).

The related scientific sources can be reviewed in two aspects:

(1) Using QFD to develop the standards of meat and meat products:

Among the studies, the application of QFD to develop the processes of meat and meat products can be observed in only 2 cases: The study of Dalen (1996) on the possibility of the implementation of OFD in meat industry, and the study of Park et al. (2012) on a detailed identification of the customers' demands for a food made from beef.

(2) 4-matrix approach of QFD

On the other hand, to find out the customers' needs of the foodstuff products, the 4-matrix technique has been used just in one study (Kowalska et al., 2015).

Perhaps, the scientists of foodstuff industries are not well aware of the principal aims of QFD and its potential in foodstuff products development (Costa et al., 2000). While more than half a century has been passed from the birth of QFD concept, the research and studies on foodstuff field still is limited (Benner et al., 2003).

In the literature there is probably no practical study to identify the customers' needs and expectations from fast foods , especially hamburgers, using the 4-matrix approach of QFD and to produce a product in accordance with the customer's taste. Whereas the consumption of these kinds of products shows a growing trend among various countries of the world (Bowman and Vinyard, 2004; Chiang et al., 2011; Jeffery et al., 2006; Majabadi et al., 2016).

The present study seeks to obviate the lack of a study that practically and operationally converts the opinions of a kind of fast food's consumers into the technical features and characteristics of the product. The aim of this study is thus to improve the production process of special 60% red meat-hamburger with the inclusion of customers' demands and to enhance its quality level by combining QFD and GAHP techniques.

2. Materials and methods

2.1. QFD and its 4-Matrix model

While there are differences between various QFD models, the logic and philosophy of all of them are the same and they have no aim but creating a clear and transparent relation between what the customers expect from a product and the product/service process. The 4-matrix approach will be able to answer all the questions about designing and production of a product. This approach can comprehensively review the features and characteristics of the required materials and components and also the manufacturing processes of a product. Besides, the requirements related to planning and control of manufacturing processes can be identified by this approach.

Considering that 4-Matrix model is more common than other models, this model is used in present study. Especially it provides a coverage on different stages of production (Revelle et al., 1998).

The weak point of QFD is that the numbers entered in its matrices may be inconsistent. However, if these numbers be calculated through Group Analytic Hierarchy Process (GAHP), we will not see this inconsistency.

2.2. Group Analytic Hierarchy Process (GAHP) and Its Methodology

Analytic Hierarchy Process is one of the multi-criteria decision making techniques. The technique was first introduced by Thomas L. Saaty (Saaty, 1980) .In this method, first a hierarchical structure of the problem is formed. In this structure, the aim is at the top and indices and alternatives are on other levels. After formation of the hierarchical structure, the indices undergo weighing observation in form of paired comparison first with each other and then with the alternatives. For judging about weighing paired comparison matrix, the quantitative value of 1 to 9 is used. After weighing observation, the weights must be normalized. Then, arithmetic means (relative weight) of all rows of normalized matrices of the paired comparisons are achieved. At the end, the resulted relative weights are multiplied by the arithmetic mean of the alternatives. Now, we can arrange alternatives in a list of priorities. In this technique, Inconsistent Rate (IR) of decision can always be calculated and the decision can be judged to be good or bad, and acceptable or

unacceptable. In every system, the degree of acceptable inconsistency depends on its decision maker, but if the inconsistency degree is more than 1%, the judgments shall be reconsidered. Therefore, calculation of inconsistency degree of paired comparisons matrix too is necessary. For this purpose, paired comparisons matrix (A) is multiplied by the relative weights (W). The resulted vector is called Weighted Sum Vector (WSV). Then, Consistency Vector (CV) is resulted to be as follows (Golden et al., 1989):

$$WSV = A \times W \quad (1)$$

$$CV = \frac{WSV}{W} \quad (2)$$

We call the average of consistency vector elements λ_{max} (the greatest Eigen Value of paired comparison matrix). Having λ_{max}, the Inconsistence Index (II) and inconsistence rate for the group comparisons can be defined as follows (Golden et al., 1989):

$$II = \frac{\lambda_{max} - n}{n - 1} \quad (3)$$

$$IR = \frac{II}{IIR} \quad (4)$$

The variable n represents number of customers' demands which enter into QFD 4-matrix models (n=14) (Eq. 3). In scientific sources, the values of Inconsistency Index Random matrix (IIR) of differentn's have been calculated. Here, for n=14 the value of IIR is 1.57 (IRR=1.57) (Golden et al., 1989) . In case we want to have the opinion of two or more decision makers about paired comparison, we have to resort to group decision making method. Saaty and Aczel have showed that geometric mean method is the best method for consolidating the judgments on group Analytic Hierarchy Process. Thus, geometric mean of the opinions of different experts $a_{ij}^{'}$ is defined as follows (Aczél and Saaty, 1983) (Eq. 5).

$$a_{ij}^{'} = \left(\prod_{l}^{k} a_{ijl}\right)^{\frac{1}{k}} \quad (5)$$
$$l = 1,2,\dots,k;$$
$$i,j = 1,2,\dots,n; i \neq j$$

In Equation 5, l is the code of the decision maker, k is number of decision makers, and i, j are the compared alternatives.

2.3. Stages of the Research

The steps taken in this research have been demonstrated in Figure 1.

2.4. Case Study

The case study for this research was made in Pak Taliseh Foodstuff Industries Company in year 2019. This company started its activities in year 1979 under trade name 202 and at present is one of the biggest producers of meat products in Iran.

2.4.1. Recognizing the Customers and Their Demands

At the beginning, QFD team was formed independently beyond the framework of Pak Taliseh Company organization. The duty of the team at first step was recognizing the base of desired customer. Holding several meetings with the experts and managers of the Company, the high volume of selling and expansion of activities were considered as criteria and as a result chain stores, supermarkets, sellers of ready-to-use foodstuffs, and restaurants were selected as base of desired customer. In continuation of studies, 25 demands that the customers expect from 60% red meat-hamburger were recognized. Then, the demands which were contrary to legal requirements and internal rules of the Company were eliminated, and so only 14 demands were selected for being entered into the House of Quality Matrix.

Table 1. Demands of the customers of 60% red meat-hamburger

Demands	Classification
In-between Paper of the Hamburger be separable Easily	Packaging Feature
The box of the purchased hamburger be not damp (has no wet patch)	
Production series be written on the box	
Packed in different weights	

It shall be fresh	Reliability Feature
It shall have high durability	
It shall be delicious	Qualitative Features
It shall not have bad color when cooked	
It shall not be ill smelling	
It shall be crisp and juicy	
It shall not shrink when is being cooked	
Not being harmful to body	
The surface of raw hamburger shall be smooth	Appearance Features
The surface of raw hamburger shall not be dark	

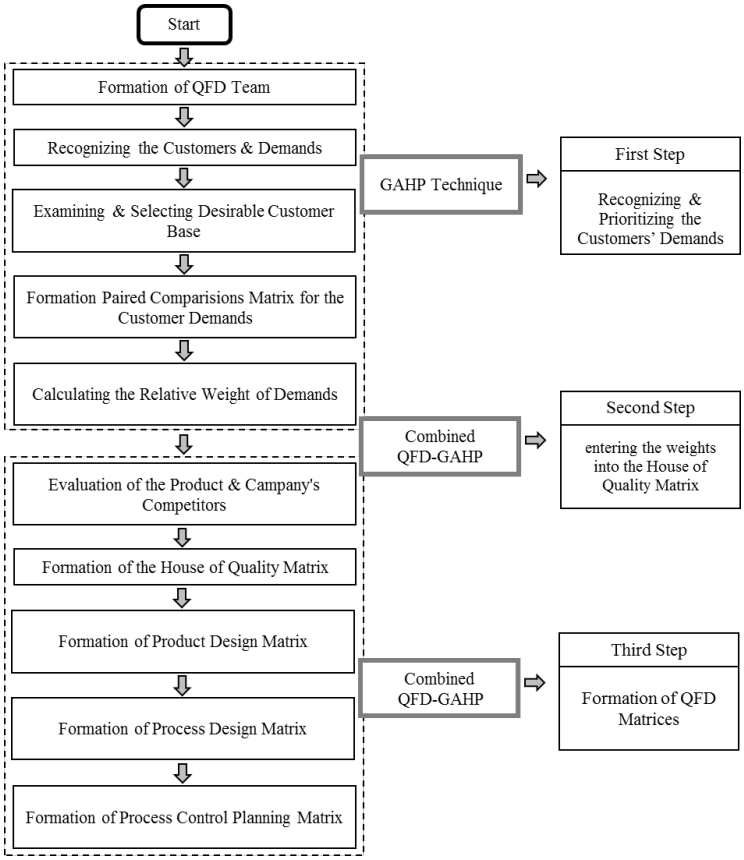

Figure 1. Steps of Combined QFD-GAHP Technique

2.4.2. Calculation of Relative Weight of the Customer's Demands

For allocating weights to the demands of customers, three proficient experts were consulted. The opinions of these three experts were equally considered. In the study, the paired comparisons were made in Expert Choice software and by using the qualitative values from 1 to 9. Each expert provided separately one table for the paired comparisons of 14 demands of the customers (totally, 3 tables were provided). The final comparison table of the demands of the customers was drawn up by combining the comparison tables in geometric mean method. For brevity, only the final table is presented here. As an example, the number 0.5848 in Table 2 (first row, column 14) has been as displayed below (Eq. 6).

$$a_{ij}^{'} = \left(\prod_{l}^{k} a_{ijl}\right)^{\frac{1}{k}} = \left(\frac{1 \times 1 \times 1}{5}\right)^{\frac{1}{3}} = 0.5848 \qquad (6)$$

Table 2. Final table the customers' demands comparison

	In-between Paper of the Hamburger be separable Easily	The box of the purchased hamburger be not damp (has no wet patch)	Production series be written on the box	Packed in different weights	It shall be fresh	It shall have high durability	It shall be delicious	It shall not have bad color when cooked	It shall not be ill smelling	It shall be crisp and juicy	It shall not shrink when is being cooked	Not being harmful to body	The surface of raw hamburger shall be smooth	The surface of raw hamburger shall not be dark
In-between Paper of the Hamburger be separable Easily	1	0.6300	0.8736	0.5848	0.1366	0.1598	0.1208	0.4368	0.1882	0.5503	0.7937	0.1383	1	0.5848
The box of the purchased hamburger be not damp (has no wet patch)	1.5874	1	3.1072	2.4101	0.1376	0.1682	0.1529	0.7469	0.1926	0.7211	0.9086	0.1314	1.9129	1.2051
Production series be written on the box	1.1447	0.3218	1	1.4422	0.1682	0.1771	0.1682	0.4368	0.1598	0.2811	0.4368	0.1257	0.3218	0.4368
Packed in different weights	0.6300	0.4149	0.6934	1	0.1314	0.1609	0.1156	0.3150	0.1598	0.2154	0.2554	0.1111	0.3684	0.2554
It shall be fresh	7.3186	7.2685	5.9439	7.6117	1	1.2164	1	6.0822	1	3.2711	7.2304	0.2811	6.6039	6.6039
It shall have high durability	6.2573	5.9439	5.6462	6.2145	0.8221	1	0.3420	5.3133	0.7937	4	4.3089	0.1682	3.9149	4.8203
It shall be delicious	8.2768	6.5421	5.9439	8.6535	1	2.9240	1	7.5595	1	1.5874	1.5178	0.2811	6.6494	5.3133
It shall not have bad color when cooked	2.2894	1.3389	2.2894	3.1748	0.1644	0.1882	0.1323	1	0.1598	1	1	0.1257	1	1.2599
It shall not be ill smelling	5.3133	5.1925	6.2573	6.2573	1	1.2599	1	6.2573	1	6.2573	5.5934	0.1949	5.6462	5.6462
It shall be crisp and juicy	1.8171	1.3867	3.5569	4.6416	0.3057	0.2500	0.6300	1	0.1598	1	1	0.1257	1	1
It shall not shrink when is being cooked	1.2599	1.1006	2.2894	3.9149	0.1383	0.2321	0.1812	1	0.1788	1	1	0.1352	1.4422	1.2599
Not being harmful to body	7.2304	7.6117	7.9581	9	3.5569	5.9439	3.5569	7.9581	5.1299	7.9581	7.3986	1	8.2768	7.3986
The surface of raw hamburger shall be smooth	1	0.5228	3.1072	2.7144	0.1514	0.2554	0.1504	1	0.1771	1	0.6934	0.1208	1	1
The surface of raw hamburger shall not be dark	1.7100	0.8298	2.2894	3.9149	0.1514	0.2075	0.1882	0.7937	0.1771	1	0.7937	0.1352	1	1

Further, the inconsistency rate of the comparisons too shall be calculated. For this purpose, MATLAB software was used and λ_{max} = 14.9 was resulted. According to Equations 3 and 4 we have IR = 0.044. As interpretation of the value resulted for IR, one may say that since 0.044 is less than 0.1 thus the paired comparisons are consistent (Golden et al., 1989). Then, the weights were normalized. For normalization, each weight was divided by the total of the weights of same column. At the end, the arithmetic mean of each row of the new table was calculated and by so doing, the relative weight of each customer's demand was obtained. The relative weights were entered into the table 3 in order of their importance. Second step was entering the weight of each demand into the House of Quality Matrix. Then we started third step.

Table 3. Final table the customers' demands comparison

Demands of Customers	**Relative Weight**
Not being harmful to body	0.2683
It shall be delicious	0.1367
It shall be fresh	0.1338
It shall not be ill smelling	0.1249
It shall have high durability	0.1024
It shall be crisp and juicy	0.0382
It shall not shrink when is being cooked	0.0308

It shall not have bad color when cooked	0.0304
The box of the purchased hamburger be not damp (has no wet patch)	0.0294
The surface of raw hamburger shall be smooth	0.0289
The surface of raw hamburger shall not be dark	0.0269
In-between Paper of the Hamburger be separable Easily	0.0196
Production series be written on the box	0.0167
Packed in different weights	0.0130

2.4.3. Formation of QFD 4-Matrix Models

At first stage of third step, the QFD team evaluated 60% red meat-hamburger produced by 2 companies, that is, Pak Taliseh Company and BA Company, with due regard to the demands of the customers and the qualitative requirements of the product expected by customers. For comparing the product of Pak Taliseh Company with product of BA Company, a scale from 1 (the worst) to 5 (the best) was used. Attention shall be made that Table 4 will enter into Evaluation of the Company column (Column N) and Table 5 in Evaluation of the Company's Competitors column (Column O) of Table 6. For instance, the number 3.3019 in 12th row has been extracted from Table 4 by $(3\times4\times3)^{(1/3)}$ = 3.3019 operation. After prioritization of the customers' demands and completion of Tables 4 and 5, the 4-matrix models were prepared while the principles governing their formation were regarded (Tables 6,7,8,9)(Revelle et al., 1998). In these tables, the symbol • represents number 9, symbol o represents number 3, and symbol Δ is representative of number 1.

Table 4. Evaluation of the hamburger produced by Pak Taliseh Company

Demands of Customers	Relative Weight
In-between Paper of the Hamburger be separable Easily	4
The box of the purchased hamburger be not damp (has no wet patch)	3.9149
Production series be written on the box	4.6416
Packed in different weights	1.2599
It shall be fresh	4.6416
It shall have high durability	4.3089
It shall be delicious	4
It shall not have bad color when cooked	5
It shall not be ill smelling	4.6416
It shall be crisp and juicy	5
It shall not shrink when is being cooked	3.3019
Not being harmful to body	5
The surface of raw hamburger shall be smooth	4.6416
The surface of raw hamburger shall not be dark	5

Table 5. Evaluation of the hamburger produced by BA Company

Demands of Customers	Relative Weight
In-between Paper of the Hamburger be separable Easily	3.3019

The box of the purchased hamburger be not damp (has no wet patch)	5
Production series be written on the box	3.3019
Packed in different weights	1.5874
It shall be fresh	2.5198
It shall have high durability	4.6416
It shall be delicious	2.5198
It shall not have bad color when cooked	2.7144
It shall not be ill smelling	3.9149
It shall be crisp and juicy	2.8845
It shall not shrink when is being cooked	3.9149
Not being harmful to body	4.6416
The surface of raw hamburger shall be smooth	3.3019
The surface of raw hamburger shall not be dark	2.2894

Table 6. the House of Quality Matrix of 60% red meat-hamburger

															A	N	O	P	B	C	D	E
Engineering Characteristics / **Customer Requirements**	Food grade paraffin	Horse back-white cardboard sheet	Industrial storage system & GSP	National standard requirements	Supervision on food and drug organization	Frozen meat	Storage conditions	Spice	Fillers	Fat percentage	Rusk flour	Stabilizer	Washing, antiseptic	Production conditions	Importance degree	Appraisal of organization	Appraisal of the competitors of the organization	The organization' s program	Improvement ratio	Correction coefficient	Absolute weight	Relative weight
In-between Paper of the Hamburger be separable Easily	•			o	o									o	0.0196	4	3.3019	5	1.25	1/2	0.0294	1.8790
The box of the purchased hamburger be not damp (has no wet patch)		•	o	o	o		•							Δ	0.0294	3.9149	5	4	1.0217	1/2	0.0360	2.3009
Production series be written on the box			•	Δ	o										0.0167	4.6416	3.3019	3	0.6463	1	0.0107	0.6838
Packed in different weights				o	•										0.0130	1.2599	1.5874	3	2.3811	1	0.0309	1.9749
It shall be fresh			•	o	•	•	•	•	o	Δ	•	Δ		o	0.1338	4.6416	2.5198	5	1.0772	1/5	0.2161	13.8118
It shall have high durability			o	•	•	•	•	o	•	o	o	•	•		0.1024	4.3089	4.6416	5	1.1603	1/5	0.1782	11.3894
It shall be delicious				•	o	•		•	•	•	•	o			0.1367	4	2.5198	5	1.25	1/5	0.2563	16.3811
It shall not have bad color when cooked			•	Δ	Δ	•	•	o	o	•	o	o	Δ		0.0304	5	2.7144	5	1	1/2	0.0364	2.3264
It shall not be ill smelling			Δ	o	o	•	o	•	•	•	•	•	o	o	0.1249	4.6416	3.9149	5	1.0772	1/5	0.2018	12.8978
It shall be crisp and juicy				o		•			•	•	•	•			0.0382	5	2.8845	5	1	1/5	0.0573	3.6622
It shall not shrink when is being cooked				o		o	o		•	•	o	•		•	0.0308	3.3019	3.9149	4	1.2114	1/2	0.0447	2.8569
Not being harmful to body	•	o	•	•	•	•	•	•	•	•	•	•	•	•	0.2683	5	4.6416	5	1	1/5	0.4024	25.7190
The surface of raw hamburger shall be smooth		o	o			o	•		o					•	0.0269	4.6416	3.3019	4	0.8617	1/2	0.0298	1.9046
The surface of raw hamburger shall not be dark			o	Δ	Δ	•	o	o	o	o	o	o		•	0.0289	5	2.8494	5	1	1/2	0.0346	2.2114

Target Value		10-15 gr/cm	150-450 gr				In accordance to standards	-18 °C	radiated	Water absorption at rate higher than 3 times more	Maximum 16%	60-80 Carbohydrate	Preventing size shrinkage	Lowering the stuff to less than 1000 pieces in each sample	Subzero temperature in semi-processed product	Total		Total	1.5646	100
Weight	Absolute weight	2.5911	1.1502	4.9069	5.8117	5.6987	7.9455	5.8746	6.4584	6.9717	6.1914	6.8946	5.8032	3.7414	5.2536	75.2293				
	Relative weight(%)	3.4414	1.5276	6.5171	7.5687	10.5528	7.8023	8.5777	9.2594	8.2231	9.1570	7.7075	4.9691	6.9775	6.9775	100				

Table 7. Product design matrix of 60% red meat-hamburger

features of the product	Target Value	Importance degree	Protein	Fat	Carbohydrate	Salmonella	Staphylococcus Aureus	Mold	E.coli	Humidity
Frozen meat	60%	10.5528	●	o	Δ	●	Δ		Δ	o
Rusk flour	6%	9.1570			●					o
Fillers	32%	9.2594	●		o					o
Stabilizer	1.5%	7.7075	●		o					o
Spice	0.5%	8.5777						●		●
Production conditions	hall temperature below 35°C	6.9775					●		o	o
		Target Value	Minimum 11.5%	Maximum 16%	Maximum 6.5%	In 25-gram sample, negative	1000 pieces per gram of sample	1000 pieces per gram of sample	In 25-gram sample, negative	Maximum 66%
		Absolute weight	247.6773	31.6584	143.8665	94.9752	73.3503	77.1993	31.4853	208.1619
		Relative weight (%)	27.2660	3.4852	15.8378	10.4555	8.0749	8.4986	3.4661	22.9159

Table 8. Process design matrix of 60% red meat-hamburger

			Inspection and entrance control						During-process control					
Features of the ingredients	Target Value	Importance degree	Chemical test of meat	Microbial test of meat	Determining fat of meat	Determining the protein of meat	Microbial test of the surface	Determining the carbohydrate of consumable materials	Microbial test of semi-processed product	Chemical test of semi-processed product	Microbial sampling of the surfaces	Measuring temperature of semi-processed product	Surveillance on the temperature of production hall	Surveillance on the freezing Tunnel

Protein	Minimum 11.5%	27.2660	•		o	•		o		•				
Fat	Maximum 16%	3.4852	•		•	o		o		•				
Carbohydrate	Maximum 6.5%	15.8378	o		o	o		•		•				
Staphylococcus Aureus	1000 pieces per gram of sample	8.0749	o	•			•		•		•	o	o	•
Salmonella	In 25-gram sample, negative	10.4555	o	•			o		•		o	o	o	•
	Target Value		In accordance to standards	Salmonella negative in 25-gram sample	5-6 %	18-22 %	Maximum 1000 pieces per gram of sample	60-80% carbohydrate in rusk flour	Salmonella negative in 25-gram sample	In accordance to standards	Stuff less than 1000 pieces in each sample	Subzero temperature	Temperature below 15°C	Temperature below 35°C
	Absolute weight		379.8654	166.7736	160.6782	303.3630	104.0406	234.7938	166.7736	419.3010	104.0406	55.5912	55.5912	166.7736
	Relative weight (%)		16.3906	7.1960	6.9330	13.0896	4.4892	10.1310	7.1960	18.0921	4.4892	2.3987	2.3987	7.1960

Table 9. Process control planning matrix of 60% red meat-hamburger

process stages	key needs of the process	Importance degree	evaluation of operation					planning needs								
			difficult control	frequency of potential problems	Intensity	ability in recognition	multiplication	Preventive measures	Corrective measures	error avoidance methods	Surveillance on the process	Data analysis	drawing up needed instructions	training	promoting hygienic conditions of workhouse	executive methods
During-process control	Surveillance on the temperature of freezing tunnel	7.1960	3	2	2	1	12	√	√	√					√	
	Surveillance on the temperature of production hall	2.3987	1	1	1	1	1	√	√						√	
	Measuring temperature of semi-processed product	2.3987	1	1	1	1	1				√					
	Microbial sampling of the surfaces	4.4892	2	2	2	1	8			√	√	√		√	√	√
	Chemical test of semi-processed product	18.0921	1	1	1	1	1			√	√	√	√			√
	Microbial test of semi-processed product	7.1960	3	3	2	1	18			√	√	√	√			√

Inspection and	Determining the carbohydrate of consumable materials	10.1310	1	1	1	1	1					√	√			√
	Microbial test of the surface	4.4892	2	2	2	1	8	√	√		√		√		√	√
	Determining the protein of meat	13.0896	1	1	1	1	1			√		√	√			√
	Determining fat of meat	6.9330	1	1	1	1	1					√	√			√
	Microbial test of meat	7.1960	3	3	2	1	18			√	√	√	√			√
	Chemical test of meat	16.3906	1	1	1	1	1			√	√	√	√			√

3.Results and discussions

In this section, the results of using Combined QFD-GAHP approach are presented in Figure 1.

3.1. The Results Earned from the House of Quality Matrix

In the first matrix, called the House of Quality Matrix (Table 6), in the column of "the Degree of Importance" the highest weight was for "It shall not be harmful to body" with 0.2683. This requirement is strongly linked to 13 items out of totally 14 items of engineering characteristics**.** Among engineering characteristics, "frozen meat" with absolute weight of 7.9455, was the most important. It is highly related to the qualitative features of "It shall be fresh", "It shall have high durability", "It shall not have bad color when cooked", "It shall not be ill smelling", "It shall be crispy and juicy", "It shall not be harmful to body", "It shall be delicious", and "The surface of raw hamburger shall not be dark". Therefore, improvement of the quality of frozen meat has a great effect on meeting these eight customer needs.

3.2. Results of the Product Design Matrix

In product design matrix (Table 7), improvement of quality of "frozen meat" depends on the levels set for "protein" and "salmonella". In this matrix, the most important feature of hamburger relates to "protein" (with absolute weight of 247.6773) and the least important one relates to "E.coli" (with absolute weight of 31.4853).

3.3. Results of the Process Design Matrix and the Process Control Planning Matrix

In process design matrix (Table 8), the most important in-process controlling feature relates to "chemical test of semi-processed product" (with absolute weight of 419.301) and the least important ones are "surveillance on the temperature of production hall" and "measuring the temperature of semi-processed product" (each with absolute weight of 55.5912. Since in process control planning matrix (Table 9), both "microbial test of meat" and "microbial test of semi-processed product" have an appraisal level of 18 (column of multiplication), therefore performing these two tests are more necessary than other requirements. For "surveillance on the temperature of freezing tunnel", "surveillance on the temperature of production hall" and "microbial test of surfaces", corrective and preventive actions are also needed.

Considering the fourth QFD matrix, using error avoidance methods for issues such as surveillance on the temperature of freezing tunnel, microbial sampling of the surface, chemical test of semi-processed product, microbial test of semi-processed product, determining the protein of meat, microbial test of meat, and chemical test of meat is unavoidable. Similarly, the necessary actions for surveillance on the process, data analysis, drawing up needed instructions, training, promoting hygienic conditions of workhouse, and "executive methods" maybe defined.

3.4 Findings of Other Studies

The findings of Dalen's study (1996) indicated that QFD technique is a suitable tool for converting the customers' needs to technical features of the final product. The findings of Waisarayutt and Tutiyapak (2006) indicate that more detailed needs of customers may be discovered with help of 4Cs and then enter them into QFD matrices.. There is only one study (Mattsson and Helmersson, 2007) that has analyzed and prioritized the customers'

feedbacks about the Hamburger consumption. In this article, the Customers' opinions are classified into 4 clusters and 2 general categories of "taste" and "ingredients" of the product. The results represented that the customers' opinions are largely in accordance with the concept of what is known as the standard hamburger.

The articles of Dalen's (1996); Waisarayutt and Tutiyapak (2006); and Mattsson and Helmersson, (2007), have reported QFD as a suitable technique to identify customers' needs and expectations from products, but none of these articles has even designed and completed its matrices. Meanwhile, there was only one article (Mattsson and Helmersson, 2007) about hamburger. In this research, although surveying and clustering the consumers' views about a type of hamburger were studied, but no approach was proposed in order to produce and develop a product based on customer expectations. On the contrary, the present study could well develop the product designing to production stage, by the QFD-GAHP mixed approach based on the requirements of customers.

Among the reviewed articles, only two papers (Sayadi et al., 2017) (Kowalska et al., 2015) have provided the QFD matrices. Sayyadi et al, has provided only the first matrix, and kowalska et al. (2015), has completed all of the four matrices. This is despite the fact that the research field of both of these studies was not the meat industry and products. Instead, the present paper has formed all of the four matrices of QFD in accordance with the technical principles and hygienic standards laid down in the meat products industry. On the other hand, the simultaneous use of the opinions of some experts in completing the paired comparisons matrix and eliminating the inconsistency of them was another feature of this study.

4. Conclusions

In present study, for promoting the quality of 60% red meat-hamburger, a combined QFD-GAHP technique was used. This approach had a significant impact on the designing to production stages of new hamburgers, with QFD 4-Matrix Models and group analytical hierarchy by the elimination of inconsistency in the pairwise comparison matrix.

The demand of "It shall not be harmful to body" and the engineering characteristics of "frozen meat" were respectively the most important requirement and the engineering characteristic of hamburger. The results of this study showed that the improvement of the "protein" and "salmonella" characteristics would be two of key factors in improving the quality of "frozen meat". Also, "microbial test of meat" and "microbial test of semi-processed product" were reported as the most important control characteristics during the process.

Finally, the point which is noteworthy is that QFD 4-Matrix Model was able to consider the qualitative requirements of customers in all stages of hamburger production from designing to production. The study made valuable information about the customers and engineering features of hamburger accessible to QFD team engaged in our case study. The method used in this research, may be used successfully in development and enhancing the quality of other meat products. Mitigating the risk of product rejection by customers, more selling in market, and decreasing the costs such as quality costs and costs of reworks are among the benefits of using QFD in the foodstuff researches.

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ANTICANCER EFFECT OF PROBIOTIC SACCHAROMYCES BOULARDII SUPERNATANT ON HUMAN CACO-2 CELLS; AN IN VITRO STUDY

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ABSTRACT

Colon cancer is an important worldwide cause of death in human which is treated commonly by chemotherapy, radiotherapy and surgery methods with different side effects. Natural treatment such as microbial cell wall extract is suggested to be used as an effective alternative of chemical drugs for treatment of colon cancers without any side effect. *Saccharomyces boulardii* is used in probiotic foods and supplement capsules in viable and yeast cell wall extract forms. At the present study, we in vitro investigate the anticancer properties of *S. boulardii* supernatant (SBS) on colon cancer cells. We found that, SBS without dilution after 72 hours successfully killed the colon cancer cells. Also, this treatment induced apoptosis and down-regulated the expression of survivin gene significantly. However, effects of SBS without dilution after 24 and 48 hours were considerable. Downregulation of survivin gene expression by functional compounds in SBS induced apoptosis and killed the colon cancer cells successfully. However, future in vivo and in vitro investigation of anticancer effects of SBS on other cancer cells are suggested to be implemented.

1.Introduction

Cancer is the worldwide public health challenge and the second disease leading to death. In the year 2019, 1,762,450 new cases and 606,880 deaths because of cancer diseases were reported just in the united states. Colon cancer also is known as one of the most probable cancer disease and cause of death in human recently around the world. In the united states, 101,420 new cases and 51,020 deaths because of this cancer was reported last year (Siegel, Miller, & Jemal, 2019). Radiotherapy, chemotherapy and surgery are methods commonly used for treatment of cancer cells and promotion of health status of the cancer patients (Garrett, 2019). Oxaliplain, leucovorin, and 5-fluorouracil are commonly used as chemotherapy regimen for colon cancer treatment; however, hand numbness, tingling, dizziness, weakness and loss of muscle tone are the prominent side effects of these chemical drugs (Grothey et al., 2018). Researchers have always been studying to find efficient alternatives of strategies of cancer disease treatments because of different side effects of these drugs and methods. There are many anticancer herbal and natural drugs developed in

recent decades to improve cancer diseases without any considerable side effects (Board, 2019). Some of these anticancer drugs are plant-derived or microbial cell wall extract. Killing activity and inducing apoptosis effects of these natural drugs have been investigated and proved in vitro and in vivo for many cancer cells such as breast, colon, lung, and prostate cancer cells (Benson et al., 2017).

Saccharomyces boulardii is a yeast which has been extensively used as a probiotic with different health promotion properties in probiotic foods and supplement capsules. *S. boulardii* are employed as a beneficial yeast in viable and cell wall extract forms in foods and drugs (Altmann, 2017). *S. boulardii* supernatant (SBS) or cell wall extract of this yeast is produced by ultra-centrifugation of yeast biomass yielded from activated cells (Brun et al., 2017). There are many researches showed considerable antioxidant, anti-inflammatory, anticancer, and killing activities on cancer cells for SBS. Also, SBS inhibited and treated many chronological disorders such as inflammatory bowel disease, Crohn`s disease, and ulcerative colitis successfully (Warila & Hoover, 2017). However, viable form of *S. boulardii* had preventive effects on some diarrheal pathogens such as Clostridium difficile and Citrobacter rodentium. Inhibitory effect of this probiotic yeast also has been showed for fungal pathogens as well as Candida albicans (Warila & Hoover, 2017). It is worthy to be noted that anticancer activity of SBS on some cancer cells have been investigated by some researchers and they found this treatment effective and efficient for killing cancer cells. Mannan, Beta Glucan, and bioactive peptide compounds contribute to cancer cell killing activity of SBS (Warila & Hoover, 2017). The aim of this study was in vitro investigation of killing, inducing apoptosis and down-regulation of survivin gene expression activities of SBS on caco-2 cell line as colon cancer cells.

2. Materials and methods

2.1. Yeast cell wall extract of *S. boulardii*

Sacharomyces boulardii was purchased from a local drug store in Qazvin, Iran as commercial lyophilized yeast in a dietary supplement capsule (®YOMOGI, Germany). The strain was mixed with DMEM culture 100 mg/mL then incubated overnight at 37 °C. Incubated yeast cells were centrifuged at 20,000 g for 15 min then the supernatant was collected. The collected supernatant was filtered through 0.22 µm filters (Sigma-Alrrich, Budapest) to remove the residual yeast cells. Also, the filtered supernatant was diluted 1:2 with DMEM. Supernatants without dilution and diluted 1:2 with DMEM were used as SBS treatments for activated caco-2 cells.

2.2. Caco-2 cell culture and treatments

Caco-2 cell line (Pasteur Institute, Iran) were cultured and activated in DMEM culture (Gibco, MD, USA) by incubating at 37 °C with 5% CO2. Cells were passaged once a week and subcultured into 96-well plates at 80% confluence. After formation of cell monolayer for 48 hours, cells were treated with SBS without dilution and 1:2 diluted. All treated cells were analyzed for viability, cell apoptosis and survivin gene expression after 24, 48, and 72 hours.

2.3. MTT assay

MTT assay (m 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to measure viability of treated cells. Caco-2 cells were cultured in DMEM at 37 °C with 5% CO2 then subcultured into 96-well plates at 80% confluence. After formation of cell monolayer within 48 hours, cells were treated with SBS without dilution and diluted 1:2 for 24, 48, and 72 h. For MTT assay, each well was renewed with the medium containing 0.5 mg/mL MTT then incubated for 4 h. After incubation, medium was discarded and dimethyl sulfoxide (DMSO; Merck, Germany) was added into each well. Because of enzymatic reaction of tetrazolium MTT (yellow) to formazan (purple at 570 nm) in viable cells, absorbance of each well was

measured by microplate reader instrument model Elx808 (Bio-Tek, Vinooski VT, USA) to evaluate the viability of the treated cells. Percentage of cell viability was calculated as:

Cell viability (%) = Ae-An/Ac-An × 100

While Ae is the absorbance of the experiment group, An is the absorbance of the blank group, and Ac is the absorbance of the control group (Warila & Hoover, 2017).

2.4. Cell apoptosis analysis

Annexin V-FITC and propidium iodide (PI) staining using cell apoptosis kit (Ebioscience, San Diego, USA) was used to measure and detect cell apoptosis in treated caco-2 cells. According to the manufacturer`s instructions, 1 × 10^6 cells/well seeded in 6-well plates then treated with diluted and complete SBS for 24-hour intervals until 72 hours. After treatments, cells were harvested then washed using phosphate-buffered saline (PBS) and incubated in Annexin V-FITC and PI for 30 and 5 min respectively at room temperature in a dark place. After staining, expression of Annexin and PI were measured employing the FACS-Calibur flow cytometer instrument (Becton, Dickinson Immunocytometry system, CA, USA).

2.5. Measurement of survivin gene expression

Relative expression of survivin gene was measured in treated cells using quantitative reverse transcriptase real-time PCR (qRT-PCR) method. First, total RNA of each well was extracted using tissue total RNA extraction kit (GeneAll, Korea) according to the manufacturer`s instructions. Then the extracted RNA was reverse transcribed to synthesize cDNA using RT-PCR kit (GeneAll, Korea) based on manufacturer`s instructions and the PCR thermal-cycler instrument ABI model 9092 (ABI, Applied biosystems, USA). Quality and quantity of the extracted RNA and the synthesized cDNA were measured using Nanodrop 2000c spectrophotometer (ThermoFisher Scientific, USA). Survivin gene expression implemented using quantitative RT-PCR of synthesized cDNA. For this evaluation, 25 µL reaction mixture were mixed with 12.5 µL qRT-PCR SYBR green ROX master mix (Ampliqon, Denmark), 1 µL of each primer (5µM/µL), 2 µL of template cDNA and deionized sterilized water to 25 µL as the final volume of the reaction. Primers sequences were synthesized by Sina Colon company (Sina Colon, Tehran, Iran) and used to measure the expression of survivin gene as follows: Forward; 5`-ATG GCA CGG CGC ACT TT-3` and Reverse; 5`-TCC ACT GCC CCA CTG AGA A-3`. qRT-PCRs were performed in a RotorGene real-time PCR instrument model 6000 (QiaGen, USA) with 15 min at 95 °C as initial denaturation, followed by 40 cycles including 15 s at 95 °C and 1 min at 60 °C. After determining cycle threshold (CT) for each reaction, the relative quantification of survivin gene expression was measured as previously described by Motawi et al. (2014) (Motawi, Bustanji, EL-Maraghy, Taha, & Al-Ghussein, 2014).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used to measure significant ($P < 0.05$) differences among the groups of data employing SPSS software version 22 (SPSS Inc., Chicago, IL, USA). All measurement and experiments were done in triplicates.

3.Results and discussions

Effects of SBS (without dilution and diluted 1:2 w/w) for different duration treatment on viability of colon cancer cell line, caco-2, were investigated using MTT assay (FIGURE 1). As it is shown in FIGURE 1, treatment after 48 and 72 hours with SBS without dilution significantly more killed the caco-2 cells. However, the killing activity of the treatment with SBS without dilution after 24 hours was considerable, but the killing activity of this treatment was significantly lower than that of other duration treatments of without diluted SBS. Consequently, in vitro cytotoxic activity of SBS on caco-2 cells are shown at the present study. However, dilution of SBS significantly affects the killing activity of this treatment.

FIGURE 2 illustrates analysis of flow cytometry of the caco-2 cells treated with 1:2 diluted (A) and without dilution (B: 24, C: 48, and D: 72 hours) SBS. The proportion of viable, early apoptotic and late apoptotic/necrotic cells are shown in Q4, Q3, and Q2 quadrants respectively. The proportions of apoptotic cells, including early and late apoptosis, for caco-2 cells treated with 1:2 diluted SBS was 3.75. However, these proportions for cells treated with SBS without dilution after 24, 48, and 72 hours were measured 18.28, 12.92, and 53.2% respectively. Also, the percentage of viable cells in caco-2 cells treated without dilution after 72 hours (Q4 = 37.6%) was significantly lower than that in cells treated with other treatments. Thus, these results indicate that treatment with SBS without dilution after 72 hours induced significant apoptosis in caco-2 cells and decreased cell viability in comparison with other treatments. However, SBS treatment without dilution after 24 and 48 hours induced apoptosis (18.28 and 12.92% respectively) considerably higher than all 1:2 diluted treatment.

Caco-2 cells treated with diluted and without dilution SBS were subjected to analysis of survivin gene expression (all synthesized cDNA was quantitively and qualitatively appropriate for qRT-PCR) by qRT-PCR and the results are illustrated in FIGURE 3. Similar to results of MTT assay and apoptotic analysis of treated cells, treatment with SBS without dilution after 72 hours decreased the survivin gene expression significantly higher than other treatments as can be seen in FIGURE 4. Also, other duration times of without dilution treatments were more considerably effective than 1:2 diluted treatments in reduction of survivin expression. Inducing apoptosis decrease the survivin expression which shows lower cell viability in MTT assay. As a result of the study, treatment with SBS without dilution after 72 hours successfully killed the colon cancer cells (caco-2 cell line). This killing activity was proved by investigation of inducing apoptosis, decreasing cell viability, and survivin expression in the treated cells.

At the present study, we investigated killing activity of *S. boulardii* supernatant or SBS without dilution and diluted 1:2 on viability of colon cancer cells (caco-2). The results indicated that SBS without dilution after 72 hours significantly killed more caco-2 cells than other treatments; consequently, this treatment can be considered as an efficient and new colon cancer cell killer which is studied in vitro for caco-2 cells.

Yeast cell wall extract consists of many bioactive compounds with different activities including antifungal, antioxidant, immunomodulating, anti-inflammatory, and anticancer activities. Also, cell wall extract or supernatant of *S. boulardii* biomass as a probiotic yeast showed many functional and bioactive properties as mentioned previously (Datta, Timson, & Annapure, 2017). It is previously found that Mannan and Beta Glucan compounds presenting in cell wall extract of *S. boulardii* and other yeast cells usually kill the tumor cells and inhibit the growth effectively. However, these polysaccharides as well as Beta Glucan with antitumor activity was extracted from other sources such as some fruit and vegetables (Suryavanshi et al., 2013). Also, a varied set of complex phenolic compounds present in SBS which are associated with the antioxidant and anticancer activity of *S. boulardii* cell wall extract. There is a strength association between antioxidant and anticancer activity of a bioactive compound (Ryan et al., 2011). Gallic acid and catechin are the most prevalent phenolic compounds in SBS. Therefore, a complex interaction of antioxidant compounds (phenolic acids) and polysaccharides (mannan and beta glucan) explain killing activity of cancer cells for SBS. However, it is reported that these compounds are heat stable with less than 1 KD molecular weight (Murzyn, Krasowska, Stefanowicz, Dziadkowiec, & Łukaszewicz, 2010).

We found that SBS after 72 hours induce apoptosis in caco-2 cells. Probiotic supernatant and cell wall extract up-regulate the expression of apoptosis gene in cancer cells. Nouzari et al. (2019) have shown that supernatant of

Lactobacillus paracasei biomass as a probiotic bacterium induced the apoptosis regulation in caco-2 cells which decreased the viability of these cells effectively (Nozari et al., 2019). Shyu et al. (2014) also reported that probiotic supernatant significantly increases gene expression regulation of apoptosis in HCT116 and HT29 cell lines (Shyu, Oyong, & Cabrera, 2014). Huang et al. (2016) illustrated anticancer effect of Lactobacillus species supernatant on HT29 cells. They extracted proteins from supernatant of probiotic bacteria and showed a strong association between cancer cell killing activity of cell wall extract and the protein content (Huang et al., 2016). However, there are many studies showed anticancer effect of bioactive peptides presented in protein content of the cell wall extracts. Also, it is proved that the protein fraction of the probiotic supernatant induces apoptosis and contribute to death in cancer cells. This protein fraction affects the signaling pathway of apoptosis in carcinoma colon cells (Naimah, Al-Manhel, & Al-Shawi, 2018).

Survivin gene is categorized in a unique group of protein recently described as inhibitors of apoptosis (IAP) which inhibit the induced apoptosis in cell. Survivin expresses in mitosis step in the cell cycle, also expression of this gene is inhibited and reduced during the apoptosis (Li et al., 2018). Consequently; every treatment reducing the expression of survivin gene induces and increases the apoptosis in cell (Arafat et al., 2017). At the present study, SBS without dilution treatment after 72 hours decreased the survivin gene expression in caco-2 cells therefore killed them successfully. Schwab et al. (2009) showed that butyrate and mesalazine down-regulated and decreased expression of IAP proteins such as survivin gene in caco-2 cells successfully. They indicated that down-regulation of the survivin gene inducing apoptosis in caco-2 cells is caused by interaction of butyrate and mesalazine with IAP proteins such as survivin (Schwab, Reynders, Steinhilber, & Stein, 2009). Hwang et al. (2015) also indicated decreasing effect of Shogaol, a fraction if ginger plant, on survivin gene expression as an antiapoptotic protein in caco-2 cells. It could be considered that the main reason of colon cancer killing activity of SBS is probably because of down-regulation effect of this treatment on expression of IAP protein as well as survivin in caco-2 cells (Hwang, Lee, Oh, Lee, & Kwon, 2015). There are some researches about the effects of SBS on signaling and growth rate of many cancer cells; however, the effects of SBS on caco-2 cells have not been investigated previously and now it is studied at the present study. Most of researchers believe that Beta Glucan and some proteins in yeast cell wall extract describe the killing activity of SBS on caco-2 cells (Fortin, Aguilar-Uscanga, Vu, Salmieri, & Lacroix, 2018). Cancer treatment activity of some drugs such as naproxen and cromolyn also is associated with reducing surviving gene expression in cancer cells as well as caco-2 cell line (Henry, D'hondt, Andre, Holemans, & Canon, 2004). In addition to anti-inflammatory and antioxidant activities of SBS, cancer treatment function of yeast cell wall extract of probiotic *S. boulardii* is suggested to be employed by some researchers recently (Chen et al., 2009). Fortin et al. (2018) investigated preventive effect of cell wall extract of *S. boulardii* in vivo on colon cancer in rats treated with Dimethylhydrazine. They found that SBS successfully prevented and treated the colon cancer in rats (Fortin et al., 2018). Fatemi et al. (2019) also demonstrated inhibitory effect of SBS on breast cancer induced by Dimethylbenza-Anthracene in rats. They found that SBS reduced the tumor size and spread of breast cancer cells by inducing apoptosis (Fatemi, Ghandhari, & Karimi, 2019). At the present study, we in vitro killed the colon cancer cells by SBS without dilution treatment after 72 hours. We found that SBS down-regulated the expression of survivin gene and induced apoptosis to kill the caco-2 cells successfully.

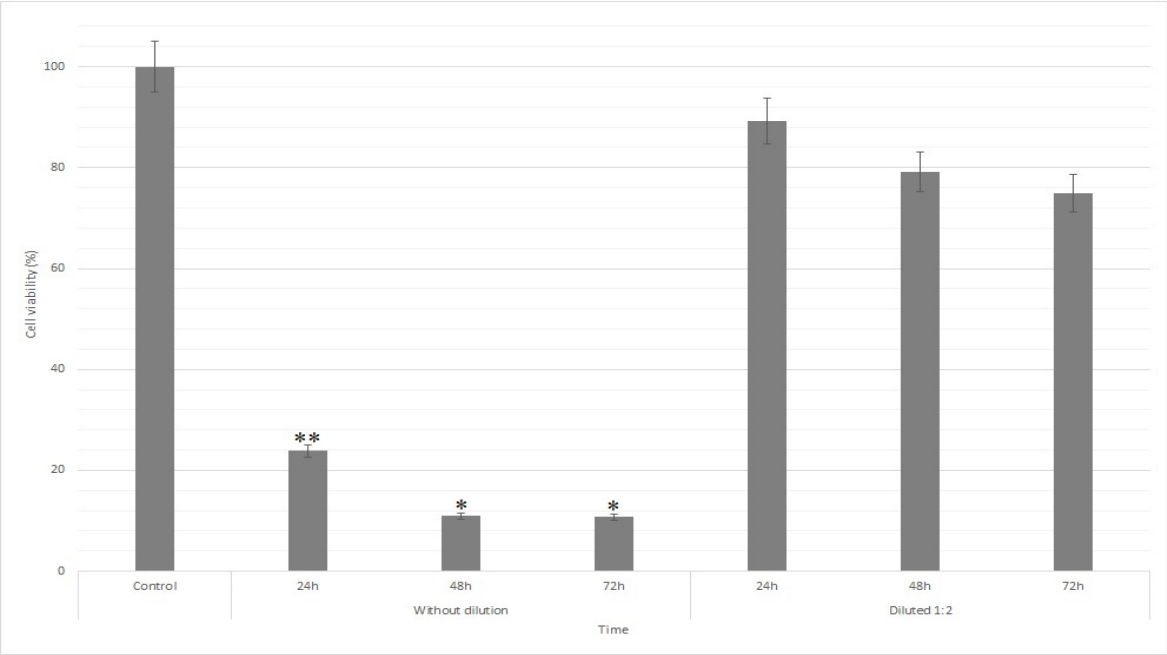

Figure 1. Cytotoxicity determination of SBS on Caco-2 cells by MTT assay.
* and ** indicates significant difference

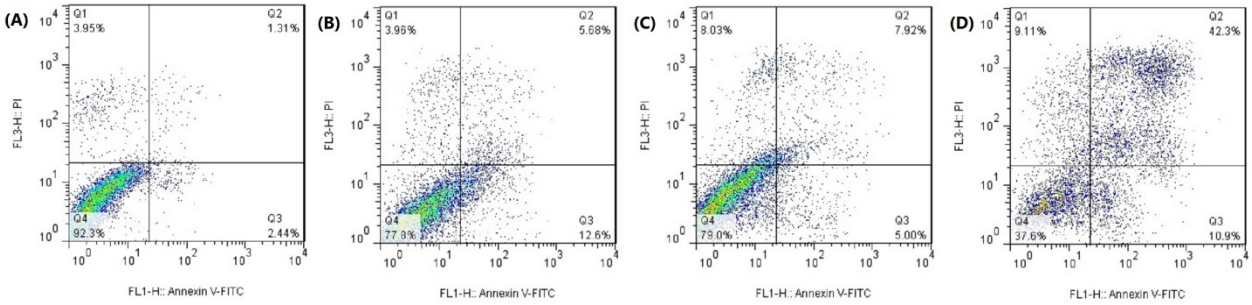

Figure 2. Analysis of SBS (Diluted 1:2 (A); and without dilution for 24h (B), 48h (C), and 72h (D)) on Caco-2 cells with apoptosis determination by flow cytometry.

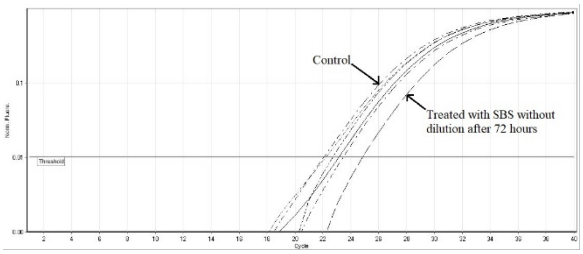

Figure 3. Normalized fluorescence curves of qRT-PCR of survivin gene mRNA for treated caco-2 cells with SBS.

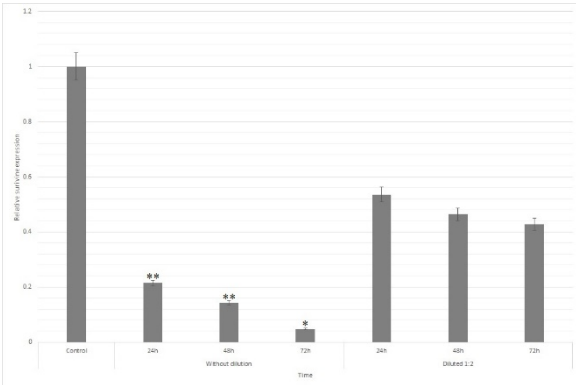

Figure 4. Relative Survivine gene expression in Caco-2 cells treated with SBS.
* and ** indicates significant difference

4. Conclusions

At the present study, we investigated the killing activity and in vitro anticancer properties of yeast cell wall extract of *S. boulardii* on colon cancer (caco-2) cells. We found that SBS without dilution significantly killed caco-2 cells after 72 hours; however, the effect of this treatment after 24 and 48 hours were considerable. SBS without dilution treatment after 72 hours reduced the survivin expression in caco-2 cells and induced 53.2% apoptosis. Functional compounds such as Beta Glucan, Mannan and bioactive peptides in protein fraction of SBS contribute to inhibitory effect on survivin gene expression and against caco-2 cell viability. We strongly suggest SBS to be employed as a colon cancer treatment; however, further in vivo investigations and studying the killing activity of colon cancer cells with cell wall extract of other yeasts are necessary to be implemented consequently.

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CHEMICAL AND MICROBIOLOGICAL QUALITY DURING STORAGE: HALF-DRIED SALTED ROUND SCAD (*DECAPTERUS MARUADSI*)

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ABSTRACT

Half-dried salted round scad can be stored for only a short time due to high-moisture content. The objective of this study was to evaluate the quality changes during storage under 3 packaging; air (T1), vacuum (T2), and air with oxygen absorber (T3). Moisture content, pH, TVB value, TMA value, TBA value, total viable count as well as yeast and mold of half-dried salted round scad significantly increased along with the longer storage time ($p<0.05$). The vacuum packaging had the lowest effect on the quality changes of half-dried salted round scad. When stored at room temperature)(30±2 ^{0}C) half-dried salted round scad gained higher amounts of yeast and mold than the standard of TISI. On day 15 of storage, yeast and mold of the sample were 6.4×10^2 CFU/g When stored under chilling condition (4±1 ^{0}C)for 30 days, half-dried salted round scad gained higher TMA value as well as yeast and mold values than the standard of TISI at 11.48 mg/100 g and 5.2×10^2 CFU/g, respectively. Therefore, the shelf life of half-dried salted round scad packed under vacuum condition were 10 and 25 days for samples stored at room temperature and cold storage, respectively.

1. Introduction

Half-dried salted fish is one of the most popular traditional products. It has high nutrition, soft texture, unique taste and flavor. It is also a great source of high protein which contains all essential amino acid. Half-dried salted fish is different from normal salted fish in term of the moisture and salt content in their final products. For salted fish, the a_w is usually under 0.85 and the salt content is over 10% (Thai industrial standards institute; TISI. 312/2006). For half-dried salted fish, it is a high-moisture content product and the salt content is less than 10% (light cure). Moreover, the main characteristics of salted fish affecting consumer buying decision are light salty taste, special flavor without stinky smell and absence of fish bone (Sukjuntra, 2014). Due to the lower salt content in half-dried salted fish, food spoilage bacteria and food-born pathogenic bacteria can easily grow in this product which leads to the product's shorter shelf life. Nowadays, snake-head fish and sepat siam, freshwater fish, are commonly used as half-dried salted fish products.

Among the sea water fish species, the round scad (*Decapterus maruadsi*), a fatty fish, has been chosen as the raw material for half-dried salted fish production because of its appearance and taste which are better than those of lean fish. Half-dried salted round scad also has soft texture during to the low salt added which leads to no denaturation of fish protein (Ana and Rui, 2010; Magnus and Turid, 2012). Moreover, the half-dried salted round scad is healthy food for consumer, due to its low salt content (Nuwanthi

et al., 2016). For the above reasons, half-dried salted round scad is widely consumed and available in local markets especially in the lower southern part of Thailand. However, the supply of half-dried salted round scad are still low because of its traditional sun drying process with the lack of proper technology to improve the production. Furthermore, during the rainy season, the half-dried salted round scad is not available in the market because of the sun drying process. The factors determining the half-dried salted round scad product's quality are raw material quality, salt quantity and quality, good and hygienic processing, packaging and suitable storage condition (Koral, 2013; Lorentzen *et al*., 2016).

In order to improve the quality and extend the shelf life of half-dried salted round scad, appropriate packaging is crucial. Nowadays, there are many studies on various packaging for shelf life extension of fish and fishery products including vacuum packaging, oxygen absorber, moisture absorber and modified atmosphere packaging (Mohan *et al*., 2009). The vacuum packaging is a widely used method in the market as its efficiency and low cost. This packaging can prevent oxidative rancidity, and inhibition of the growth of aerobic bacteria and spoilage bacteria, especially *Macrococcus* and *Aeromonas* (Masniyom, 2011; Renato, 2012; Pinar, 2013; Kumar and Ganguly, 2014).

Appropriate packaging is able to increase the shelf life of half-dried salted round scad, increase the marketing competition capacity and also enhance reputation of this product. Thus, the objectives of this study were to select the practical packaging methods conforming to entrepreneur in the lower southern part of Thailand for increasing the shelf life of half-dried salted round scad. Samples were stored at room temperature (30±2 ^{0}C) and at cold storage (4±1 ^{0}C) for 15 and 30 days, respectively. The changes of physical, chemical and microbiological properties of sample were determined at 5 days interval.

2. Materials and methods

2.1. Round Scad Preparation

The A grade to low B grade of round scad (*Decapterus maruadsi*) (8-12 fish/kg) according to EU grade was beheaded, gutted, butterflied and deboned. After that, the butterflied fish was soaked in 6% (w/v) brine solution for 20 min at a ratio of 1:1 (fish: brine solution). Then the soaked fish was dried in a solar dryer (Electricity Generating Authority of Thailand; SD-050 model) until a_w of fish fresh ranged from 0.85 to 0.90. Then, the fish was stored in low density polyethylene bags (LDPE) under 3 types of packaging; air (T1), vacuum (T2) (VAC-STAR S220, Switzerland), and air with an iron-based oxygen absorbent (T3) which utilized in sachet form and meant to be used in products with maximum water activity (a_w) of 0.85 and minimum oxygen absorption of 300 ml. Afterwards, half-dried salted round scad was stored at room temperature (30±2 ^{0}C) for 15 days, chilled in condition (4±1 ^{0}C) for 30 days, and sampled every 5 days for evaluation of the shelf life.

2.2. Quality Analysis

Chemical Analysis

The pH measurement was determined by homogenizing samples with distilled water at ratio of 1:5 (w/v), as described by Manthey et al. (1988). First, pH values of homogenate were measured by using a pH meter (Schott, model G 0840). Also, moisture content was determined using an oven method (Association of official Analytical Chemists, 2000). Then, salt content was analyzed using AOAC method. Moreover, the Total volatile base nitrogen (TVB) and Trimethylamine (TMA) value were analyzed using conway unit (Siang and Kim, 1992) by grinding 2 g of grounded sample with 8 ml of 4% (w/v) Trichloroacetic acid (TCA) in a mortar and then filtrating the texture through filter paper (Whatman No. 41). The volume of filtrate was adjusted to 10 ml using 4% (w/v) TCA. After that, 1 ml of sample extract was pipetted into outer ring of conway unit. Then 1 ml of inner ring solution (boric acid in ethanol) was

pipetted into inner ring of conway unit. Subsequently, 1 ml of saturated K_2CO_3 was pipetted into another side outer ring of conway unit. Moreover, The Trimethylamine value was carried out according to the methodology of TVB with a slightly different. After the sample extraction, the sample extract, inner ring solution and saturated K_2CO_3 were added into conway unit. Then, 1 ml of 10% (v/v) Formaldehyde solution was pipetted into the sample extract portion. After conway unit was closed, both of the TVB and TMA portion were gently mixed and incubated at 37 ^{0}C for 45 - 60 min, respectively. After incubation, the inner ring solution was titrated with 0.02 N Hydrochloric acid (HCl) using micro-burette until its green color turned pink color. The blank test was done using 1 ml of 4% (w/v) TCA instead of sample extract. TVB and TMA value was calculated as follows.

$$\text{TVB (mg/100mg)} = \frac{\text{N} \times 14 \times (\text{A} - \text{C}) \times 10 \times 100}{\text{W}} \quad (1)$$

$$\text{TMA (mg/100mg)} = \frac{\text{N} \times 14 \times (\text{B} - \text{C}) \times 10 \times 100}{\text{W}} \quad (2)$$

Where; N = Normality of HCl

A = Titration volume (ml) of 0.02N HCl for sample extract (TVB)

B = Titration volume (ml) of 0.02N HCl for blank

C = Titration volume (ml) of 0.02N HCl for sample extract (TMA)

W = Weight of sample (g)

Besides, the Thiobarbituric acid (TBA) value was determined by a distillation method (Egan *et al*., 1981). First, 10 g of sample was homogenized with 50 ml of distilled water, then adding 4 N HCl. After that, the mixture was heated with steam distillation until 50 ml of distillate was collected. A 5 ml of distillate was mixed with 5 ml of TBA reagent and incubated in boiling water for 35 min. After cooling, the absorbance of the solution was read at 538 nm. The blank test was done using distilled water instead of sample. TBA value was calculated as follows.

$$\text{TBA(mg MAD/kg)} = 7.8 \times \text{A} \quad (3)$$

Where A; Absorbance of the solution was read at 538 nm

Microbiological analysis

A microbiological analysis was carried out by examining total plate count (TPC) as well as yeast and mold according to the technique recommended by Bacteriological Analytical Manual (2001). *Staphylococcus aureus* and *Escherichia coli* MPN (3 tubes method) were analyzed using the technique recommended by Bacteriological Analytical Manual (2002).

2.3. Statistical analysis

The experiment was carried out with a completely randomized design (CRD). Data obtained from chemical parameters were analyzed by analysis of variance (ANOVA) using computer software. Duncan's New Multiple Range Test (DMRT) was used to compare the means with a significance level of 95%.

3.Results and discussions

3.1. Chemical quality changes

pH value

The result of this study indicated that the storage at room temperature (30±2 ^{0}C) had affected the pH value of half-dried salted round scad. pH values of the samples significantly increased with the increasing storage time. All 3 samples packed in air (T1), vacuum (T2) and air with oxygen absorber (T3) had the pH values of 5.98 on day 0 and their pH values reached to 6.73, 6.63 and 6.74 at the end of storage (day 15), respectively ($p>0.05$) (data is not shown). Corresponding to the result of Farid *et al.* (2014) who found that the pH values of 2 dried salted snake-head fish products stored at room temperature (27-30 ^{0}C) increased from 6.3 and 6.5 (day 0) to 8.1 and 7.9 (day 150). For half-dried salted round scad stored under chilling

condition) 4±1 ^{0}C (in the present study, the pH value used for indicating fish quality of all 3 samples slightly increased with the longer storage time ($p>0.05$) (data is not shown). During the post-mortem period, the decomposition of nitrogenous compounds occured by microorganisms and formed volatile amines, ammonia, amine and trimetylamineoxide, which are basic alkalinity compounds affecting on the increasing pH value of fish (Farid *et al.,* 2014; Frangos *et al*., 2010; Lorentzen *et al*., 2015). Thus, the storage of dried salted round scad in chilling temperature is able to retard microorganism activities leading to the minor changes in pH.

Moisture content

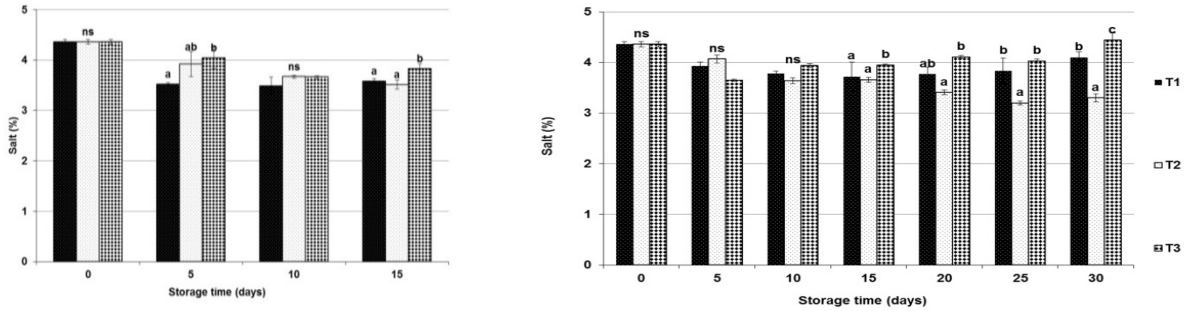
Moisture content of half-dried salted round scad stored at room temperature significantly increased with the longer storage time ($p<0.05$). The moisture content of samples packed in air (T1), vacuum (T2) and air with oxygen absorber (T3) were 54.93% on day 0 and increased to 61.27, 57.43 and 58.39% on day 15 of storage, respectively (data not shown). From these results, the moisture content of the sample stored in non-vacuum packaging (T1) was higher than other packaging. ($p<0.05$). The increase in moisture content of half-dried salted round scad was due to the moisture absorption of products an environment with from higher humidity (Farid *et al*., 2014). This is in agreement with the study of Adenike (2014), Ikutegbe & Sikoki (2014) and Reza *et al*. (2015) who found that moisture content of smoked catfish, dried chela *(Laubuka dadiburjori*) and smoked long-neck croaker (*Pseudotolithus typus*) increased with increasing the storage time ($p<0.05$). The moisture content of smoked catfish increased from 62.52% to 69.13% (on week 6), the moisture content of dried chela increased from 5.88% to 10.83% (on day 30) and the moisture content of smoked long-neck croaker increased from 10.90% to 13.10% (on week 4).

The result of half-dried salted round scad stored in chilling condition indicates that the changes of moisture content in the final products were lower than those stored at room temperature. The moisture content of sample stored in air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging was 54.93% on day 0 and increased to 59.87, 58.68 and 58.77% on day 30 of storage, respectively (data not shown). This was because dried fish slowly re-absorbed moisture from the relatively humidity condition like a chilling storage. However, moisture content of half-dried salted round scad is a factor of shelf life because it affects microbial growth and chemical changes including oxidation reaction and browning reaction. Normally, high moisture food is highly perishable.

Salt content

The salt content analysis of half-dried salted round scad stored at room temperature showed that the salt contents of samples packed in air (T1), vacuum (T2) packaging significantly decreased on day 5 of storage with a constant value on day 5-15 of storage. Whereas, the salt content of samples packed in air with oxygen absorber (T3) was stable during 15 days of storage ($p>0.05$). On day 15, salt content of 3 half-dried salted round scad were 3.38, 3.51 and 3.83% respectively (Figure 1(a)). In refrigerator storage (4±1 ^{0}C) condition, salt contents of all 3 samples varied with increasing storage time and showed the difference of salt content on day 15 of storage (Figure 1(b)). It can be seen that a decrease in salt content of products corresponded to the increased moisture content similar to Oyarekua (2014) who found that the salt content of smoked catfish slice decreased with an increase in storage time ($p<0.05$). The salt content decrease from 16.66% to 12.05% on week 6 of storage. In order to increase the shelf life of salted fish with 55-58% moisture content, Loannis (2014) demonstrated that salt content of salted fish should be in the range of 18-21%. From this study, high moisture content with 0.85 -0.90 of a_w and low salt content (less than 5%)

in half-dried salted round scad promoted the growth of microorganism and has effects the on short shelf life of this product.

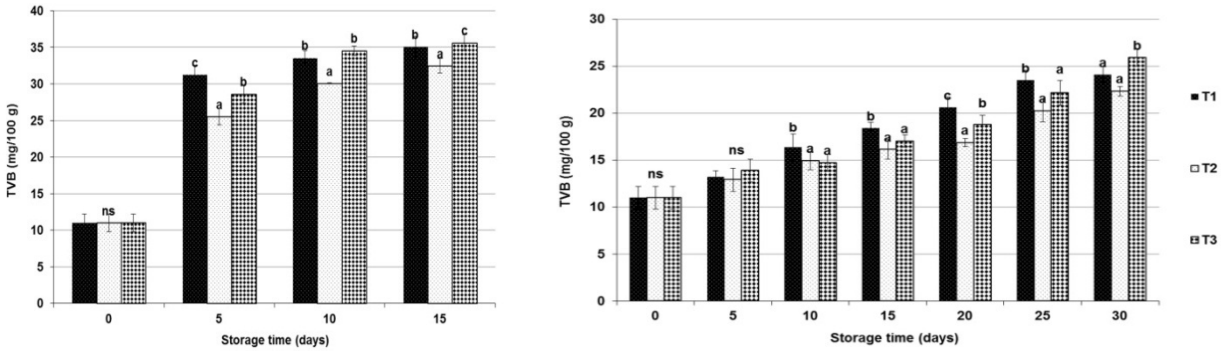


(a) **(b)**

Figure 1. Salt content of half-dried salted round scad stored at (a) room temperature and (b) chilling condition under air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging

Total volatile base nitrogen (TVB)

The TVB value in half-dried salted round scad stored at room temperature significantly increased along with the longer storage time ($p<0.05$). After 15 days of storage, TVB value in sample packed by vacuum packaging (T2) was lowest at 32.47 mg/100 g as shown in Figure 2(a). Moreover, the TVB value in half-dried salted round scad stored in refrigerator significantly increased with the longer storage time ($p<0.05$). The results showed that TVB value increased from an initial value of 10.99 mg/100 g on the day 0 to 24.24, 23.33 and 25.92 mg/100 g on the day 30 of storage in T1, T2 and T3, respectively (Figure 2(b)). Similar results were reported by Latifa et al. (2014) about refrigerated storage (4 ^{0}C) of smoke-dried chapila (*Gudusia chapra*) that TVB value increased from 8.84 to 20.04 mg/100 g after 9 months of storage. In addition, Farid et al. (2014) reported that TVB value in sun-dried salted shoal fish at room temperature (27-30 ^{0}C) storage increased from 4.89 to 30.86 mg/100 g after 5 months of storage. TVB is a group of biogenic amine produced by bacteria including ammonia, dimethylamine and trimethylamine. This increasing values correspond to the increasing fish spoilage; therefore, TVB is used for indicating the spoilage of fish and the consumer acceptance. The acceptability level of TVB in salted fish is 35 - 40 mg/100g. Above that level, fish products are considered unsuitable for human consumption (Bilgin and Degirmenci, 2019).



(a) **(b)**

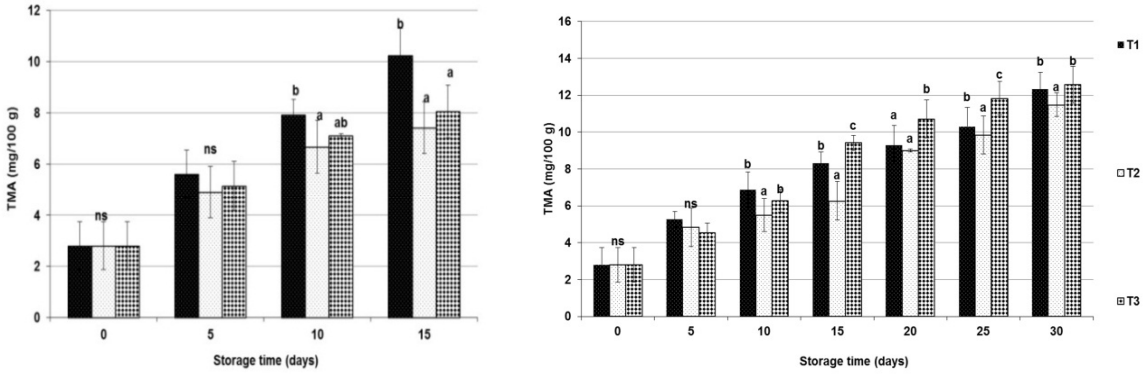
Figure 2. Total volatile base (TVB) values of half-dried salted round scad stored at (a) room temperature and (b) chilling temperature under air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging

The results of this study also showed that the different packaging effects on the growth of spoilage microorganism in fish products. Sample stored in air packaging had the highest spoilage. Whereas, sample stored in vacuum condition was not able to inhibit the growth of aerobic bacteria (Fuentes *et al*., 2011). In addition, the storage under chilling condition was able to prevent the growth of microorganism and enhance the shelf life of product. This study suggested that samples packed in air (T1), and air with oxygen absorber (T3) conditions and stored at room temperature had TVB value over the limitation of 35 mg/100 g. on day 15 of storage at 35.09 and 35.61 mg/100 g, respectively. On the contrary, TVB values of all 3 samples stored at chilling condition were within the standard of 35 mg/100 g TVB during the storage time. These results indicated that the quality of half-dried salted round scad stored at chilled condition was acceptable.

Trimethylamine (TMA)

The TMA value of half-dried salted round scad stored at room temperature significantly increased along with the longer storage time ($p<0.05$). After 15 days of storage, TMA value of sample packed by vacuum packaging (T2) was lowest at 7.42 mg/100 g (Figure 3(a)). Likewise, the values of half-dried salted round scad stored in refrigerator significantly increased along with the longer storage time ($p<0.05$). The results showed that TMA value increased from an initial value of 2.81 mg/100 g on the day 0 to 12.23, 11.48 and 12.57 mg/100 g on the day 30 of storage in T1, T2 and T3, respectively (Figure 3(b)). Similar results have been report by Bilgin and Degirmenci (2019) during refrigerated storage (4±1 ^{0}C) of hot-smoked meager (*Argyrosomus regius*). TMA value increased from 1.35 to 5.57 mg/100 g after 56 days of storage. TMA was derived from non-protein nitrogen compounds (trimethylamine oxide; TMAO) in fish by trimethylamine oxide reductase and degraded to TMA, dimethylamine (DMA), formaldehyde (FA) and ammonia (Benjakul *et al*., 1997). Thus, the increasing TMA value stimulates the increase of TVB value. The fresh fish normally has TMA value of 10-15 mg/100 g (Venugopal, 2006). TMA value of sample packed in air (T1) condition and stored at room temperature was >10 mg/100

g on day 15 of storage. Whereas, TMA value of samples stored under chilling condition was >10 mg/100 g on day 25, 30 and 20 of storage for samples packed in air (T1), vacuum (T2) and air with oxygen absorber (T3) conditions, respectively. Therefore, the vacuum packaging of half-dried salted round scad is the best packaging in slowing down the growth of aerobic bacteria.

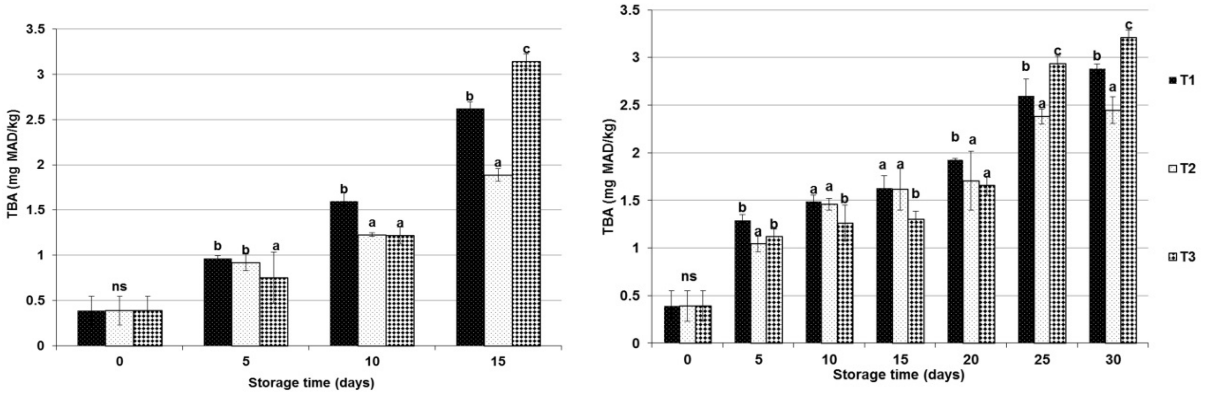


(a) **(b)**

Figure 3. Trimethylamine values (TMA) of half-dried salted round scad stored at (a) room temperature and (b) chilling condition under air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging

Thiobarbituric acid (TBA)

The TBA value of half-dried salted round scad stored at room temperature significantly increased along with the longer storage time ($p<0.05$). The sample packed under vacuum condition (T2) had the lowest increasing TBA value with an initial of 0.39 mg MAD/kg and increased to 1.89 mg MAD /kg on day 15 of storage. The TBA value of samples stored under chilling condition significantly increased with an increased storage time ($p<0.05$) with 2.45 mg MAD /kg on day 30 of storage. The increase in TBA led to the oxidation reaction of products and degraded to malonaldehyde. The increased TBA of half-dried salted round scad also has effects on food spoilage, nutrition and consumer health safety. Comparing to striped snaked-head fish, catfish and snapper queen fish, fatty fish with highly unsaturated fat, such as round scad, tend to have more oxidation reaction (Oyarekua, 2014; Lohalaksanadech and Sujarit, 2016). Moreover, Oxygen was a factor leading to oxidation reaction. So half-dried salted round scad packed in vacuum condition had the lowest TBA value according to the result of Antonios and Michael (2007) who found that the packaging of mackerel *(Scomber japonicas*) under vacuum condition resulted in the lowest increase of TBA when comparing to modified atmosphere packaging and air packaging. However, the limitation of TBA in fish was lower than 8 mg MAD /kg and fresh fish has <3 mg MAD /kg. Among fishery products, the TBA of 3-4 mg MAD /kg indicates the low quality product. When considering the TBA in dried salted round scad, the results indicated that half-dried salted fish still had acceptable levels of TBA even at the end of storage time.



(a) **(b)**

Figure 4. Thiobarbituric acid values (TBA) of half-dried d salted round scad stored at (a) room temperature and (b) chilling condition of air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging

3.2. Microbiological quality changes

Total plate count (TPC)

An initial number of TPC in half-dried salted round scad was 1.6×10^2 CFU /g in room temperature storage, and reached 2.9×10^6, 2.2×10^5 and 3.0×10^5 CFU /g on day 15 of storage for 3 samples packaging, respectively. Whereas, The TPC in samples stored in refrigerator reached 3.3×10^6, 2.0×10^5 and 3.5×10^5 CFU /g on day 30 of storage for samples packed in air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging respectively (Table 1-2). The highest numbers of TPC were observed in dried salted round scad packed in air packaging and stored at room temperature and refrigerator. Meanwhile, the lowest number of TPC was observed in samples packed in vacuum packaging and stored at room temperature and refrigerator. Even though, the Thai Community Products Standards does not define the TPC in sun-dried fish (Thai industrial standards institute; TISI. 298/2006), the TPC is still necessary for indicating the spoilage of products, especially the high moisture and low salt content products. If salt content in salted products is above 10% and their moisture content is below 40%, toxigenic-halophilic amine which forms bacteria will be inhibited (Koral *et al*., 2013).

Escherichia coli* and *Staphylococcus aureus

Escherichia coli (E. coli) and *Staphylococcus aureus* (*S. aureus)* of all 3 half-dried salted round scad packaging stored at room temperature and refrigerator were less than Thai Community Products Standards for sun-dried fish. The standard limitation of *E. coli* is less than 50 MPN/g and *S. aureus* is less than 200 CFU/g (data not shown). *E. coli* is used as a hygienic indicator for food processing plants, indicating the fecal contamination from animals and humans. *S. aureus* is a toxigenic microorganism which is harmful to consumers

Table 1. The microbial count of half-dried salted round scad stored at room temperature (30±2 ºC)

Storage time (days)	Total plate count (CFU/g)			Yeast and mold (CFU/g)		
	Air (T1)	Vacuum (T2)	Absorber (T3)	Air (T1)	vacuum (T2)	absorber (T3)
0	1.6×10^2	1.6×10^2	1.6×10^2	2.0×10^2	2.0×10^2	2.0×10^2
5	1.9×10^3	1.8×10^3	1.9×10^3	$1.6x10^2$	1.7×10^2	2.6×10^2
10	2.0×10^6	1.3×10^4	2.2×10^4	2.6×10^2	1.5×10^2	1.6×10^2
15	2.9×10^5	2.2×10^5	3.0×15^5	2.7×10^3	6.4×10^2	5.4×10^2

Table 2. The microbial count of half-dried salted round scad stored in chill condition (4±1ºC)

Storage time (days)	Total plate count (CFU/g)			Yeast and mold (CFU/g)		
	Air (T1)	Vacuum (T2)	absorber (T3)	Air (T1)	vacuum (T2)	absorber (T3)
0	1.6×10^2	1.6×10^2	$1.6x\times10^2$	2.0×10^2	2.0×10^2	2.0×10^2
5	1.3×10^3	1.4×10^3	1.8×10^3	1.9×10^2	1.8×10^2	2.5×10^2
10	1.1×10^4	1.6×10^4	2.3×10^4	1.7×10^2	2.0×10^2	3.0×10^2
15	2.4×10^4	1.9×10^4	2.8×10^4	2.9×10^2	3.4×10^2	2.1×10^2
20	2.9×10^5	2.2×10^4	3.0×10^4	2.7×10^2	3.7×10^2	2.3×10^2
25	3.1×10^6	2.7×10^4	3.2×10^4	6.9×10^2	3.5×10^2	5.5×10^2
30	$3.3x10^6$	$2.0x10^5$	$3.5x10^5$	$7.2x10^2$	$5.2x10^2$	$5.1x10^2$

4. Conclusions

Half-dried salted round scad stored in vacuum packaging showed the lowest quality changes comparing to samples stored in air and air with oxygen absorbers packaging. The chilling storage was able to increase the shelf life of half-dried salted round scad. It can be concluded that, the shelf life of half-dried salted round scad packed in air condition was 5 and 10 days length when stored at room temperature and chilled condition, respectively. On the other hand, the shelf life of half-dried salted round scad stored in vacuum package was 10 and 25 days under room temperature and chilled condition, respectively. For the greater amount of half-dried salted round scad as an industrial product, food additives are recommended to extend the product's shelf life.

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EFFECT OF DIFFERENT TYPES AND CONCENTRATIONS OF HYDROCOLLOIDS ON PASTING, HYDRATION AND SURFACE ACTIVE PROPERTIES OF PIGEON PEA FLOUR

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ABSTRACT

Present investigation was intended to find the effect of different hydrocolloids on the functionality of pigeon pea flour. Different hydrocolloids viz. guar gum, xanthan gum, pectin, alginate and carrageenan were added in pigeon pea flour at 1%, 2 % and 3 % and their influence on the pasting, hydration and surface active properties was evaluated. Pasting temperature increased in case of guar gum, carrageenan and alginate and it was contrary in case of xanthan gum and pectin. Peak viscosity increased in case of guar gum and xanthan gum and decreased with the addition of pectin, alginate and carrageenan. Similar trend was observed for hold, final, breakdown and setback viscosity as well. Water absorption capacity increased and water solubility index decreased with the increase in the inclusion of hydrocolloids except for the gaur gum which showed contrary results. All hydrocolloids decreased the oil absorption capacity of pigeon pea flour. Swelling capacity of pigeon pea flour increased linearly with the addition of all hydrocolloids. Inclusion of hydrocolloids resulted in improving the foaming properties of the blends whereas no effect on emulsification capacity was observed with decrease in the emulsion stability.

1. Introduction

Hydrocolloids are important class of food additives that are widely utilized to confer good techno-functionality to the food products. Hydrocolloids, particularly gums have high molecular weight and miscibility in water (Şahin and Özdemir, 2007; Gomez et al, 2007) and thus results in conferring good mouth feel and viscosity owing to the good interaction of its polymeric chain (Bet et al., 2018). Thus, hydrocolloids owing to its viscoelastic nature can also be utilized for the formulation of various gluten free products where it tends to mimic the function of gluten to impart rheological characteristics to the food system (Rojas et al., 1999). In addition to altering the rheological properties to the food products, hydrocolloids also confer stability by minimising the retrogradation and thus providing good freeze thaw stability (Davidou et al., 1996).

Pigeon pea is an important legume having good nutritional profile and can be explored as an important source of protein for the fortification and formulation of various food products. Besides being an important source of protein, pigeon pea also contains substantial amount of starch which confers it functional properties owing to its pasting behaviour. Pasting properties of any flour is important for characterization of its starch for its commercial utilization due to the peculiar features it imparts to the food by virtue of its amylose and amylopectin content, utilization of various moisture and temperature regimes and interaction with other food constitutes and

additives in the food systems (Torres et al., 2013). Gelatinization of starch involves a phase transition in which starch loses its crystallinity due to swelling by heating in excess of water with simultaneous leaching of amylose (Bet et al., 2018). However, the inclusion of hydrocolloids can result in interaction of hydrocolloids with the hydroxyl bonds of starch and change in its pasting properties (Fu et al., 2015). In addition to its affect on the pasting properties, hydrocolloids have tendency to interact with proteins and alter various functional properties like foaming and emulsification behaviour of the flour-hydrocolloid blend (Xie & Hettiarachchy, 1998; Mohammadian & Alavi, 2016).

The aim of this study was to determine the effect of different hydrocolloids on the functionality of the pigeon pea flour at different concentrations that are commonly employed in the food industry to confer good functionality to the various food systems. Functionality of the flour-hydrocolloid blend was studied by evaluating the effect of different hydrocolloids and their concentrations on the pasting characteristics, hydration properties, foaming and emulsification properties.

2. Materials and Methods

2.1. Materials

Pigeon pea was procured from local market of Ludhiana and grinded using Cemotech Mill (Model- 3303 Perten, Finland). Milled flour was sieved to obtain particle size of 40 µm. Food grade hydrocolloids viz. guar gum (GG), xanthan gum (XG), pectin (P), alginate (A) and carrageenan (C) were procured from Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

2.2. Preparation of blends

Hydrocolloid-pigeon pea blends were prepared by inclusion of aforesaid hydrocolloids at 1, 2 and 3 %, followed by thorough mixing and sifting the blends from 40 µm twice to allow even mixing.

2.3. Pasting properties

Pasting properties of flour blends were studied using followed by heating upto 95 ^{o}C (heating rate 6 ^{o}C/min) for 7.5 min and holding at same temperature for 4.8 min and cooling down to 50 ^{o}C (cooling rate 6 ^{o}C/ min). Pasting parameters viz. pasting temperature, peak viscosity, hold viscosity, final viscosity, breakdown viscosity and setback viscosity were recorded.

2.4. Functional properties

Water absorption capacity and water solubility index was determined by the procedure followed by Anderson *et al*. (1969). Oil absorption capacity was determined by the procedure followed by Sosulski *et al*. (1976). Emulsification and foaming properties were determined by the procedure followed by Elkhalifa & Bernhardt (2010) and Khattab & Arntfield (2009) respectively.

2.5. Statistical analysis

Samples were analysed in triplicate using One-way analysis of variance and means were compared by post hoc Tukey HSD test. P-value< 0.05 was considered significant. The data obtained was analyzed statistically using SPSS software (Version 22.0, IBM Corporation, NY, USA) to determine statistical significance of the treatments.

3. Results and Discussion

3.1. Pasting properties

The pasting properties of pigeon pea flour and hydrocolloid system at different concentration of hydrocolloids are presented in Table 1. The pasting properties of pigeon flour are profoundly influenced by the addition of different hydrocolloids. Suspension of pigeon pea flour and hydrocolloid is a complex system of starch, protein and hydrocolloids that gels to form a paste. The pasting properties of the different pigeon pea flour and hydrocolloid systems are influenced by the interaction of different hydrocolloids with pigeon pea flour and this interaction is dictated by swelling characteristic of pigeon pea flour, morphological characteristic of hydrocolloid

gel matrix and the resultant interaction between the starch and protein molecules of pigeon pea flour and gum matrix based on their thermodynamic compatibility depending on the molecular weight and heterogeneity (Biliaderis et al., 1997; Chaisawang & Suphantharika, 2006; Sikora et al., 2010).

Table 1. Effect of different hydrocolloids on pasting properties of pigeon pea flour

Hydrocolloid	Conc. (%)	Peak Viscosity (cp)	Hold Viscosity (cp)	Final Viscosity (cp)	Breakdown Viscosity (cp)	Setback Viscosity (cp)	Pasting Temp. (^oC)
Guar Gum (GG)	0	764^a	718^a	986^a	46^b	268^a	90^a
	1	804^b	761^b	1073^b	43^b	313^b	90.7^b
	2	951^c	928^c	1328^c	23^a	400^c	91.5^c
	3	1058^d	1039^d	1491^d	19^a	452^d	91.6^c
Xanthan Gum (XG)	0	764^a	718^a	986^a	46^a	268^b	90^d
	1	1382^b	1127^b	1358^b	255^b	231^a	87.5^c
	2	2841^d	2090^d	2394^d	751^c	304^d	85.1^b
	3	2485^c	1653^c	1938^c	832^d	285^c	84.1^a
Pectin (P)	0	764^d	718^d	986^d	46^a	268^b	90^b
	1	697^c	610^c	754^c	87^b	144^a	90^b
	2	655^b	550^b	694^b	105^c	144^a	89.2^a
	3	599^a	472^a	611^a	87^b	139^a	89.3^a
Alginate (A)	0	764^c	718^c	986^c	46^c	268^b	90^a
	1	618^b	594^b	818^b	24^b	224^a	91.5^b
	2	529^a	523^a	745^a	6^a	222^a	-
	3	519^a	518^a	747^a	1^a	229^a	-
Carrageenan (C)	0	764^c	718^c	986^c	46^a	268^b	90^a
	1	643^b	599^b	803^b	44^a	204^a	91.1^b
	2	571^a	528^a	714^a	43^a	186^a	91.6^c
	3	566^a	521^a	721^a	45^a	200^a	92.2^d

Values are represented as mean (n=3). a,b,c,d The means within a line followed by different superscripts are significantly different at $p < 0.05$ by Tukey's test

3.1.1. Guar gum

Inclusions of guar gum resulted in increasing the pasting temperature, peak viscosity, hold viscosity, final and setback viscosity, whereas the breakdown viscosity of the starch paste decreased. The pasting temperature of flour reflects the ease of cooking of the starch which is directly correlated with the ease of ingress of water molecules in the starch granule for the gelatinization of starch. The inclusion of guar gum resulted in decreasing the available water for the gelatinization of starch due to competitive absorption of water molecules by guar gum (Khanna & Tester, 2006) and resulted in increasing the pasting temperature of pigeon pea-guar gum paste. Moreover, inclusion of guar gum can also dictates the degree of amylose leaching from the starch granules and further restricting the water availability (Christianson, 1882). However, the increase in the peak viscosity could be due to interaction between the leached out amylose and guar gum, whereas the increase in the final viscosity could be attributed to the interaction between the guar gum and amylopectin (Funami et al., 2005). Hold viscosity of the pigeon pea flour linearly increased with the increase in the concentration of the GG and thus suggests the improved stability of hot paste on cooking with the increase in the level of GG. Overall, the results suggest that inclusion of GG will result in improving the swelling and slightly increasing the cooking temperature. However, increase in gum concentration will result in increasing the propensity of pigeon pea-guar gum paste to retrogradation as depicted by higher values of setback viscosity. The susceptibility of pigeon

pea-guar gum paste to increased retrogradation is due to thickening effect of the guar gum and increase in the concentration of low molecular weight amylose in the matrix (Funami et al., 2005a). Thus, pigeon pea-guar gum blends can be successfully employed for the formulation of noodles and pasta based products but less congenial for the formulation of the frozen products. Sikora et al. (2010) also reported increase in the brabender and setback viscosity for cereal and tuber starches with the inclusion of guar gum.

3.1.2. Xanthan gum

The inclusion of xanthan gum also increased peak, hot paste and final viscosities, however the linear increase was observed upto 2 % addition of xanthan gum unlike the incremental increase in these viscosities with inclusion of guar gum. This discrepancy can be attributed to the way the two gums behave at the molecular level; GG matrix tends to form entanglement solution whereas XG exhibits property of weak gel (Achayuthakan & Suphantharika, 2008). Paste containing XG exhibited high value of peak, hot paste and final viscosities by virtue of bridging flocculation where xanthan gum tend to adsorb on the surface of the starch granule and results in the synergistic interaction between swollen starch granules and XG (Sikora & Krystyjan, 2009). This interaction allows the proximity of starch granules by keeping them close and allows them to form better paste by easy ingress of the water that resulted in better swelling of starch granules (Mandala & Bayas, 2004). The effect of adsorption of xanthan gum on the starch granule can also be reflected on the incremental decrease in pasting temperature of pigeon pea-xanthan gum blends due to ease of ingress of the water molecule in the starch granule facilitated by the absorbed XG. However, the value of peak, hot paste and final viscosity decreased at 3 % concentration, which could be attributed to higher concentration of XG in the paste matrix that might resulted in poor paste formation due to poor interaction of starch molecules due to high electrostatic repulsion and poor gelation by competing with starch granules for water. No definite pattern for setback viscosity was observed with the increase in the concentration of XG. However, inclusion of 1 % XG exhibited lower value of setback in comparison of pigeon pea flour. The lower value for pigeon pea- xanthan gum blend paste can be attributed to reduced interaction of amylose chains among each other due to interaction between amylose and xanthan molecules (Leite et al., 2012). Therefore, the data suggests that inclusion of XG at 2 % in pigeon pea flour can confer better pasting properties to it and can be utilized for profoundly improving visco-elastic characteristic of the pigeon pea flour based dough. Also, the noodles and pasta based products formulated using pigeon pea- xanthan gum blend will have reduced cooking time with the higher inclusion of gum.

3.1.3. Pectin

Contrary to guar gum and xanthan gum, inclusion of pectin resulted in decreasing pasting temperature, peak, hot paste and final viscosities whereas the breakdown viscosity increased with the increase in the concentration of pectin. Propensity of pectin molecules to form hydrogen bonds with amylose molecules (Correa et al., 2013; Gałkowska et al., 2013) and covering the surface of starch granules (Ma et al., 2019) with resultant inhibition in the swelling of starch granules and subsequent leaching of amylose to thicken the paste resulted in low peak viscosity. Decrease in the hold viscosity and increase in the breakdown viscosity of pectin-pigeon pea blend can be ascribed to the destruction in the bonding of starch and amylose molecules due to shearing forces that resulted in easier deformation of the starch paste (Zheng et al., 2018). Similarly low value of setback viscosity can also be attributed to the bonding between pectin and amylose molecules that prevented the interaction of amylose chains with each other and decreased the retrogradation tendency of starch paste. Ma et al. (2019) observed decrease in the peak viscosity of corn starch paste with the inclusion of 0.5 and 1 % pectin, whereas Zheng et al. (2018) observed the similar progression of

increase in the breakdown viscosity of lotus root starch and pectin blend. Above results suggests that pectin has positive influence on improving the freeze thaw stability of products by virtue of prevention of the tendency to retrograde and therefore can be utilized in the formulation of frozen food products from pectin-pigeon pea blend. However, it might not be congenial for the products which require the starch to form stable matrix during product processing and exhibit high pasting in noodle and pasta products. However, it can be efficiently utilized in the gruel based products.

3.1.4. Alginate

Similar pattern of decrease in the pasting temperature, peak, hot paste and final viscosities was observed with the addition of alginate. However, contrary to pectin decrease in the value of setback viscosity was observed with the addition of alginate. Increased hydration of alginate during pasting of starch rendered less water available for the starch to imbibe and swell and therefore resulted in increase in the pasting temperature (Li et al., 2017). Poor swelling of starch due to competitive water binding of alginate also resulted in reduced leaching of amylose from the starch granule due to its poor swelling and therefore resulted in decreasing the peak viscosity of the paste. Similarly, the decreased value of breakdown viscosity is attributed to poor swelling of the starch granules and the viscosity of the paste was only attributed to the hydrated alginate molecules which tend to endure the shear forces during the pasting cycle and resulted in low value of breakdown viscosity. The value of setback viscosity was decreased with the addition of alginate, however further increase in concentration after 1 % has not shown any significant change in the value of setback viscosity. Interaction of leached amylose and alginate are responsible for decreasing the retrogradation tendency of pigeon pea-alginate blend. Similar effect of increased pasting temperature and decreased value of setback viscosity was observed by Li et al. (2017) on normal corn starch. Zhao et al. (2015) also observed the increased peak temperature and decreased breakdown and setback viscosity with the addition of sodium alginate to wheat starch.

3.1.5. Carrageenan

Carrageenan on the other hand also exerted similar aforesaid effects on pasting temperature, peak, hot paste and final viscosities whereas no effect on the breakdown viscosity was observed. These effects could be attributed to the electrostatic interaction of proteins and carrageenan as well the entrapment of the carrageenan in the starch granules that resulted in change in the surface characteristics of starch granule and restricted its swelling and decreased the aforesaid pasting viscosities (McHugh, 1987; Huc et al., 2014).

3.2. Hydration properties

Hydration properties are important characteristics of flour for the formulation of food products and depict the functionality of the flour in the resultant food product. Hydrocolloids by the virtue of its affinity for binding water can profoundly improve the functionality of food system. Table 2 highlights the effect of different hydrocolloid concentrations on the hydration properties of pigeon pea- hydrocolloid blends. Water absorption capacity (WAC) of all the pigeon pea- hydrocolloid blends were found to be increasing with the increase in the concentration of the hydrocolloid except for the pigeon pea-GG blend that exhibited linear decrease in the WAC with the increase in the GG concentration. The WAC of pigeon pea flour is attributed to the presence of high amount of protein that imbibes the water and exhibits good hydration. However, presence of GG hinders the absorption of water by proteins present in pigeon pea flour. Moreover, the unexpected decrease in WAC due to addition of GG can be ascribed to rate of hydration of guar gum. Usually for practical applications, hydration rate of two hours is essential for proper imbibition (Mudgil et al., 2014). Therefore, decreased WAC is attributed to

lower hydration time employed in evaluation of WAC.

Water solubility index decreased with the increase in the concentration of all hydrocolloids except for guar gum. Decreased WSI can be attributed to water imbibed by the hydrocolloids and increase in the viscosity of the blend that tends to trap the solids and prevent their leaching. However, slow hydration of GG resulted in lesser viscosity of blend and resulted in high WSI. As aforesaid, the WAC of pigeon pea flour is predominantly the function of proteins present in it whereas the swelling capacity of flour is predominant function of starch gelatinization. The incorporation of hydrocolloids resulted in the increase in the swelling capacity of pigeon pea flour by the virtue of gelation and resultant entrapment of water in the paste matrix. XG exhibited linear increase in the WAC whereas GG, A and C exhibited maximum SC at 1 % and pectin exhibited maximum SC at 2 %. Further increase in the concentration of hydrocolloids resulted in comparatively lower SC due to competitive water binding by hydrocolloids and decreased swelling of starch. Leaching loss is important property for the formulation of noodle and pasta products and low leaching loss is related to better product quality and behaviour during cooking. Incorporation of all the hydrocolloids except for alginate resulted in reducing the leaching loss. Reduced leaching loss is attributed to the better entrapment of solids in the viscous matrix of gelled starch-hydrocolloid matrix. XG and pectin exhibited linear decrease in the leaching loss with the increase in the concentration of hydrocolloid whereas GG and carrageenan exhibited minimum leaching loss at 2%. Incorporation of alginate exhibited increase in the leaching loss, however, the leaching loss showed no significant effect of hydrocolloid concentration after 1 %.

Table 2. Effect of different hydrocolloids on hydration properties of pigeon pea flour

Hydrocolloid	Conc. (%)	Water Absorption Capacity (g/g)	Water Solubility Index (%)	Oil Absorption Capacity (g/g)	Swelling Capacity (g/g)	Leaching Loss (%)
Guar Gum (GG)	0	1.178 ± 0.005^{d}	21.83 ± 0.12^{a}	1.142 ± 0.007^{c}	4.554 ± 0.009^{a}	35.18 ± 0.09^{c}
	1	1.115 ± 0.004^{c}	22.98 ± 0.17^{b}	1.128 ± 0.004^{b}	6.136 ± 0.012^{d}	34.23 ± 0.06^{b}
	2	1.061 ± 0.007^{b}	23.57 ± 0.09^{c}	1.088 ± 0.004^{a}	5.872 ± 0.009^{c}	33.86 ± 0.11^{a}
	3	0.988 ± 0.002^{a}	23.53 ± 0.03^{c}	1.085 ± 0.005^{a}	5.808 ± 0.011^{b}	35.84 ± 0.03^{d}
Xanthan Gum (XG)	0	1.178 ± 0.005^{a}	21.83 ± 0.12^{d}	1.142 ± 0.007^{d}	4.554 ± 0.009^{a}	35.18 ± 0.09^{d}
	1	1.594 ± 0.013^{b}	20.76 ± 0.10^{c}	1.115 ± 0.004^{b}	5.530 ± 0.012^{b}	33.08 ± 0.12^{c}
	2	2.065 ± 0.011^{c}	19.70 ± 0.00^{b}	1.088 ± 0.006^{a}	6.146 ± 0.011^{c}	32.83 ± 0.12^{b}
	3	2.617 ± 0.004^{d}	19.31 ± 0.00^{a}	1.122 ± 0.004^{b}	8.116 ± 0.013^{d}	31.42 ± 0.12^{a}
Pectin (P)	0	1.178 ± 0.005^{a}	21.83 ± 0.12^{d}	1.142 ± 0.007^{d}	4.554 ± 0.007^{a}	35.18 ± 0.09^{d}
	1	1.253 ± 0.010^{b}	19.68 ± 0.15^{c}	1.139 ± 0.011^{d}	5.496 ± 0.007^{b}	33.64 ± 0.04^{c}
	2	1.311 ± 0.011^{c}	19.23 ± 0.09^{b}	1.067 ± 0.005^{c}	5.878 ± 0.011^{d}	32.66 ± 0.07^{b}
	3	1.406 ± 0.005^{d}	18.88 ± 0.16^{a}	1.046 ± 0.005^{b}	5.764 ± 0.009^{c}	32.05 ± 0.11^{a}
Alginate (A)	0	1.178 ± 0.005^{a}	21.83 ± 0.12^{a}	1.142 ± 0.007^{d}	4.554 ± 0.007^{a}	35.18 ± 0.09^{a}
	1	1.185 ± 0.003^{b}	23.15 ± 0.12^{b}	1.095 ± 0.012^{a}	5.656 ± 0.023^{d}	36.25 ± 0.13^{b}
	2	1.199 ± 0.007^{c}	23.17 ± 0.09^{b}	1.111 ± 0.009^{b}	5.628 ± 0.014^{c}	36.07 ± 0.21^{b}
	3	1.227 ± 0.004^{d}	23.64 ± 0.02^{c}	1.211 ± 0.007^{c}	5.482 ± 0.012^{b}	36.13 ± 0.14^{b}
Carrageenan (C)	0	1.178 ± 0.005^{a}	21.83 ± 0.12^{b}	1.142 ± 0.007^{d}	4.554 ± 0.009^{a}	35.18 ± 0.09^{d}
	1	1.434 ± 0.004^{b}	20.93 ± 0.07^{a}	1.161 ± 0.004^{c}	6.136 ± 0.011^{d}	34.42 ± 0.07^{c}
	2	1.451 ± 0.012^{c}	20.83 ± 0.13^{a}	1.084 ± 0.006^{b}	5.872 ± 0.012^{c}	33.64 ± 0.11^{a}
	3	1.473 ± 0.015^{d}	20.87 ± 0.09^{a}	1.075 ± 0.004^{a}	5.808 ± 0.007^{b}	34.04 ± 0.09^{b}

3.3. Surface active properties

Data pertaining to the effect of different hydrocolloids on surface active (foaming and emulsification) properties is presented in Table 3. Foaming and emulsification properties of pigeon pea flour are attributed to its proteins. Foaming and emulsification properties of any flour are important for dictating its functionality in various processed food products where emulsification and foaming is vital for the formulation of food product. Food proteins are exposed to various processing conditions of temperature, pH, water activity, shear forces and various additives are added to it that alters the functionality of proteins during processing (Sahni et al., 2018). Therefore, hydrocolloids are added with proteins to improve their foaming properties and particularly to enhance the stability of foam (Mohammadian & Alavi, 2016). The value for foaming capacity and stability are 81.33 and 23.66 % and for emulsion capacity and stability are 61.76 and 60.29 % respectively. The values are higher than that reported by Oshodi and Ekperigin (1989). Higher protein solubility is prerequisite for the good foaming capacity and stability (Xie & Hettiarachchy, 1998). The enhancement in the foaming capacity was attributed to increased protein solubility and higher stability of the protein-hydrocolloid system can be ascribed to formation protein-polysaccaride complex by interaction with each other via interactions viz. electrostatic, H-bonding, hydrophobic and steric. (Kruif & Tuinier, 2001). Maximum foaming capacity and stability was observed at 3% for GG, P and A, whereas it was 1 % for C and foaming capacity remained same after 2 % in case of XG. Foaming capacity was found to be higher in case of anionic hydrocolloids in comparison to neutral GG. This could be justified on the basis of formation of soluble complex by protein- hydrocolloid interaction. Anionic hydrocolloids bind with cationic proteins and resulted in the formation of insoluble protein-hydrocolloid aggregates due to neutralization of charges (Schmitt et al., 1998) and further binding of anionic hydrocolloids to the neutral protein-hydrocolloid aggregates confers them negative charge and enhance their solubility (Ghosh & Bandyopadhyay, 2012). However, reduction in the foaming capacity after 1 % in case of carrageenan could be attributed to its highly anionic nature that might have resulted in electrostatic repulsion at higher concentration of gum.

Stability of food foams is dictated by the viscosity of interfacial liquid film that controls the rate of diffusion of entrapped air from the foam (Ghosh & Bandyopadhyay, 2012). Studies have reported that inclusion of hydrocolloids results in increasing the viscosity of solution with subsequent improvement in the foaming stability (Xie & Hettiarachchy, 1998; Mohammadian & Alavi, 2016). However, this increase in the viscosity of protein-hydrocolloid system is function of nature and structure of hydrocolloid (Xie & Hettiarachchy, 1998). GG, P and A exhibited highest foaming capacity at 3 % whereas XG and C exhibited highest foaming capacity at 1 %. Decrease in the foaming capacity after 1 % in case of XG and C can due to formation of excessively viscous solution and high electronegativity respectively that resulted in diffusion of air from the foams. Inclusion of hydrocolloids resulted in no effect on the emulsification capacity of the blend, however linear decrease in the emulsion stability was observed with the increase in the hydrocolloid concentration. Decreased stability could be attributed to thermal gelation of the hydrocolloids that resulted in diffusion of the oil entrapped in the emulsion.

Table 3. Effect of different hydrocolloids on surface active properties of pigeon pea flour

Hydrocolloid	Conc. (%)	Foaming Capacity (%)	Foaming Stability (%)	Emulsification Capacity (%)	Emulsion Stability (%)
Guar Gum (GG)	0	81.33 ± 0.57^{a}	23.66 ± 2.51^{a}	61.76 ± 0.00^{a}	60.29 ± 0.00^{b}
	1	87.66 ± 1.15^{b}	42.33 ± 2.08^{b}	60.78 ± 1.69^{a}	60.29 ± 0.00^{b}
	2	92.00 ± 0.00^{c}	70.33 ± 1.52^{c}	61.76 ± 0.00^{a}	59.80 ± 0.84^{b}
	3	99.33 ± 0.57^{d}	76.33 ± 1.15^{d}	62.74 ± 1.69^{a}	39.70 ± 0.55^{a}
Xanthan Gum (XG)	0	81.33 ± 0.57^{a}	23.66 ± 2.51^{a}	61.76 ± 0.00^{a}	60.29 ± 0.00^{c}
	1	86.00 ± 0.00^{b}	64.66 ± 2.08^{d}	61.76 ± 0.00^{a}	58.82 ± 0.00^{b}
	2	100.00 ± 1.73^{c}	58.33 ± 1.52^{c}	62.74 ± 1.69^{a}	58.82 ± 0.00^{b}
	3	97.33 ± 1.52^{c}	48.33 ± 2.51^{b}	61.76 ± 0.00^{a}	11.76 ± 1.89^{a}
Pectin (P)	0	81.33 ± 0.57^{a}	23.66 ± 2.51^{a}	61.76 ± 0.00^{a}	60.29 ± 0.00^{c}
	1	124.33 ± 1.15^{b}	114.33 ± 1.52^{b}	61.76 ± 0.00^{a}	60.29 ± 0.00^{c}
	2	125.66 ± 0.57^{b}	113.33 ± 2.51^{b}	61.76 ± 0.00^{a}	52.80 ± 1.67^{b}
	3	138.00 ± 0.00^{c}	124.00 ± 2.64^{c}	61.76 ± 0.00^{a}	44.26 ± 1.54^{a}
Alginate (A)	0	81.33 ± 0.57^{a}	23.66 ± 2.51^{a}	61.76 ± 0.00^{a}	60.29 ± 0.00^{b}
	1	105.00 ± 0.00^{b}	83.00 ± 2.00^{b}	60.78 ± 1.69^{a}	47.05 ± 0.54^{a}
	2	114.00 ± 0.00^{c}	88.33 ± 1.52^{c}	61.76 ± 0.00^{a}	47.45 ± 0.79^{a}
	3	120.66 ± 0.57^{d}	97.33 ± 1.15^{d}	61.76 ± 0.00^{a}	46.80 ± 1.13^{a}
Carrageenan (C)	0	81.33 ± 0.57^{b}	23.66 ± 2.51^{a}	61.76 ± 0.00^{a}	60.29 ± 0.00^{c}
	1	124.33 ± 0.57^{d}	102.66 ± 1.15^{d}	61.76 ± 0.00^{a}	50.58 ± 1.83^{b}
	2	107.66 ± 1.52^{c}	94.33 ± 1.52^{c}	61.76 ± 0.00^{a}	51.23 ± 1.63^{b}
	3	77.66 ± 0.57^{a}	60.66 ± 2.51^{b}	61.76 ± 0.00^{a}	38.34 ± 0.94^{a}

Values are represented as mean ± standard deviation. a,b,c,d The means within the line followed by different superscripts are significantly different at $p < 0.05$ by Tukey's test

4. Conclusions

Inclusion of different hydrocolloids altered the functionality of the pigeon pea flour. Pasting properties of different hydrocolloid-flour blends exhibited variations owing to the type of hydrocolloid added. Guar gum and xanthan gum were found to be potent hydrocolloids for improving the pasting properties of the pigeon pea flour and can be utilized for the formulation of pigeon pea flour based gluten free bakery, pasta and noodle products. Xanthan gum exhibited highest effect on the pasting properties and can be utilized even at low concentration of 1 %. However, it resulted in higher values for breakdown with the increase in the concentration of xanthan gum. Inclusion of pectin and alginate reduced the retrogradation tendency of paste and can be utilized in the formulation of frozen food products. However, these hydrocolloids are not congenial for the formulation of noodle and pasta products and can only be used in gruel based products. Hydrocolloids had positive influence on the water absorption and swelling capacity of flour, however incase of guar gum proper hydration time is required for exhibiting good functionality. Hydrocolloids decreased the OAC of pigeon pea flour and can be utilized for the low fat food formulations. Pigeon-pea hydrocolloid blends showed no synergism incase of emulsification capacity and decreased the emulsion stability. Therefore, hydrocolloid-pigeon pea combination is not suitable for the emulsion based products but can be used for improving the formulation of gluten free bakery and pasta products to development of healthy pigeon pea based formulations.

5.References

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