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CONTENT

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SWEET MARJORAM (*ORIGANUM MAJORANA* L.) AS A MAGICAL BIO-PROTECTIVE AGENT AGAINST FOOD SPOILAGE: A REVIEW

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ABSTRACT

Origanum majorana L. is an important member of *Lamiaceae* family. It is native to Cyperus though it is largely found in Himalayan mountain range. It is well known for its traditional use as a medicinal herb to treat gastro-intestinal, sleep and nervous system related issues. In the recent era, the use of synthetic antimicrobial agents for food safety has been questioned as consumers all over the world want to develop alternatives to antimicrobial treatments based on natural ingredients. This article compiles and justifies use of *Origanum majorana* L. as a potent preservative in food industry.

1. Introduction

Commercialization and globalization of food industry is facing main task of increasing importance of food safety and quality. Food products specially packaged food is vulnerable to contamination by various environmental and food borne microorganisms which are responsible for producing unwanted and unpleasant changes in food taste and odour. Some of the microorganisms produce harmful toxins spreading illness in consumers. A study shows that approximately 20% of eatable vegetables and fruits collected for population consumption are vanished due to storage spoilage caused by these micro-attackers (Barth et al, 2009). It is proved that amount of high water present in these food items provide a better home for microbes to feed and grow on them (Jay, 2003). Abundant amount of minerals, vitamins and fibres can be obtained from fresh fruits and vegetables at a lower cost (Nagy and Shaw, 1985). Due to the attack of spoilage microbes like bacteria and fungi the adequate supply of fresh items is hampered with the changing weather in surrounding (Ihekoronye and Ngoddy, 1985). For avoidance of this weather dependent microbial spoilage farmers opt for sun drying of vegetables and fruits which leads to loss of some important vitamins and minerals in them (Gallali et al., 2000). Similarly illness leading to death in some cases, caused by food borne pathogens is one of the main issues in undeveloped countries.The microorganism mainly responsible for deterioration and spoilage of food all over the world is fungi (Ihekoronye and Ngoddy, 1985; Gallali et al., 2000).

Increased incidences of foodborne toxicity and illness simultaneously accounting for financial losses to farmers, suppliers and consumers put strain on production of safe and good quality food in fresh as well as packaged form. (Beraha et al., 2009; Jay, 2003). It is therefore needed to use strong preservative methods to maintain the quality and availability of nutrients (vitamins and minerals) in fresh fruits and vegetables. The international standards released by FAO, also directs to use safe preservatives to maintain food quality (processed or raw) that it should be nourishing and contaminants free (Jay, 2003; Ihekoronye and Ngoddy, 1985).

Therefore in past years use of synthetic chemicals was increased as a source to protect microbial activity thus preventing microbial enzymes from spoiling fresh and packaged food. It has been advocated that some chemical based preservatives are harmful for consumers as they are able to convert some ingested items into toxic substances and carcinogens as reported in some cases (Nagy and Shaw, 1985). Hence, in the recent decade producers and stakeholders are constantly facing significant pressure to reduce or stop use of chemical based preservatives. As a result of this pressure and concern, antimicrobials from natural sources have received attention from manufacturers for their potent use as antimicrobial additive for food items.

2. Plants as natural preservatives

Plant products have been proved as important natural remedy for treatment of many health and beauty issues of human being. Many plant based products are in market and attracted a huge population due to their safer and strong desirable properties. Among these marketable products, green antimicrobials are in recent trends for use as antimicrobial and anti-oxidative agents for preservation of food items. Though their traditional use has already occupied our kitchens as taste enhancers or texture improvers, they always possessed antimicrobial properties (Beraha et al., 2009; Jay, 2003). Some of the plant products have been used in various cosmetic productions due to their pleasant aroma. Recently, use of essential oils has increased for their curative effects in aromatherapy (Nagy and Shaw, 1985). Essential oils form various plant parts are largely being utilized in pharmaceutical and other related drug developmental programmes.

Extracts and essential oils of many plants have been known to exhibit antagonist effect against a variety of food spoilage microbes (Amit et al., 2017). On the other hand these herbs have been proved to have substances that are capable of enhancing the taste and odour of regular food items. Some herbs possessing bactericidal or bacteriostatic properties are used as preservative in packaged meat (Dyankova et al., 2009). Since decades, chemical antioxidants are extensively used to protect food quality mainly by oxidatioon of lipid components of food items. Synthetic antioxidants such as butylated hydroxylanisole (BHA) and butylated hydroxyltoluene (BHT) are among mainly used inhibitors of lipid peroxidation which is capable of maintaining stability of lipids in food-stuffs (Moure et al., 2001; Tohma et al., 2016). However, as consumers have become aware of their carcinogenic nature, the use of synthetic antioxidants is now restricted by law (Stone et al., 2003; Bae et al., 2016). In this context, natural products have again gained interest of researchers and industrialists as they seem to be healthier as and safer (Topal et al., 2015a; Oztaskin, et al., 2015; Polat et al., 2015). In the recent years, spices and herbs have been used as antioxidants in processed foods as a promising alternative of synthetic antioxidants (Topal et al., 2015b).Various studies have demonstrated that essential oils obtained from different plant parts of *Origanum* spp. express a range of different biological activities, such as cytotoxic, antifungal, antioxidant, anti-inflammatory, antibacterial and insecticidal (Dyankova et al., 2009).

2.1.*Origanum majorana* as food spoilage protective agent:

The sweet marjoram (*Origanum majorana* L.) is a member of notably important family of economically and industrially useful plants in all forms i.e. fresh and dried. Since decades, it has been widely used as a household herb for treatment of various disorders and health issues (Table 1). These properties are attributed due to presence of many compounds such as terpenoids, phenols, esters etc. in different derivative forms.

These compounds are synthesized and stored in various plant parts such as leaves, flowers, stem etc. (Table 2). Its leaves are rich in aroma; flowers are used with other spices as condiment to enhance flavour and odour of many food items. It is commonly known as sweet marjoram and murua because of its

pleasant smell. Essential oil extracted from whole plant is also being used as flavouring agent in various commercially sold food stuff and beverage.

Table 1. Medicinal use of different parts of *O. majorana*

S.No.	Plant part used	Medicinal property
1.	**Leaves**	Antimicrobial, antiseptic, carminative, antitussive and used for gastrointestinal disorder, unilateral facial paralysis, headache, epilepsy, cataract, weakness of sight, ear pain, dyspnea, cardiac pain, spondylolisthesis, groin pain, back pain, fatigue, freckle, migraine, bronchial coughs.
2.	**Essential oil**	Flatulence, nervousness, diuretic, sedative toothache, muscular pain, asthma.

Table 2. Main compounds attributing properties of *O. majorana*

S.No.	Class of compounds	Main compounds present
1.	**Monoterpenoids**	a-pinene, beta-pinene, sabinene, myrcene, a-terpinene, y-terpinene, paracymene, terpinolene, a-phellandrene, beta-phellandrene
2.	**Sesquiterpenoids**	beta-caryophyllene, a-humulene
3.	**Monoterpenols**	linalool, terpine-1-ol-4, terpine-1-ol-3, a-terpineol, cis-thuyanol-4, trans-thuyanol-4
4.	**Terpenic esters**	linalyl acetate, terpenyl acetate, geranyl acetate
5.	**Phenol-methyl-ethers**	trans-ethanol
6.	**Aroma components**	Bicyclic monoterpene alcohol, cis-sabinene hydrate, a-terpinene, 4-terpineol, a-terpineol, terpinenyl-4-acetate, and 1, 8-cineol

3. Food preservative quality of sweet marjoram has been accessed on following points:

3.1. As Antioxidant

There have been several lab reports for its antioxidant activity against commonly found food spoilage. It is well known that the main cause of food deterioration is oxidative rancidity due to which finally rejected by consumers as spoiled food has bad odour, taste and texture (Embuscado, 2015b). Therefore, Some herbs and spices, such as oregano, thyme, ginger, black pepper, clove, marjoram, basil, peppermint, balm, fennel, parsley, cinnamon, cumin, nutmeg, garlic and coriander are added to lipid containing foods as they possess anti-oxidative properties which however stabilize lipids in food products by reducing their oxidation (Yanishlieva et al., 2006). Sweet marjoram is reported to be rich in compounds like beta-carotene, beta-sitosterol, caffeic acid, carvacrol, eugenol, hydroquinone, linalyl-acetate,rosmarinic acid, terpinen-4-ol, beta-carotene, caffeic acid, tannin, phenol, trans-anethole,ursolic acid and oleanolic acid which makes it a good scavenger of free radicals present in food items (Embuscado, 2015a and b).

This antioxidative property has attracted many researchers to analyse its activity in in-vitro and in-vivo. Amarowicz (2009) extracted phenolic compounds from thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.) and marjoram (*Origanum majorana* L.) with solvent ethanol and observed that antioxidant activity of extracts of marjoram was highest.

It is also observed that marjoram essential oil added with oils of rosemary has been proved to control and reduce lipid oxidation in beef patties (Mohamed and Mansour, 2012). Fried mullet and fish fillets coated with mixture of thyme (2.5, 5%) and marjoram (2.5, 5%) showed strong effects against species of family *Enterobacteriaceae* (Yasin and Abou talab, 2007). Lucera and colleagues (2012) also concluded this study in their review analysing preservative properties of some spices specially marjoram and thyme. Antioxidant property of thyme, sage and marjoram extract was examined in an experiment by using DPPH assay and results revealed that thyme, marjoram and sage presented stronger antioxidant activity in comparison to BHT and BHA (Hussein et al., 2012). Saeed and colleagues reviewed work of (Goni et al., 2009) on marojarm and oregano and observed that marjoram along with oregano essential oil is very effective antagonist for gram positive and negative bacteria. Activity of ethanol and hexane extracts of some herbs and spices including sweet marjoram have been assayed and reported upto 50% reduction in growth of food spoilage (Witkowskaet al., 2013).

Gutierrez et al., (2008) tested efficacy of essential oils in different combinations and found additive effects against *E. coli* and *P. aeruginosa*, when oregano was used in combination with thyme, marjoram or basil. Nessrien (2007) investigated deleterious effect of marjoram and thyme on the microbes spoiling the quality of semi fried mullet fish fillets during cold storage. Marjoram is well known to have antioxidant activity as well as therapeutic properties as assayed in various studies (Vagi et al., 2005; Tsai et al., 2007; Lopez et al., 2007; Yazdanparast and Shahriyary 2008; Al-Howiriny et al., 2009; Abdel-Massih et al.,2010; Viuda-Martos et al., 2010; Mossa and Nawwar 2011; Hossain et al., 2012). Anti-oxidant potential of this plant has been tested by various researchers (Ayari and Landoulsi, 2013; Erenler et al., 2016; Baatour et al., 2011) and they strongly recommend industrial application of this plant as food preservative.

3.2. As Antimicrobial Agent

In recent years its antimicrobial property has also attracted researchers and commercialist to use it as anti-spoilage agent in packaged food stuff. From Egypt it has been reported that a moderate activity was observed for all tested concentrations against common food borne bacteria viz. *Bacillus cereus* (Selim et al., 2013). In a different study it was found that it was highly active against *Bacillus subtilis*, followed by *Staphylococcus aureus* (Joshi et al., 2009). Fungistatic and fungicidal effect of essential oil of this plant has been reported on *Verticillium dahliae* and *Penicillium aurantiogriseum*; well-known disastrous fungi. These fungi are notoriously known for their post-harvest spoilage and deleterious effect on storage food (Rus et al., 2015). The antibacterial effect of essential oils (EOs) *Origanum majorana*, and some other plants have been investigated on two food borne bacteria viz. *Bacillus cereus* and *Escherichia coli* and the most effective antibacterial activity was obtained with marjoram and clary sage oil, alone and in combination (Tserennadmid et al., 2010). The antibacterial potential of marjoram along with some other herbs was screened against food borne pathogens including *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Salmonella Typhimurium* and *Bacillus cereus* and observed differential susceptibility of pathogens towards the extracts used.

It has also been reported in the same study that essential oils of marjoram contains alcoholic terpenoids which gives it potent antagonistic nature against food borne microbes (Shenawy et al., 2015). Antagonist effect of *Origanum majorana* L. (marjoram) essential oil has been reported against cultures of *Salmonella*

spp., Shigella, Proteus, E. coli, Citrobacter and *Pseudomonas* species isolated from poultry meat. This study claims that the essential oil of marjoram was more effective in comparison to conventionally used antibiotics such as amoxiclav (amoxicillin / clavulanic acid) against which these strains showed high resistance. Even *Salmonella* strain was more susceptible in comparison to other tested strains to antimicrobial action of marjoram essential oil (Marquesa et al., 2015). It was also recommended that essential oil and other extracts of marjoram could be used as a potent bio-protective agent for preservation of refrigerated meat. Thus, marjoram essential oil can play an important role as antimicrobial agent in refrigerated minced meat and potentially it might be used as a natural preservative ingredient for longer periods without the need to use hazardous preservatives in food industry (Omara et al., 2014). Extracts of plants specially spices have been traditionally used as an ingredient of food items but essential oil of spices has also been proved to work as preservative due to their antimicrobial activity against various food borne pathogens (Marquesa et al., 2015). The most commonly found and most notorious food borne pathogen responsible for usual intoxication and consequent illness is *Staphylococcus aureus*. Extracts of marjoram have been found to possess strong antagonistic effect against this bacterium also (Gottardi et al., 2016).

In a different study it was observed that different extracts of *Origanum majorana* L. (from various solvents) showed notably strong deleterious effect on *Staphylococcus aureus*, protozoa *Pentatrichomonas hominis* and six *Candida* sp. strains used (Srinivasan, 2014). Soxhlet extract of n-hexane, aqueous-ethanol extract and ethanolic- ammonia extracts were assessed in *in-vitro* assay for their activity against twenty pathogenic strains. It was found that n-hexane extract exhibited the highest antagonist activity and was capable to inhibit growth of maximum number of *Staphylococcus aureus* strains tested. The other bacteria showed resistance to almost all extracts, but *Acinetobacter baumannii* indicated a little growth inhibition by aqueous-ethanol extract. The extracts were also vigorous against three out of six *Candida* sp. strains tested, likewise ethanolic-ammonia extract was notably capable to reduce viability of *P. hominis*trophozoites by 50% at 160 μg/ml concentration the remaining extracts were found considerably less active (Kozlowska et al., 2010).

It is also found that essential oil of marjoram plant and coriander has capability to stop *Aspergillus flavus* infestation in chickpea seeds upto 50% (Prakash et al., 2012). *Origanum majorana* is also found effective against food borne pathogens *B.subtilis, E.coli* (Leeja and Thopil, 2007), *P.aeruginosa, S .aureus* and *A.niger* (Srinivasan, 2014). Potential of many spices including sweet marjoram against food spoilage microbes has been assessed and the results showed that spices such as marjoram possess substantial antimicrobial activity against food spoilage bacteria like *Bacillus subtilis* and *Pseudomonas fluorescens* as well as against food borne pathogens like *Staphylococcus aureus* and *Vibrio parahaemolyticus*.

It is also evident that oils of this plant are deleterious for harmful fungi like *Aspergillu sflavus* and also reported that its essential oil shows activity against antibiotic resistant microorganisms also such as methicillin resistant *Staphylococcus aureus* (Liu et al., 2017). Marques et al., (2015) also reported the antimicrobial activities of the EOs of marjoram against *S. aureus* isolated from poultry meat. In an antimicrobial investigation it was observed that *Candida albicans* was significantly susceptible to the action of marjoram essential oil in comparison to commercial antifungal agent (Badee et al., 2013).

Table 3. Microorganisms susceptible to contents of *O.majorana* extract

Bacteria	**Fungi**
Escherichia coli	*Candida rugosa*
Pseudomonas aeruginosa	*Debaryomyces hansenii*
Salmonella poona	*Kluyveromyces marxianus*
Helicobacter pylori	*Rhodotorula glutinis*
	Rhodotorula minuta
	Saccharomyces cerevisiae
	Trichosporon cutaneum
	Yarrowia lipolytica
	Zygosaccharomyces rouxii

Broth microdilution and agar diffusion methods were used to evaluate the antibacterial activity of *Origanum majorana* L. oil collected from Tunisia against 10 bacteria. The observation showed that this oil was highly vigorous against all of the tested strains. The most susceptible were *Streptococcus group A, Salmonella enteritidis, Shigella dysenteria* and *Escherichia coli.* Some of the bacteria were less susceptible to the tested concentrations of this oil; the least susceptible bacterium was *Pseudomonas aeruginosa* (Ben Ezzeddineet al., 2001).

In *In vitro* experiments it is showed that essential oil of *O. majorana* shows highly effective antimicrobial and antifungal activity against five bacteria and one yeast strain studied (Stefanakis et al., 2013).

An effective mixture was prepared which corresponded to 28%, 30% and 42% *O. compactum*, *O. majorana* and *T. serpyllum*, respectively and tested against *Bacillus subtilis* and *Staphylococcus aureus*.

This mixture depicted inhibition of *E. coli* at 75% and 25% of *O. compactum* and *O. Majorana* essential oils respectively. This finding clearly advocates use of *O. majorana* in successful application as natural preservatives in foods (Ouedrhiri et al., 2016). Similarly *Staphylococcus aureus* and *Enterococcus faecalis* have been found to supressed at concentrations of 0.4 to 3.2 mg ml^{-1} of essential oil of this plant. However, leaves and stems have shown maximum antimicrobial effects. The studies on antimicrobial activity of *Origanum majorana* L. essential oil have showed that it possess an important antagonist activity against various bacterial strains (Ibrahim et al., 2017). Mith and colleagues (2014) tested essential oil of sweet marjoram enriched with Terpinene-4-ol (24.21), a-terpinene (8.44), sabinene (7.12) and observed its excellent antagonistic action against Gram-negative bacteria than Gram-positive bacteria and stated that these materials can served assignificant natural alternative to control bacterial growth in food products. Chouhan *et al.,* (2017) explained in their comprehensive review on present status and future prospective of plant antimicrobials in food preservation and stated that the essential oil of marjoram can be successfully used to prevent growth of *Clostridium perfringens* strain (Radaelli et al., 2016).

Phenol compounds extracted and identified from sweet marjoram has attracted researchers in recent years because of its strong biological activities especially antimicrobial, antifungal and antioxidant. These properties make it a suitable replacement of conventionally used food preservatives in food industry (Baatour et al., 2011; Baatour et al., 2013). Carvacrol, terpinen-4-ol, andterpinen-4-ol and thymol are the majorly found compounds in sweet marjoram EO (Busatta et al., 2007). These compounds express high antimicrobial property in in-vitro as exposure of bacterial cells to compounds like results in increased membrane fluidity facilitating seepage of protons and potassium ions which finally lead to collapse of the membrane and ultimately cell death (Baatour et al., 2013; Freire et al., 2011).

Conversely, Cox et al., (2000) also confirmed that terpinen is also among the major compounds of marjoram EO which can inhibit oxidative respiration, a lethal action causing cytoplasmic membrane damage. It is also observed in some studies that marjoram essential oils are rich in phenolic compounds which attributes its potential both antioxidant and antimicrobial (Leeja and Thoppil, 2007 ; Sagdic et al., 2010). In other studies also antimicrobial properties of sweet marjoram have been evaluated which makes it a suitable substitute of chemical based preservatives (Table 3).

4. Conclusions

In conclusion, it is shown in this review that this plant possess many biological activities such as antibacterial, antifungal and antioxidant, which make it a suitable replacement of commonly used chemical based food preservatives. It is clear from tis review that many researchers have strongly recommended use of essential oil of *O. majorana* as a natural preservative ingredient in food industry. All parts of this plant have been proven to possess antioxidant and antimicrobial compounds so these might present important supplementary sources for use in food industries.Though, investigations are required to perform on its toxicity and other pharmacological aspects before its prominent use in food industry.

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DEVELOPMENT OF EDIBLE COATING WITH *SPIRULINA PLATENSIS* AND BY-PRODUCTS OF CASSAVA AND ORANGE

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ABSTRACT

The use of by-products from orange and cassava processing and the *Spirulina platensis* biomass was studied to prepare edible coating, using a Simplex-Centroid design. The formulations were evaluated regarding physical, optical, solubility and barrier properties. There was no significant difference among the samples in relation to water activity, grammage and swelling in acid solution (30 min) ($p>0.10$). The addition of *S. platensis* caused lower rates of swelling in $NaHCO_3$ solution. The cassava bagasse incorporation affected positively the density, which can be related to the starch content of this by-product. The ternary mixture of the components studied had the highest values for the color parameter a*. The results showed potential application of the films produced in dehydrated and green products.

1.Introduction

Population growth has triggered investigations into sustainable alternatives to meet the increasing need for food, mitigate losses and maximize the use of raw materials so as to value production chains. The researches on edible coating have been motivated by both increasing consumer demand for safe, convenient and stable foods (Konuk Takma and Korel, 2019). Edible coatings perform multiple functions; they may avoid moisture migration (active packaging), create barriers against external elements, retain aromatic compounds, improve mechanical characteristics of foods – providing structural integrity – and they have antioxidant and antimicrobial potential (Basumatary *et al.*, 2018; Hanani, Yee and Nor-Khaizura, 2019). In order to improve coating performance, applicability and functionality, several composites have been developed through fiber reinforcement (Sobral, 2015). Interaction among components of the formulations reflects on the final properties of the resulting matrices (Brodhagen *et al.*, 2015).

Starch has been the most common raw material used for making edible coatings due to its availability, biodegradability, even though it has high hygroscopicity. Otherwise, agro-industrial by-products have become promising alternatives as materials for coating production making possible the valorization of productive chains with sustainable technologies (Dantas *et al.*, 2015; Sobral, 2015). Brazil is the largest producer of orange and the second largest producer of cassava worldwide, which generates high amounts of by-products after juice and starch production, respectively (Bussolo *et al.*, 2018). The use of cassava bagasse is interesting due to its high starch content, while the flour produced from orange albedo has high pectin content (Maria *et al.*, 2012). Both by-products are rich in polymeric materials and have

potential film forming properties (Bussolo *et al.*, 2018). Addition of *S. platensis* may be original since this biomass has effects on health promotion and its antioxidant and antimicrobial activity, however, its behavior in the edible coating production must be evaluated (Lanlan *et al.*, 2015). The aim of this study was to develop edible coatings using cassava and orange by-products, as well as *S. platensis*, characterizing them in relation to their properties to enable their potential application.

2. Materials and methods

The components used in the edible coating production were: cassava starch (Yoki®, Paranavaí, Paraná, Brazil), commercial *S. platensis* (Fazenda Tamanduá® 0023/2014, Santa Terezinha, Paraíba, Brazil) cassava bagasse supplied by Lorenz® (a cassava processing industry located in Umuarama, Paraná, Brazil), orange bagasse (obtained in the food engineering laboratory of the State University of Ponta Grossa/ Brazil from orange albedo after juice extraction), glycerol (P.A Reactif®) and water. The by-products were submitted to dehydration in an oven (Tecnal TE-394/2, Piracicaba, Brazil) at 60 ºC for 24 h. The dried materials were grinded in a mill (Ika Werke M20, Wilmington, USA) and the particle size separation was performed through metallic sieves (Bertel, Caieiras, Brazil). For characterization analyses were used anhydrous calcium chloride ($CaCl_2$), sodium chloride (NaCl), hydrochloric acid (HCl) and sodium bicarbonate ($NaHCO_3$).

The Simplex-Centroid design was chosen for planning with three components, resulting in 10 experiments. The coating formulations were produced using the mixture of *S. platensis* (X1), cassava bagasse (X2), orange bagasse (X3) totalizing 2% (Table 1), cassava starch (4%), glycerol (1%) and water to bring the volume to 100%. The fixed concentrations of starch and glycerol were established in preliminary tests. Coatings solutions were obtained by casting, which consists of dispersion, solubilization and gelatinization in the solvent of the raw materials (Lopes et al., 2014). Formulations were placed in the RVA (Rapid Visco Analyser®, Newport Scientific, Narabeen, Australia), where gelatinization took place at temperatures from 45°C to 90°C, for 13 min, at constant mechanical stirring (160 rpm). After processing, the solutions were poured on acrylic plates and placed in a forced-air oven (Tecnal TE-394/2, Piracicaba, São Paulo, Brazil) at 42°C for 3 h. After drying, the coatings were put in a desiccator with NaCl-saturated environment (75% RU) at room temperature (Maria et al., 2012).

Table 1. Simplex-Centroid Design increased to mix components to edible coatings formulation

Formulation	**Coded variables**			**Real variables (g/100 g)**		
	S	CB	OB	S	CB	OB
A	1.00	0.00	0.00	2	0	0
B	0.00	1.00	0.00	0	2	0
C	0.00	0.00	1.00	0	0	2
D	0.50	0.50	0.00	1	1	0
E	0.50	0.00	0.50	1	0	1
F	0.00	0.50	0.50	0	50	50
G	0.66	0.17	0.17	1.32	0.34	0.34
H	0.17	0.66	0.17	0.3417	1.3266	0.3417

I	0.17	0.17	0.66	0.3417	0.3417	1.32
J	0.33	0.33	0.33	0.66	0.66	0.66

Note: S – ***Spirulina platensis***; CB – cassava bagasse; OB – orange bagasse

The moisture was determined according to Ghasemlou et al. (2011). The water activity (A_w) was analyzed by Aqualab3 TE (Decagon, Aqualab Inc, Pullman, WA, USA). Density was evaluated as the ratio between the mass and the volume of the coating. The thickness was measured by a digital micrometer (Mitutoyo, Tokyo, Japan). Grammage was determined by the ratio between the mass and the area of the material under analysis, while the final result was obtained by calculating the mean of the triplicates (Sobral, 1999).

Samples of the same size obtained through the use of a caliper, which had been previously dried, were immersed in distilled water (50 mL) and agitated for 24h at room temperature (25 ºC). The solubilized mass was quantified by filtration of the solution, dried in an oven at 105 ºC (Nova Ética®, Botucatu, São Paulo, Brazil) for 24h and, then, weighed (Gontard et al., 1992). Edible coating solubility in water (MS) was expressed as percentage, as shown by Equation 1, where $M_{initial}$ is the amount of initial dry material and M_{final} is the dry mass after a 24-hour period.

$$MS = \frac{(\text{M initial} - \text{M final})}{\text{M initial}} X\ 100 \quad \text{(Eq. 1)}$$

The water vapor permeability was determined gravimetrically, according to the ASTM E96 test, which was used by Duong et al. (2015). About 5 g of anhydrous calcium chloride was placed into a glass container called permeability capsule. The container was then sealed with the edible coating whose area was 0.00144545 m². Capsules were stored in a desiccator whose relative humidity was 75% (saturated solution of NaCl). The desiccator was kept in an BOD incubator (Tecnal, Piracicaba, São Paulo, Brazil) at 25 °C. Moisture gain in grams (W) was determined by successive weighing every 24h. Saturation pressure of water vapor (ΔP) at 25 °C was expressed as Pascal (Pa). Permeability was calculated by Equation 2.

$$\text{Permeability} = \frac{(W \times T)}{(A \times \Delta P)} \text{ (Eq. 2)}$$

Color determination was carried out by a direct reading colorimeter (Hunterlab, Miniscan EZ, Reston, VA, USA) in the central part of the material. Equal-area samples of edible coating were immersed into an acid solution (HCl, pH 1.8) and into an alkaline solution ($NaHCO_3$, pH 8.3). Samples were withdrawn every 10, 30 and 60 min, removing the excess of solution with absorbent paper, and after they were weighed. Quantification of swelling was made by using the Equation 3, where SI (%) is the swelling index, Wi is the weight of the wet film and Ws is the weight of the dry coating.

$$\frac{\text{SI} = (\text{Wi} - \text{Ws})}{\text{Ws}} X 100 \quad \text{(Eq. 3)}$$

Mechanical tests were performed in triplicate, with adaptations of the method described by De Carvalho and Grosso, (2004). Maximum tensile strength (MPa) and elongation at break (%) were determined using a Shimadzu® AG-I 10KN (Shimadzu, Kyoto, Japan) operating according to the standard method ASTM D882-02, with adaptations. The films were cut forming specimens of 0.10 m in length and 0.025 m in width, suffering a traction at a speed of 8 x 10^{-4} m / s, and the initial distance between the clamps being 0.050 m. The breaking stress can be calculated from Equation 4 and the breaking elongation according to Equation 5:

$$\text{Tensile maximum strength} = \frac{F_{max}}{A_{min}} \text{ (Eq. 4)}$$

Where:

F_{max} = maximum force (N)

A_{min} = minimum initial sample area (mm^2).

$$\text{Elongation at break} = \frac{Ar}{DG} x100 \quad \text{(Eq. 5)}$$

Where:

Ar = elongation (mm)

DG = initial distance between clamps (mm)

Statistical tests were performed to verify both normality of data (Kolmogorov-Smirnov) and homogeneity of variance (Levene). Data were adjusted to obtain the mathematical model and submitted to the analysis of variance (ANOVA), at 90 % level of significance, to check the statistical difference between the samples and the model significance. The general equation for modeling can be seen in Equation 6, where Y is the estimated response, β1, β2 and β3 are the regression coefficients for the linear model, β12, β13 and β23 are the regression coefficients for the quadratic model and β123 for the special cubic model. The quality of the adjustment was measured by the normality of residues (Kolmogorov-Smirnov). Response surfaces and their mathematical model that do not represent the totality of the data (R2 value below 0.7) are not shown. In the equations it was used S for *S. platensis* (X1), CB for cassava bagasse (X2) and OB (X3) for orange bagasse.

$$Y = \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 12X1X2 + \beta 13X1X3 + \beta 23X2X3 + \beta 123X1X2X3 \quad \text{(Eq. 6)}$$

3. Results and discussions

Moisture analysis is fundamental to understand the coating behavior, since low water contents may form brittle coatings whereas high water contents may develop an environment which favors microbial growth. In this study, moisture ranged between 11.69 and 16.31 g/100 g (Table 2).

This parameter was adjusted to obtain the corresponding mathematical model (Eq. 7) and the contour plot (Figure 1A). The model was adjusted and followed normal distribution of data (p=0.29). The contour plot confirms the tendency that moisture has to increase due to the presence of orange bagasse.

$$\text{Moisture} = 13.18(S) + 13.81(CB) + 17.95(OB) - 12.40(S.CB) - 14.16(S.OB) - 9.40(CB.OB) + 68.35(S.CB.OB) \text{ (Eq. 7)}$$

The water activity values ranged from 0.59 to 0.61, with no significant difference among the formulations. According to Corrêa et al. (2001), values below 0.80 decrease the velocity of most enzyme reactions as well as growth inhibition of fungi and pathogens.

The use of cassava bagasse affected positively the density of the formulations studied. This by-product, obtained after starch extraction, has high percentage of starch (64%) (Dos Santos Abrahão, Do Prado, Marques, Perotto, & Bernardo Lugão, 2006), depending on the efficiency of the extraction process and it has lower fiber content than that found in orange bagasse (78%) (Neres et al., 2016). It is known that high amounts of fiber form films with low densities, i. e., low compaction, which enables empty spaces to form (Wollerdorfer and Bader, 1998).

The velocity at which solvents disseminate into the polymeric matrix depends on the compaction level of the chains and on the number of molecules of the permeate. Therefore, the increase in density may be related to the high molar mass of starch (Almeida, Woiciechowski, Wosiacki, Prestes, & Pinheiro, 2013). This parameter may be associated with the moisture content of coatings, as shown in Table 2. Low densities enabled higher moisture permeation into intermolecular empty spaces. Denser films have higher grammage, making the polymeric matrix more compact and less permeable. The parameter density was adjusted to obtain the corresponding mathematical model (Eq. 8) and the contour plot (Figure 1B).

$$\text{Density} = 0.99(S) + 0.91(CB) + 0.74(OB) + 1.45(S.CB) + 0.59(S.OB) - 0.41(CB.OB) - 9.1(S.OB.CB) + 1.59\,(S.CB).(S - CB) - 1.8(S.OB).(S - OB) \quad \text{(Eq. 8)}$$

The model of the parameter density explains 97.5% of the data. There is no lack of adjustment, a fact that can be confirmed by the normal distribution of residues (p=0.880). The contour plot (Figure 1B) illustrates the data shown in Table 2.

Table 2.Moisture, water activity, density, thickness and grammage of coating formulations

Formulation	Moisture (g/100 g)	Aw	Density ($x10^3 kg/m^3$)	Thickness ($x10^{-3}m$)
A	14.84 ± 2.38^{abc}	0.61 ± 0.10	0.88 ± 0.13^{a}	0.25 ± 0.03^{e}
B	11.69 ± 1.27^{d}	0.60 ± 0.00	1.01 ± 0.29^{a}	0.23 ± 0.03^{e}
C	14.17 ± 2.21^{bc}	0.59 ± 0.30	0.86 ± 0.11^{a}	0.26 ± 0.06^{e}
D	13.58 ± 1.29^{cd}	$0.59\pm0,00$	0.82 ± 0.11^{ab}	0.25 ± 0.02^{e}
E	14.80 ± 2.38^{abc}	0.58 ± 0.00	0.44 ± 0.06^{d}	0.42 ± 0.05^{a}
F	16.31 ± 1.08^{a}	$0,59\pm0.00$	0.65 ± 0.15^{bc}	0.30 ± 0.04^{cd}
G	14.89 ± 0.70^{abc}	0.59 ± 0.1	0.49 ± 0.09^{cd}	0.35 ± 0.04^{bc}
H	15.73 ± 2.21^{ab}	0.59 ± 0.0	0.57 ± 0.04^{cd}	0.34 ± 0.04^{abc}
I	15.37 ± 0.70^{abc}	0.58 ± 0.01	0.62 ± 0.13^{cd}	0.40 ± 0.05^{ab}
J	15.45 ± 0.23^{abc}	0.59 ± 0.00	0.57 ± 0.08^{cd}	0.27 ± 0.04^{e}
P value (*K-S)	0.25	<0.01	0.57	0.19
P value (**Levene)	0.78	0.55	0.61	0.96
P value (ANOVA)	0.04	0.15***	<0.01	<0.01
P (****K-S)	0.29	-	0.88	0.49
Formulation	Grammage (kg/m^2)	WVP ($g.m/m^2.h.Pa$)		Solubility (g/100 g)
A	0.15 ± 0.03	$8.12.10^{-7}\pm2.45.10^{-8bd}$		31.76 ± 9.66^{abcd}
B	0.16 ± 0.02	$6.14.10^{-7}\pm6.83.10^{-8d}$		34.54 ± 2.47^{ab}
C	0.13 ± 0.02	$1.37.10^{-6}\pm1.60.10^{-7a}$		38.59 ± 3.32^{a}
D	0.14 ± 0.01	$5.78.10^{-7}\pm4.49.10^{-8d}$		32.58 ± 1.47^{abcd}
E	0.14 ± 0.01	$1.09.10^{-6}\pm2.63.10^{-8abc}$		33.55 ± 7.91^{abc}
F	0.12 ± 0.02	$1.06.10^{-6}\pm2.45.10^{-7abc}$		31.49 ± 6.47^{abcd}
G	0.12 ± 0.00	$1.32.10^{-6}\pm3.75.10^{-7a}$		26.68 ± 2.84^{cde}
H	0.13 ± 0.03	$1.10.10^{-6}\pm4.39.10^{-7abc}$		24.10 ± 4.84^{e}
I	0.13 ± 0.03	$9.41.10^{-7}\pm2.66.10^{-8bcd}$		25.88 ± 3.16^{de}

J	0.12±0.01	$1.28.10^{-6}$±$2.13.10^{-7}$[ac]	27.96±1.81[bcde]
P value (*K-S)	0.01	0.82	0.30
P value (**Levene)	0.63	0.76	0.78
P value (ANOVA)	0.48***	0.01	0.06
P (****K-S)	-	0.18	0.87

Note: *Normality test; **Homoscedasticity test; ***Non-parametric Kruskal-wallis test (p<0.10); **** Normality of residues; Different letters in the same column represent a significant difference according to the Fisher LSD test (p<0.10).

The thickness values ranged from 0.23 to 0.42 x 10^{-3}m in the edible coatings produced. Lower value was found in a ternary mix studying composed of protein/chitosan/ sunflower oil ($0.0581x10^{-3}$ m) (Abugoch, Tapia, Plasencia, Pastor, & Escalona, 2015). The samples presented significant difference between them (p<0.10) and the data were adjusted to obtain a mathematical model (Eq. 9) and representative response surface (Figure 1C).

$$\text{Thickness} = -2.43(S) - 2.22(CB) - 2.43(OB) - 1.38(S.CB) + 0.79(S.OB) - 0.89(CB.OB) - 1.98(S.CB.OB) - 1.22\,(S.CB).(S - CB) + 7.52\,(S.CB).(S - CB) \quad \text{(Eq. 9)}$$

High grammage result in high tensile strength which is desirable when coatings wrap food that is sensitive to handling (Aloui, Khwaldia, Slama, & Hamdi, 2011). However, the values found in this study did not show any significant difference (p<0.10). Consequently, the model could not be adjusted. Henrique et al. (2008) reported that coatings made from modified cassava starch had grammage values about 0.32 to 0.46 kg/m^2 than that found in this study.

There was a significant difference in water vapor permeability for the formulations tested, however, the model does not represent the totality of the data (R^2<0.7) and therefore the response surface and mathematical equation are not presented. Water vapor permeability in the formulations produced was lower than that found for coatings made from native cassava starch (2 g/100 g) and glycerol (1.9 g/100 g) (Gutiérrez, Tapia, Pérez, & Famá, 2015).

Interaction between the by-products and the polymeric matrix generates coatings with higher porosity (empty spaces). As a result, they incorporate water easily and provide more soluble structures (Bouchonneau et al., 2010). It is a critical factor in food coating because of its susceptibility to deterioration as a consequence of moisture. The values of water solubility found by this study were similar to the values reported by Saberi *et al.*, (2016) for coatings made from pea starch, glycerol and guar gum.

Starch films have low resistance in aqueous media, making permeability a limiting property (Woggum et al., 2015). In most of the formulations under analysis, the higher values of water vapor permeability increased solubility, as can be observed by correlation analysis (R^2 = 0.77, p <0.05). The highest solubility was recorded for the ternary mixture with the highest proportion of cassava bagasse.

Swelling occurs when there is direct contact of the edible coating and a liquid medium. It is a relevant parameter when the material aims at application to food. It is fundamental to know the strength that the material has in solutions (Da Silva, Gomes, Ricardo, & Machado, 2016). Table 3 shows the results of swelling at different times in acid and alkaline media.

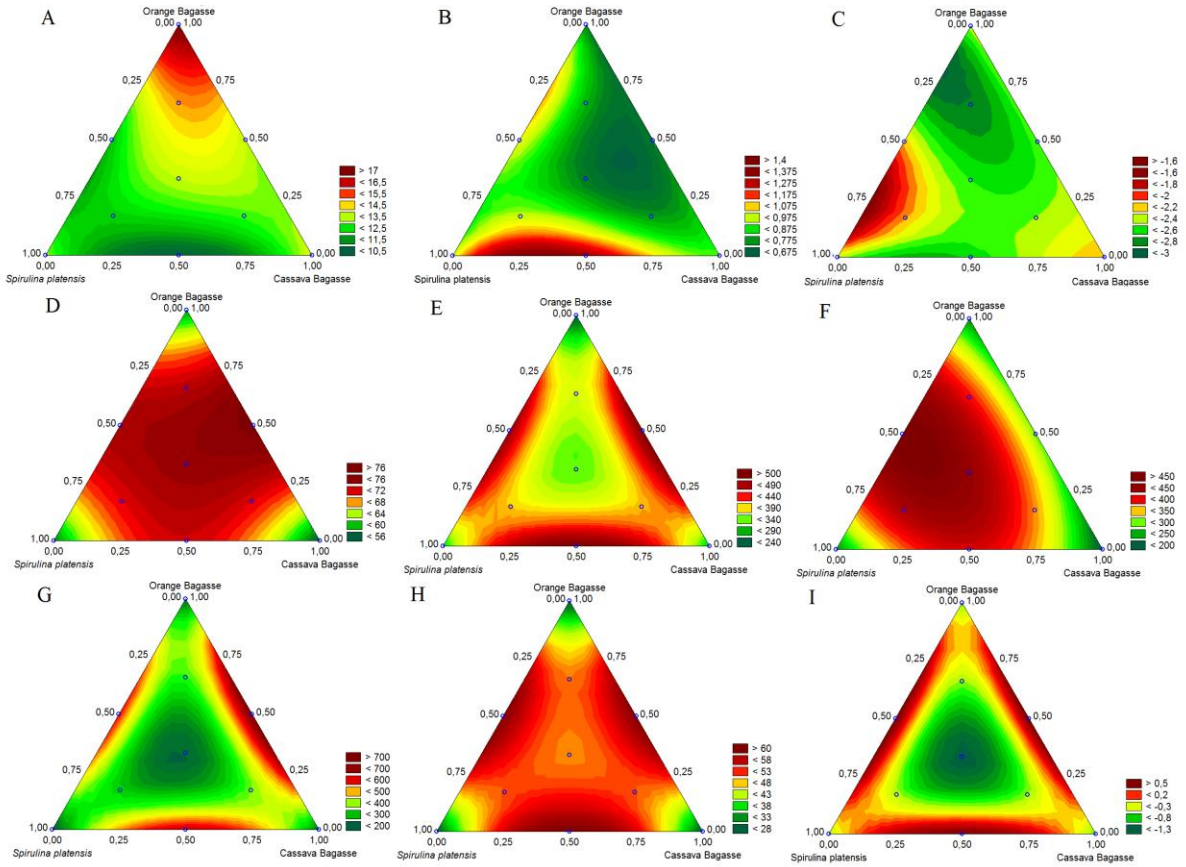

Figure 1. Response surfaces for the parameters moisture (A), density (B), thickness (C), swelling data in acid medium (SA 60 min) (D), alkaline medium (SK 10 min) (E), alkaline medium (SK 30 min) (F), alkaline medium (SK 60 min) (G), color a* (H) and color b* (I) of formulations under investigation

Table 3. Swelling index in alkaline and acid media of coating formulations under study

Formulation	S_{HCl} (%) 10 min	S_{HCl} (%) 30 min	S_{HCl} (%) 60 min
A	282.93±18.53^{cd}	433.18±57.45	634.88±35.71^{f}
B	458.18±38.35^{ab}	563.65±19.49	1007.35±65.92^{bc}
C	304.45±23.66^{c}	343.55±18.34	1173.29±13.84^{a}
D	408.93±29.12^{b}	475.25±28.55	824.76±4.48^{d}
E	253.11±25.26^{d}	386.29±5.69	795.28±23.33^{d}
F	295.75±59.80^{c}	539.95±20.67	1175.66±39.40^{a}
G	517.93±46.57^{a}	350.19±19.48	717.72±29.38^{e}
H	285.62±36.14^{cd}	329.32±9.32	1044.85±38.29^{b}
I	199.78±13.97^{e}	568.00±2.02	955.35±36.01^{c}
J	419.85±18.19^{b}	364.09±15.84	833.87±39.57^{d}
P value (*K-S)	0.48	<0.01	0.13
P value (**)	0.87	0.75	0.90

P value (ANOVA)	<0.01	<0.01***	<0.01
P (****K-S)	0.47	0.14	0.21
Formulation	S_{NaHCO3} (%) 10 min	S_{NaHCO3} (%) 30 min	S_{NaHCO3} (%) 60 min
A	278.31±35.39^{de}	211.20±22.56^{ef}	219.50±17.12^{e}
B	513.54±1.95^{a}	387.01±57.01^{ab}	661.51±68.40^{a}
C	379.76±61.79^{b}	435.67±38.18^{a}	281.81±95.06^{de}
D	331.71±24.27^{bcd}	361.67±27.06^{bc}	415.97±107.60^{bc}
E	307.36±28.56^{cd}	314.92±35.50^{cd}	448.70±17.91^{b}
F	338.53±65.24^{bcd}	443.90±69.31^{a}	617.49±46.12^{a}
G	291.24±67.84^{d}	266.62±57.55^{de}	279.63±60.70^{de}
H	364.29±60.14^{bc}	292.51±51.45^{d}	469.81±54.34^{b}
I	212.77±68.96^{e}	320.30±34.51^{bcd}	329.73±13.7^{cd}
J	366.84±25.25^{bc}	186.8±431.14^{f}	266.62±47.87^{de}
P value (*K-S)	0.68	0.92	0.31
P value (**)	0.12	0.93	0.95
P value (ANOVA)	0.01	<0.01	<0.01
P (****K-S)	0.43	0.08	0.72

Note: S – swelling; *Normality test; **Homoscedasticity test (Levene) (p<0.10); ***Non-parametric Kruskal-Wallis test (p<0.10); ****Normality of residues; Different letters in the same column represent a significant difference according to the Fisher LSD test (p<0.10).

The comparison of treatments showed that coatings with *S. platensis* addition swelled less in alkaline medium and they were more resistant. High swelling of films with both bagasse (HCl, 60 min, formulations B, C, F and H) may be due to the fact that they have significant amount of fiber. When immersed into a solution, they are able to hold higher amount of solvent in their porous channels which can be filled and make the coating swell (Bouchonneau et al., 2010). Therefore, in the case of orange bagasse, which has a large amount of pectin, the swelling index is expected to be high due to its hydrophilic nature and small number of crossed bonds (Altenhofen, Cristiane, Bierhalz, & Kieckbusch, 2009). There was no significant difference among samples in acid medium in the time of 30 min (p<0.10). The swelling data on acid (SA 60 min) and alkaline medium (SK 10, 30 and 60 min) were adjusted to obtain a mathematical model (Eq. 10, 11, 12 and 13) and response surface (Figure 1D, 1E, 1F and 1G). Equations and surfaces not shown were not representative of the data analyzed.

$$SA\ 60 = +57.40(S) + 55.08(CB) + 58.23(OB) + 62.17(S.CB) + 63.50(S.OB) + 79.62(CB.OB) - 136.50(S.CB.OB) \quad \text{(Eq. 10)}$$

$$SK\ 10 = +305.88(S) + 298.10(CB) + 230.42(OB) + 899.74(S.CB) + 903.26(S.OB) + 1017.35(CB.OB) - 7095.92(S.CB.OB) \quad \text{(Eq. 11)}$$

$$SK\ 30 = +231.23(S) + 199.97(CB) + 233.43(OB) - 1.38(S.CB) +$$

$$753.88(S.CB) + 937.11\ (S.OB) + 314.30(S.CB.OB)\ \text{(Eq. 12)}$$

$$SK\ 60 = +180.18(S) + 230.45(CB) + 218.45(OB) + 1645.58(S.CB) + 1493.35(S.OB) + 2040.32(CB.OB) - 15900.87(S.CB.OB)\ \text{(Eq. 13)}$$

In relation to the color parameters (Table 4), a* and b* values ranged from -2.73 to 0.77 and -1.24 to 16.99, respectively. The high starch content of cassava bagasse can interfere in the luminosity as observed in formulation B. The values found for the color parameters presented significant differences and were adjusted to obtain the corresponding mathematical model (Eq. 14 and 15) and the contour surface graph (Figure 1H and 1I). Equations and models not shown were not representative of the data (R2<0.7).

$$\text{Parameter L} = 28.55(S) + 28.18(CB) - 0.56(OB) + 27.88(S.CB) + 143.83(S.CB) + 138.12(S.OB) + 138.71(CB.OB) - 683.84(S.CB.OB)\ \text{(Eq. 14)}$$

$$\text{Parameter a}* = 0.48(S) - 0.61(CB) - 0.56(OB) + 5.70(S.CB) + 5.61(S.OB) + 5.74(CB.OB) - 73.12(S.CB.OB)\ \text{(Eq. 15)}$$

Table 4. Color parameters of coating formulations

Formulation	L*	a*	b*
A	26.67±0.57^{h}	-0.60±0.08^{d}	13.06±0.42^{d}
B	60.01±1.10^{a}	0.77±0.03^{a}	-1.24±0.23^{h}
C	60.35±0.53^{a}	-0.30±0.01^{c}	2.10±0.16^{g}
D	34.50±0.21^{f}	-1.81±0.03^{f}	16.99±0.07^{a}
E	37.12±1.28^{e}	-2.32±0.09^{g}	16.78±0.34^{a}
F	59.05±0.51^{b}	0.41±0.01^{b}	-0.57±0.18^{h}
G	32.58±0.43^{g}	-1.58±0.18^{e}	15.70±1.55^{b}
H	50.46±0.20^{c}	-2.48±0.06^{h}	11.69±0.36^{e}
I	50.01±0.14^{c}	-2.51±0.01^{h}	9.71±0.13^{f}
J	43.66±0.54^{d}	-2.73±0.08^{i}	14.30±0.71^{c}
P value(*K-S)	0.03	<0.01	<0.01
P value(**)	0.66	0.65	0.49
P value(ANOVA)	<0.01***	<0.01***	<0.01***
P model	0.35	0.11	0.35
P (****K-S)	0.04	0.02	0.03

Note: *Normality test; **Homoscedasticity test (Levene); ***Non-parametric Kruskal-Wallis test; **** Normality of residues; Different letters in the same column represent a significant difference according to the Fisher LSD test (p<0.10).

S. platensis has more pigments than the by-products used in the edible coatings production, such as chlorophyll, besides β-carotene and phycocianin (Liu, Huang, Zhang, Cai, & Cai, 2016; Wu, Liu, Miron, Klímová, & Wan, 2016). However, the center point corresponding to the ternary mixture showed the highest values for this color parameter (green color) as can be

observed in Figure 1I. It can be attributed to the interaction of the components of the microalga and the by-products used in the process of coating production. The coating that presented green color has potential for application in vegetables of this color.

The films developed in this study presented a low tensile strength compared to previous study by Mohammadi *et al.* (2018), where the values were about 60 times higher for this parameter. However, elongation values at rupture were relatively close (3.52-39.82 %). Data not shown indicate that the films did not have sufficient and adequate structure for this analysis.

4. Conclusions

In the edible coatings produced, the addition of *S. platensis* decreased the rates of swelling in $NaHCO_3$ solution, the orange bagasse and cassava bagasse affected positively the moisture content and the density, respectively. High density results in more compact material and fewer spaces for water incorporation, a fact that enables this coating to be applied to dehydrated products. The ternary mixture of the components studied showed the highest values for the color parameter a*, suggesting their application in green vegetables and potential incorporation of bioactive compounds due to the presence of *S. platensis*. Mathematical modeling and statistical analysis of data enabled different formulations to be compared regarding several parameters. Thus, this study provided information on the interference of various components incorporated to the polymeric matrix in coating characteristics and functionality. This study gave evidence of the viability of making edible coating with cassava and orange by-products and *S. platensis*. It is the starting point for future works on this subject using bagasse combination. The methodology and tests for larger scales must be adapted.

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INFLUENCE OF DIFFERENT EXTRACTION METHODS ON PHYSIOCHEMICAL AND BIOLOGICAL PROPERTIES OF β-GLUCAN FROM INDIAN BARLEY VARIETIES

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ABSTRACT

Currently barley has received renewed interest especially due to its high content of glucans. Since the glucan content vary greatly with genotype, four hull less barley variety (K-551, RD-2794, RD-2035 and RD-2552) commonly cultivated in northern plains of India were chosen for study. In this study we isolate β-glucan from different barley cultivars by four different methods includes alkali, acidic, hot water and enzymatic methods. Different extraction methods have significant effect on yield, recovery, functional and biological properties. Highest gum yield (4.85%) was found in samples that were extracted by hot water treatment in all barley cultivars. Although the highest recovery were achieved by enzymatic method (86.7%) followed by hot water extraction method (85.2%). Among all cultivars, RD-2552 identified to have highest glucan content (4.57%) and therefore selected to evaluate the effect of different extraction methods on functional and biological properties . Highest water binding capacity (3.82g/g) and foaming capacity (1.65g/g) was exhibited by hot water extraction method where as enzymatic method result in highest viscosity (49.1cP). The biological properties were determined in term of antioxidant and antimicrobial activities. In this study found extraction method have no significant effect on the antioxidant activity but have significant effect on antimicrobial activity. Among all method, enzymatic extraction methods give highest recovery of glucan and exhibit highest antioxidant and antimicrobial activity.

1.Introduction

Barley (*Hordeum vulgare* L.) is an important cereal grain throughout the world, belonging to the family Poaceae and the genus Hordeum (Mekonnon et al. 2015).In India, it is commonly grown in northern plains such as states of UP, Rajasthan, MP, Bihar, Punjab, Haryana, HP and J&K. They are rich source of protein, soluble fiber, vitamin, minerals and also exhibit positive physiological effects (Marwat et al. 2012).Even though,90% of barley commonly used as feeds for animals, malting and brewing (Stanca et al. 2016). Barley is one of the ancient crops and had been consumed as food since centaury however recently its functional importance has recognized due to its high nutritional value especially because of its high glucan content. β-glucans are the polymers of glucose units linked by β-1,3 and/or β-1,4linkage with or without side branches β(1,6)/β(1,2) glycosidic linkage (Barsanti et al 2011). Commercially β- glucan isolated from different sources such as oats, barley, mushrooms and some microorganisms.

Beta glucans derived from cereals are the long linear chain of glucose composed of cellatriosyl and cellatetraosyl units linked by β

(1- 3) and β (1- 4) linkages in a ratio of 3:7. Beta glucan constitutes 1 % of wheat grains, 3-7% of oats and 5-11% of barley (Skendi et al 2003). In barley, β-glucan concentrated in the cell walls of the endosperm and aleurone layer which varies with species. β-glucan has received considerable attention due to their health benefits includes promotion of colonic health, lowering the risk of constipation and colorectal cancer, reduction of serum cholesterol and regulation of blood glucose levels (Lattimer and Haub 2010; Das et al 2012).

Agencies such as Food and Drug Administration,1997 (FDA) and the scientific panel of European Food Safety Authority, 2010 (EFSA) has acknowledged the nutritional claims of β-glucan for lowering the blood glucose and cholesterol levels of individuals and recommended the intake of 3 gram of β-glucan per day for its health benefits.

In addition, β-glucans exhibits specific physiochemical properties such as viscosity, swelling capacity, foaming ability and stability that increase its potential for utilization as functional ingredients in food industry (Ahmad et al 2012).

It have been documented that the genetic background of barley is more important than environmental conditions for determining the β-glucan content of the grain. Along with β-glucan, its cell wall also contains enzymes, starch, protein, fats and minerals which interfere in the extraction of glucan.

For extraction of β-glucan various traditional to modified methods such as ultrasound-assisted extraction (UAE), response surface methodology (RSM), microwave-assisted extraction (MAE), and accelerated solvent extraction (ASE) have been developed.

Traditional methods such as alkali, acidic, hot water and enzymatic methods has been found to be simple, economical and proven to be successful in lowering blood cholesterol and glucose.

These methods have significant effect on the yield and functional properties of β-glucan which have technological role in food industry. The aim of the study was to evaluate the best extraction method on the basis of their yield, recovery and functional and biological properties. Since the glucan content vary greatly with genotype, four hull-less barley variety (K-551, RD-2794, RD-2552, RD-2035) commonly cultivated in northern plains of India were chosen for study. Four different procedures include alkali, acidic, hot water and enzymatic methods were employed to isolate the glucan.

Then the effects of different extraction methods on yield, purity, functional and biological properties of β-glucan were analyzed in order to obtain the best method of extraction.

FDA recognized barley as functional food ingredient exhibits the positive physiological effect but there are limited studies on the effect of extraction methods on its activity. Therefore, in present study antioxidant activity of different gum pellets were also analyzed.

2.Materials and Methods

2.1. Procurement and Sample Preparation

Among the different varieties of barley, four hull-less barley variety commonly cultivated in northern plain of India (K-551, RD-2794, RD-2552, RD-2035) were procured from Agro Food Industry, Jabalpur, India.

Whole barley was milled in a high-speed electric mill and sieved by a mesh size of 20mm and stored for further analysis.

2.2. Extraction and Purification of β-glucan

There are four extraction methods were used for extraction of β-glucan from whole barley grain. Four methods includes as alkaline extraction that employ NaOH, acidic extraction in which citric acid was used; hot water extraction and the fourth method was enzymatic extraction accomplish by αamylase enzyme.

Before extraction, 50 g of flour was suspended in 500ml of aqueous ethanol (80%, v/v) and stirred under reflux for 6 h at 85 ◦C to inactivate endogenous *β*-glucanases and to remove most of the lipids. A schematic outline of the extraction protocol is presented in figure 1.

Alkaline Extraction	**Acidic Extraction**	**Hot Water Extraction**	**Enzymatic Extraction**
100 g Flour	100 g Flour	100 g Flour	100 g Flour
↓	↓	↓	↓
Refluxing with 80% Ethanol for 6 hours	Refluxing with 80% Ethanol for 6 hours	Refluxing with 80% Ethanol for 6 hours	Refluxing with 80% Ethanol for 6 hours
↓	↓	↓	↓
Mixing the Flour with 1 M NaOH in 1:7 ratio	Mixing the Flour with 1 M Citric Acid in 1:7 ratio	Mixing the Flour with water in 1:10 ratio	Treated with heat stable Alpha amylase at 40 ° C
↓	↓	↓	↓
Mixing on hot plate with Magnetic Stirrer for 90 Min at 55°C	Mixing on hot plate with Magnetic Stirrer for 90 Min at 55°C	High speed stirring on hot plate with Magnetic Stirrer for 90 Min at 55°C	Centrifuge at 15,000 g for 20 Min at 40°C
↓	↓	↓	↓
Centrifuge at 15,000 g for 20 Min at 40° C	Centrifuge at 15,000 g for 20 Min at 40° C	Centrifuge at 15,000 g for 20 Min at 40° C	Supernatant was treated with Protease Enzyme at 37° C and incubated for 3 hours
↓	↓	↓	↓
Supernatant taken & mixed with 1 M NaOH in 1:3 ratio	Supernatant taken & mixed with 1 M Citric Acid in 1:3 ratio	Supernatant adjusted at PH 8.5 with sodium bicarbonate and stirred on hot plate for 30 min at 55°C	Centrifuged at 21,000 g for 25 min at 4° C
↓	↓	↓	↓
Centrifuged at 18,000 g for 20 min at 40°C	Centrifuged at 18,000 g for 20 min at 40°C	Centrifuged at 18,000 g for 20 min at 40°C	Mix Supernatant + Ethanol (80%) 1:1 and hold for 20 min
↓	↓	↓	↓
Supernatant adjusted at PH 7 with Citric Acid	Supernatant was Neutralized at PH 7 with NaOH	Supernatant adjusted at PH 7 with Citric Acid	Centrifuged at 4000 g at 4°C
↓	↓	↓	↓
Centrifuged at 21,000 g for 25 min	Centrifuged at 21,000 g for 25 min	Centrifuged at 21,000 g for 25 min	Dry Pellets in Vacuum Oven
↓	↓	↓	

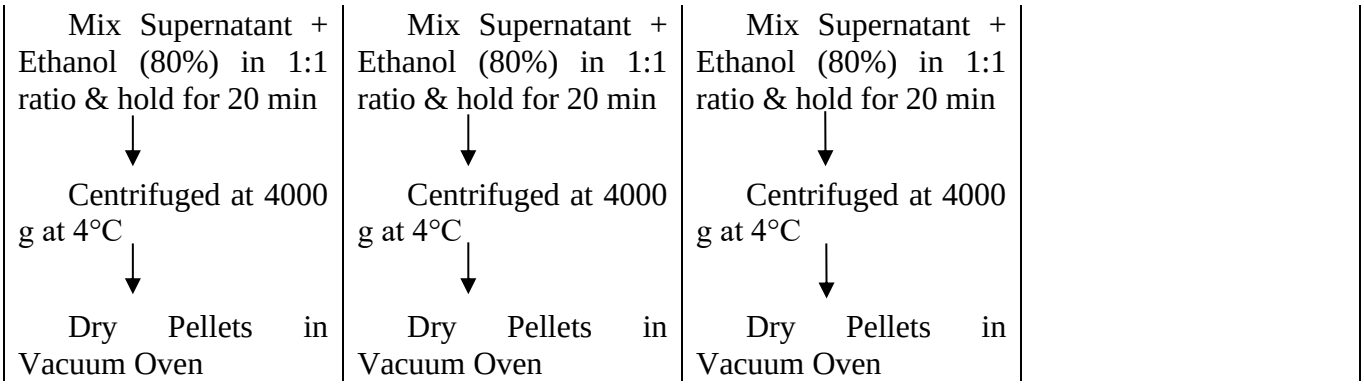

Figure 1. Procedure for extraction of β-glucan by four different methods

2.3. Estimation of β-glucan

The β-glucan content was determined using the β-glucan enzymatic assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). It determines the recovery of β-glucan from barley flour by different extraction methods.

2.4. Proximate Composition

The proximate composition includes moisture, protein, fat, starch and total dietary fiber of flour and gum pellets were determined according to the approved method of American Association of Cereal Chemists Method (AACC, 2003). Protein content of the β-glucan isolates was estimated by the method of Lowry *et al*.1967.

2.5 Physiochemical properties

The functional properties of β-glucan such as water binding capacity, swelling capacity, viscosity and foaming capacity were studied.

2.5.1. Water binding capacity

The water binding capacity (WBC) of samples was measured by the modified method described by Wong & Cheung (2005). Twenty millilitre of distilled water was added into a centrifuge tube containing 200 mg β-glucan. After which it was placed in a shaker at 25 ^{0}C for 12 h, the tubes were centrifuged at 14000 g for 30 min at 25 ^{0}C. The supernatant (unbound water) was discarded, and the amount of water held in the hydrated sample was determined by heating the pre-weighed pellet in a hot air oven for 2 h at 120 ^{0}C. The WBC of each sample was expressed as the weight of water held by 1.0 g of β-glucan sample.

2.5.2. Viscosity measurement

For viscosity measurements, 1% (w/v, as is basis) dispersion of β-glucan gum in deionised water was prepared by heating mixtures at 100 ^{0}C for 10 min followed by stirring on magnetic stirrer at 30 ^{0}C for 2 h and adjusting pH at 7. Viscosity was measured using a Rion viscometer (Rion Co., Ltd, Boston, MA, USA).

2.5.3. Swelling power

The swelling power was determined according to the method described by Okaka and Potter (1977). In 100 ml graduated cylinders, samples were filled to 10 ml mark and then distilled water was added up to 50 ml. Mix suspension thoroughly and mark the volume occupied by the sample after the 18 h.

2.5.4. Foaming capacity

The foaming capacity was studied by following the method of Temelli (1997) with slight modification. 2.5g of gum pellets were dissolved in 100 ml distilled water. The resulting solution was mixed vigorously for 2 min using a hand-held food mixer at high speed in a stainless steel bowl with straight sides and volumes were recorded before and after whipping. The percentage volume increase (which serves as index of foam capacity) was calculated according to the following equation:

Foaming capacity (%) = (volume after whipping – volume before whipping) × 100 (Volume before whipping) (Eq.1)

2.5.5. Color

The color value for β-glucan pellets were measure by using L^* a^* b^* color space with color tech, USA.

2.6. Biological activity

2.6.1. Antimicrobial activity

The antimicrobial activity was determined by well diffusion method. The bacterial culture obtained from the Department of Microbiology, Chandigarh, India. 100µl of glucan pellets(1mg/ml) extracted from different methods was added into wells. The plates were incubated for 24 h at 37 ^{0}C and zone of inhibition was measured by zone inhibitory scale.

2.6.2. DPPH radical scavenging activity

The radical scavenging activity of algal extracts was measured according to the method of Brand-Williams et al (1995) with slight modification. Briefly, fresh stock solution of DPPH (4.3 mg DPPH in 3.3 mL absolute methanol) was prepared and stored at 4 °C until use in the experiment. Concentration of 0.1 ml of glucan extract (0.5-2w/v%) was added to 150 µl DPPH solution and make up the volume to 3ml with pure methanol. The reaction mixture was incubated in the dark for 15minutes at room temperature, and the absorbance was measured at 517 nm was measured using UV–VISION spectrophotometer (UV-2450, Shmadzu, Japan). The radical scavenging activity of samples was expressed as the percentage discoloration of DPPH solution by using the following equation:

Free radical scavenging activity (%) = $\{(A_{blank}–A_{sample})/A_{blank}\}\times 100\%$ (Eq.2)

where A_{sample} was the absorbance with sample and A_{blank} was the absorbance without sample. Ascorbic acid was used as positive control.

2.6.3. Chelating ability on ferrous ions

Chelating ability was determined according to the method of Sutharut, (2012). 0.5ml of glucan extract (0.5-2 w/v%) was mixed with 1.6 ml of 80% methanol and 50µl ferrous chloride. The reaction was initiated by the addition of 0.1 ml of 5 mM ferrozine. After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against the blank. Blank was the solution with all reagents but without extract. A lower absorbance indicates a higher chelating ability. EDTA was used for reference standard.

2.7. Statistical analysis

All experiments were done in triplicates and data represent the means ± SD. The means were analyzed by one- way ANOVA and significant differences between treatments were tested using Duncan's multiple range test (DMRT). *P*-values ≤0.05 were considered significant. Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) 16.0.

3.Results and discussions

3.1. Screening of Barley Variety

3.1.1. Nutritional composition of barley varieties

Table 1 summarized the chemical composition of different variety of barley ranges 10.5-12.9% protein, 2.88-3.25% fat, 59.1-64.5% starch, 17-21.3%, total dietary fiber, and β-glucan content varied from 3.57-4.87%. The chemical composition of barley varies with environmental region and species (Zhu et al 2017). In consistent to previous study this study also that the major components of barley are starch, dietary fibre, and crude protein (Asare et al 2011;Biel et al 2013; Bleidere et al 2015 Rodehutscord et al. 2016).In all four Indian barley cultivar glucan present in good quantity varies from 3.57-4.87%. In support of this study, Irakli et al (2004) had observed β-glucan in range to 3.91% - 5.93% in the 6 barley varieties and Papageorgiou (2005) reported that the β-glucan content in barley and oat cultivars ranged 2.5% to 5.4% and 2.1% to 3.9%, respectively. Proximate composition of barley flour indicates that barley cultivar RD-2552 has highest nutritional value in term of ash (2.58%), protein (12.9%), fiber (21.3%) and β-glucan (4.57%).

Table 1. Nutritional composition of different Indian barley varieties

Barley variety	DWR-28	RD-2503	RD-2035	RD-2552
Moisture (%)	8.12 ± 0.9^{a}	8.65 ± 0.97^{b}	9.51 ± 1.32^{c}	9.43 ± 1.2^{d}
Ash (%)	2.2 ± 0.12^{a}	2.32 ± 0.143^{ab}	2.46 ± 0.16^{b}	2.58 ± 0.09^{c}
Protein (%)	10.5 ± 1.07^{a}	11.7 ± 1.1^{b}	12.4 ± 0.94^{c}	12.9 ± 1.14^{c}
Fat (%)	2.88 ± 0.21^{a}	3.17 ± 0.17^{b}	3.25 ± 0.13^{bc}	2.99 ± 0.17^{ab}
Starch (%)	59.1 ± 2.5^{a}	61.97 ± 2.7^{b}	62.34 ± 1.9^{b}	64.52 ± 2.3^{c}
TDF (%)	17.05 ± 2.5^{a}	17.95 ± 2.9^{b}	19.25 ± 3.1^{c}	21.35 ± 2.8^{d}
Glucan(%)	3.57 ± 0.19^{a}	3.73 ± 0.17^{a}	4.27 ± 0.21^{b}	4.873 ± 0.2^{c}

Values are averages of triplicates (Mean ± SD). Mean with different superscript letters in the same column are significant different ($p<0.05$)

3.1.2. Gum Yield and recovery of β glucan in different barley varieties

The result of present studies revealed that different extraction methods were found to have a significant ($P \leq 0.05$) effect on gum yield and recovery of all barley variety. Figure 2a elicited the yield of gum product (wt. of gum/100g flour) ranges from 3.1 to 5.8% in different barley variety by different extraction methods. In this support Vizhi et al (2014) and Ahmed et al (2009) documented the gum yield 4.65% and 5.4% respectively. Highest gum yield achieved by hot water method (5.85%) followed by alkaline method of extraction (5.1%). In consistent of previous research lower yield of β-glucan (3.62%) obtained under acidic extraction (Ahmed 2009, Ahmed 2010; Babu 2015). Several researchers were reported that along β-glucan small amounts of fat, protein, starch, pentosans and mineral (ash) matter also present in flour. They were extracted along with glucan as impurities and reduced the recovery of β-glucan. Consequently, in order to determine the efficiency of various extraction methods recovery of β-glucan has calculated. β-Glucan recovery represents the % ratio of weight of β-glucan in extracted gum product to the weight of β-glucan in 100 g flour. Figure 2b elicited that different methods of extraction have significant effect on glucan recovery ranges from 74.8%-86.7% in different barley cultivars. Enzymatic extraction followed by hot water extraction procedure results in highest recovery (%) in all barley variety. Among all cultivars RD-2552 attained highest recovery (86.7%) in enzymatic extraction method followed by 85.2% recovery attained in water method and lowest recovery (81.2%) obtained by alkaline method using 1M NaOH. Similarly 85%-92% of recovery in water extraction had been observed by previous studies (Irakli et al 2004; Skendi et al 2003; Papageorgiou et al 2005, Ahmed 2009). This study indicated that among all cultivars RD-

2552 identified to have highest glucan content and selected for further analysis.

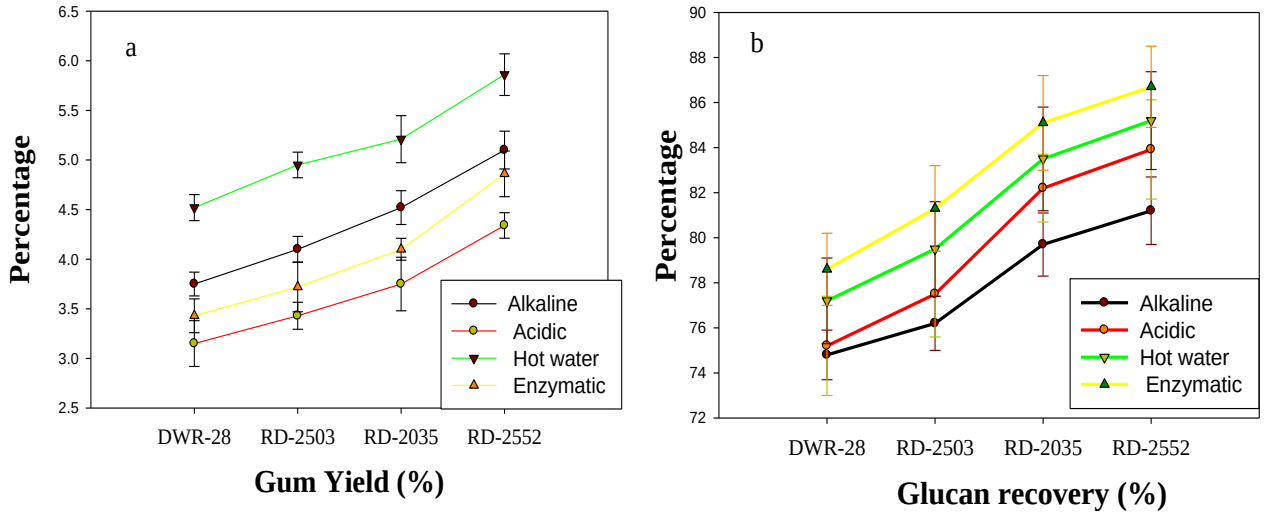

Figure 2. Yield and Recovery of β-glucan in different barley varieties

3.2. Effect of Different Extraction Methods on Selected Barley Beta Glucan

3.2.1. Chemical composition of β-glucan pellets

The chemical compositions of gum extracted from barley RD-2552 by different methods were illustrated in figure 3. All the methods have significant effect ($p \leq 0.05$) on the composition of gums includes starch, protein, lipid, fiber. These were the impurities extracted along with β-glucan and may affects its physiochemical properties and its utilization as functional ingredient in various food system (Wood, 2004; Burkus & Temelli, 2005). Among all constituent protein and starch were the major impurities vary substantially from 5.72% to 6.23% and 3.59% to 3.9% respectively. Highest protein content (6.23%) were observed in water extraction procedure shows its inefficiency to remove protein. Although, acidic treatments were found efficient to remove protein as added acid meets the isoelectric point of protein which support its precipitation and removal. This work found that substantial amount of glucan could be extracted by alkaline method but this gum have considerable starch (3.9%) and protein (6.1%) impurities resulting in impure glucan products (Cui et al 2000; Wei et al 2006;Li et al 2006). Like oats, in barley glucan compartmentalized in starch- protein matrix and therefore appeared as major impurities during extraction of β-glucan. Symons and Brennan (2004) compared extraction procedures showing that extraction with thermostable alpha-amylase yielded the purest β-glucan fraction. Lowest content of starch (3.59%) and protein (5.72%) were observed when enzymatic method was adopted. This was because enzymatic procedure were most efficient to remove impurities such as starch and proteins from extracted β-glucans due to the alpha amylase and proteinase enzyme facilitates the precipitation of starch and protein respectively (Mikkelsen,et al 2012). Although the highest content of fat (1.18%) was observed in enzymatic procedure this may undergo oxidative degradation and produces bitter flavor during processing and storage. The ash (mineral) content of glucan pellets varies from 1.23% to 1.65% with higher content in alkaline (1.65%) followed by enzymatic (1.55%) procedure. Different extraction methods significantly ($p < 0.05$) affect the total dietary fiber(TDF), soluble dietary fiber (SDF) and insoluble dietary fiber (IDF). It is well documented that high fiber reduces the risk of heart disease, diabetes, colorectal cancer, obesity and inflammation. In

this study gum extracted by enzymatic methods exhibit highest glucan content (88.18%) was comparable to previous study. The higher amounts of dietary fiber in extracted β-glucan gum makes it suitable for many industrial applications such as preparing bread, cookies and other cereal-based pasta produc

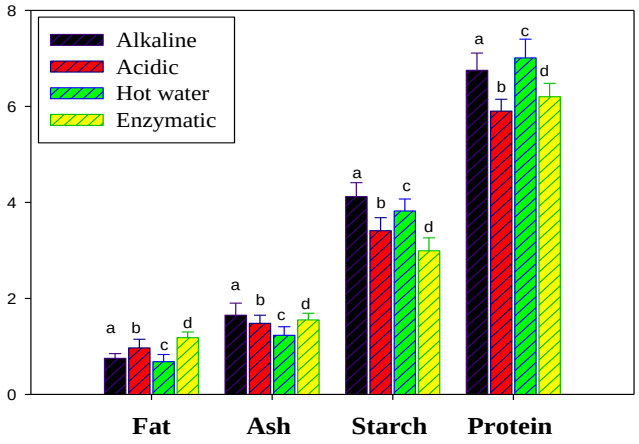

Figure 3. Chemical Analysis of β-glucan pellets extracted from different methods

3.2.2. Physiochemical Properties

3.2.2.1. Water binding capacity (WBC)

The functional properties are important determinants of β-glucan to be applied in food industry for formulation of different food products. Water binding capacity (WBC) is defined as the amount of water retained by a known weight of samples (Robertson *et al* 2000). Hot water extraction method exhibited the highest WBC (3.82 g/g dry weight) followed by alkaline (3.17 g/g dry weight) and acidic fractions (2.89 g/g dry weight). The water binding properties increases its potential for various food products such as jam, jellies, sauces and cheese as it put off the syneresis problem. In addition high water holding capacity also helps to control moisture migration and ice crystal formation; therefore increases thaw stability (Kulp and Joseph, 2014). Previous study reported that β-glucan well suited for the replacement of thickeners and to improve the consistency of food products such as drinks, dressing, and fermented dairy products due to its water binding capacity. Table 3 illustrated the significant positive correlation of WHC with starch($r = 0.77$, $p \leq 0.05$) and protein content ($r = 0.99$, $p \leq 0.05$) and negative correlation with fat ($r = -0.95$, $p \leq 0.05$).

3.2.2.2. Swelling capacity

Different extraction methods have no significant effect on swelling capacity on β-glucan . Swelling capacity of glucan varies between 8.2-7.98g/g in the following order: Alkaline>Acidic>Hot water>Enzymatic. The Pearson correlation of coefficient shows the positive correlation($r = 0.99$, $p < 0.05$) of starch with swelling capacity as starch absorb water. Proteins may lower the swelling power by being embedded in the starch granules forming a stiff matrix that limits the access of water into the starch granule and showed negative correlation with swelling power ($r = -0.30$).

3.2.2.3. Viscosity

Enzymatic method exhibits highest viscosity (50.1cP) but lowest WBC (2.32g/g), swelling capacity (7.98g/g) and foaming capacity (1.15g/g). Similarly, Panahi et al. (2007) reported that the viscosity of beta-glucan was highly preserved by the enzymatic method, which improved postprandial glycemic control when incorporated into a fiber drink. It have been reported that biological properties of glucan related to its ability to form viscous solution. Alkaline and acidic extraction methods reduced the viscosity of the extracted beta-glucan gum, because extreme pH can cause an unfavorable effect on the molecular structure,

especially on the beta-(1,3) linkages which leads to the depolymerisation of linear structure of β-glucan. The positive correlation was experienced between viscosity and soluble fiber ($r = 0.921$, $p \leq 0.05$) as presented in table 3**.**

3.2.2.4. Foaming capacity (FC)

Foaming capacity (FC) varied significantly ($P \leq 0.05$) among the flours. Foams are used to improve texture, consistency and appearance of foods (Akubor 2007). FC was calculated as % increase in volume of protein dispersion upon mixing. Highest foaming capacity (1.65g/g) was attained in water extraction procedure may be due to its high protein content which form a continuous cohesive film that trapped more air to develop more foam. This is further supported by the result of Pearson correlation coefficient (r=0.67) shows protein supports foaming ability. High foaming capacity and stability is desirable characteristic in making cakes and batters although the lowest foaming capacity exhibit in enzymatic method. Table 3 elicited the negative correlation (r= -0.99) between foaming capacity and fat content which is reasoned as fat interferes with the foaming properties

3.2.2.5. Color

The color characteristics (L*, a*, b*) of glucan pellets extracted from different methods are presented in table 2. The significant differences were observed between the different color parameters in all glucan pellets. The L* values for different glucan varied 69.2 to 75.5 indicates the lightness of product. Glucan extracted by hot water methods possessed highest L* (75.5) value and lowest a*(6.3) value. The high L^* value had a technologically advantage for the addition in various food product as it may increase product acceptance without affecting the base color. Correlation study (Table 4) indicates a highly positive correlation between L* value and soluble fibre ($r = 0.94$, $p \leq 0.05$). Highest b* value (29.4) attained by alkaline methods reflects the yellowness of glucans that increase its suitability for soups, dips and sauces. The positive correlation observed between b* value and starch content ($r = 0.85$, $p \leq 0.05$) as it has been seen that the variation in b* value among samples may be attributed to the amount of carbohydrate (Jamin and Flores 1998).

Table 2. Effects of different extraction methods on physiochemical properties

Extraction Methods	WBC (g/g)	Swelling capacity (g/g)	Viscosity (cP)	Foaming capacity (g/g)	L*	a*	b*
Alkaline	3.17^{a}	8.12^{ab}	37.12^{a}	1.52^{a}	69.2^{a}	11.7^{a}	29.4^{a}
Acidic	2.89^{b}	**8.2^{a}**	45.7^{b}	1.35^{b}	70.3^{b}	8.9^{b}	23.6^{b}
Hot Water	**3.82^{c}**	8.05^{b}	48.6^{c}	**1.65^{c}**	**75.5^{c}**	6.3^{c}	19.8^{c}
Enzymatic	2.32^{d}	7.98^{c}	**50.1^{d}**	1.15^{d}	72.8^{d}	7.5^{d}	15.7^{d}

Values are averages of triplicates (Mean ±SD). Mean with different superscript letters in the same column are significant different ($p<0.05$)

Table 3. Correlation among different parameters

	WBC	SC	VISCO	FC	L*	a*	b*
Starch	0.24	0.99	-0.99	0.38	-0.79	0.97	0.85
Protein	0.75	-0.30	-0.18	0.67	-0.12	-0.58	-0.16
Fat	-0.95	-0.58	0.48	-0.99	0.84	-0.28	-0.59
IDF	-0.299	0.792	-0.853	-0.122	-0.98	0.98	0.98
SDF	0.131	-0.877	0.921	-0.051	0.94	-0.89	-1.00

3.2.3. Anti-oxidant activity

β-glucans are the promising compounds having wide health promoting activity due to its biological activities. In present study the antioxidant activities were determined using different assays like DPPH (2, 2-diphenyl-1-picryl-hydrazyl), and metal chelating ability. In this study found that extraction method have no significant effect on the antioxidant activity of glucan. Free radicals are the major contributor degenerative diseases such as, diabetes mellitus cardiovascular disease, cancer and immune system. The radical scavenging activity was determined by DPPH assay at different concentrations is shown in Fig. 4a. Among all extraction procedures, gum extracted by enzymatic method exhibits highest free radical scavenging activity (43.3%-66.6%). No significant difference was observed in radical scavenging activity of alkaline and hot water extraction method. Transition metal ions, especially iron can stimulate lipid peroxidation and thereby perpetuate chain reaction. Chelating activity measures the ability of an antioxidant to deactivate the metals and prevents lipid peroxidation. Metal chelating activity at different concentration of gum extracted by different procedure varies from 35.9%-61.7% (figure 4b). The present result coincides with the study conducted by Kyoko kofuji et al (2012) reported that beta-glucan extracted by various methods have high scavenging activity and metal chelating activity.

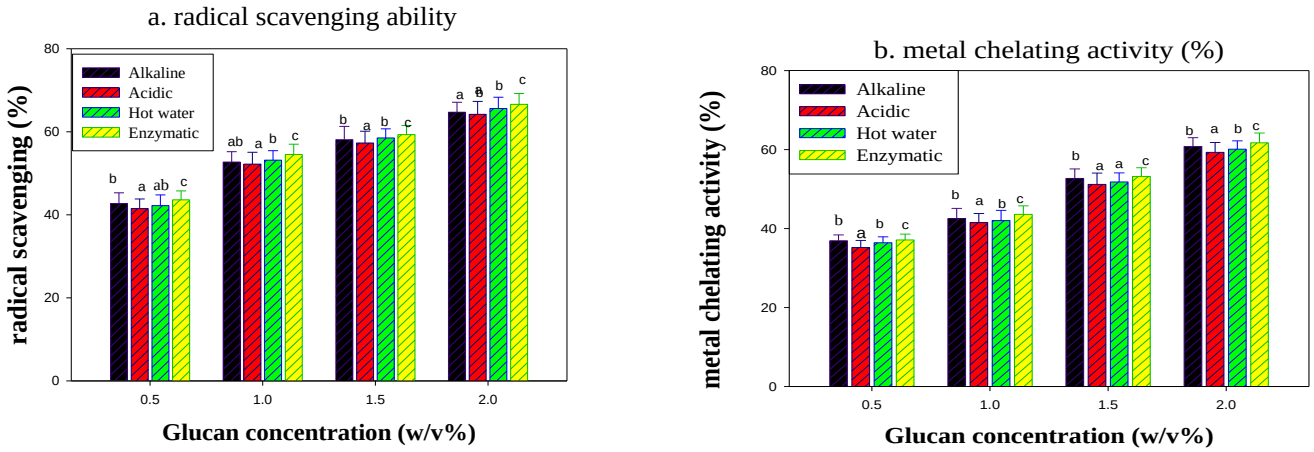

Figure 4. Antioxidant activity of β-glucan pellets extracted from different methods

3.2.4. Antimicrobial activity

In present study the antibacterial activity of glucan pellets was tested against both Gram-negative (*Escherichia coli,Pseudomonas, Shigella*) and Gram-positive bacteria (*Streptococcus aureus*) by well diffusion method. The antibacterial activity was determined by measuring zone of inhibition against standard (streptomycin), elicited in Table 4. The significant effect of extraction methods on antibacterial activity of β-glucan was observed. Among all the methods, maximum zone of inhibition was observed by enzymatic extracted glucan followed by hot water extracted glucan. The result revealed that all extract have good inhibition activity against tested bacteria. The maximum zone of inhibition was observed against *S aureus* (23mm) followed by *E coli*(21mm) and minimium zone of inhibition was observed against *Shigella* (10mm) followed by *psedumonas*(13mm). The antibacterial activity was explained due to interaction of polycations of glucan cell membrane to the negatively charged bacterial surface. This interaction alters the membrane permeability and thereby inhibiting the growth. No study was found that explain the effect of extraction methods on antibacterial activity.

Table 4. Antibacterial activity of glucan extracted by different methods

Extraction Methods	*Staphylococcus aureus*	*Pseudomonas*	*Shigella*	*E coli*
Alkaline	19^{a}	13^{b}	9^{c}	16^{d}
Acidic	20^{a}	15^{b}	11^{c}	17^{d}
Hot Water	21^{a}	17^{b}	14^{c}	19^{d}
Enzymatic	23^{a}	19^{b}	15^{c}	21^{d}

Values are averages of triplicates (Mean ±SD). Mean with different superscript letters in the same column are significant different (p<0.05%)

4.Conclusions

Various extraction procedures have been employed to extract the β-glucans from barley cultivars native of Northern plains of India. Barley variety RD-2552 found to have high nutritional value with high glucan content. The result of present study found that highest yield was attained by hot water extraction method whereas highest recovery and highest biological activity was achieved by enzymatic method followed by hot water extraction method. Enzymatic extraction method is found to be the best method of extraction on the basis of glucan recovery, functional and biological properties.

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ANTIOXIDANT AND ANTIMICROBIAL EFFECT OF SUMAC (*RHUS CORIARIA L.*) POWDER ON *E.COLI* AND *PENICILlIM NOTATUM* IN PREBIOTICS LOW FAT YOGHURT

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ABSTRACT

Yoghurt is one of the popular food diets with refreshing tasteand health benefits. The purpose of this study identify sumac powder with antioxidant and antimicrobial efficacy for controlling some food borne pathogens such as *Escherichia coli* and *Penicillium notatum.*The antioxidant and antimicrobial effects of sumac *(Rhus coriaria L.)* powder (0%, 1%, 1.5%and 5%) in prebiotic (Resistant starch type 2) low fat yoghurt, were performed respectively to the DPPH and surface cultivation during storage at 4C°.The data were expressed as mean ± SD and were tested by one-way ANOVA at α=0.05. Titratable acidity of yoghurt containing sumac powder increased whereas the pH decreased with increasing amounts of added sumac powder. Also, the antioxidant activity of yoghurt samples were significantly increased with the increasing of sumac powder from 0 to 5% ($p<0.05$). Different concentration of sumac powder reduced the number of *Escherichia coli* and *Penicillium notatum* in prebiotic low fat yoghurt during 28 days of cold storage. Addition of 5% sumac resulted the best attributes in prebiotic low fat yoghurt.

1. Introduction

Yoghurt is the most popular fermented milk products all over the world because of its high nutritive ,therapeutic values and sensory properties (Srivastava et al, 2015). The properties of yoghurt are due to the presence of lactic acid bacteria, which ferment lactose to lactic acid, so improve the nutritional values of yoghurt (Adolfsson et al, 2004).

This product is made with different fat percentages, but nowadays it is preferred to use low-fat and non-fatty dairy foods, especially those with high blood lipids and cardiovascular disease (Aghazadeh et al, 2010). But considering that the amount of total solids in the milk will greatly affect the physical properties and the texture of the manufactured yoghurt, reducing the fat content of the solids will decrease, so the produced yoghurt, of these compounds have a weak tissue. Studies have been carried out to improve rheological and the physico-chemical properties of low-fat and non-fat yoghurts using various additives. Therefore, to create such properties in low-fat products, appropriate additives such as resistant starches type 2 should be used for texture improvement which is a prebiotic ingredient (Heshmati et al, 2016). Prebiotics are nonviable food ingredients that exert a benefit on the health of the host,

linked with modulation of the intestinal flora (Cruz et al, 2010).

In spite of the growth of the food industry and the observance of health standards in the production and processing of food, a significant percentage of industrialized and non-industrialized countries are still affected by diseases caused by harmful microbes. Yoghurt as a fermented milk product is a favorite dairy food, the microorganisms cannot be survival in yoghurt. Given that most microorganisms, in particular intestinal bacteria, cannot tolerate a pH of less than 4.5, but there is evidence that some of the available yoghurts in the market may be infected with coliforms, including *E.coli* (Soomro et al, 2002). The reason for the presence of the coliform in yoghurt implies that it is contaminated with animal or human stools and indicates secondary contamination or violation in the production, storage and distribution stages (Faramarzi et al, 2012; Rad et al, 2019). Also the activity of lactic acid bacteria and high acidity as well as a relatively long duration of storage, they provide the conditions for the growth of molds, especially with the presence of *Penicillium notatum* in yoghurt. *Penicillium* notatum leading to unpleasant changes in the taste, smell and appearance of yoghurt, will remove it from the consumption cycle and bring heavy economic losses to yoghurt producer (Green & Ibe, 1987). Therefore, yoghurt can infect with bacteria and molds, cause digestive infections and food poisoning, so finding new compounds and methods to minimize growths and activity of harmful bacteria and mold in yoghurt is necessary.

Oxidative stress is caused by an imbalance between the production of free radicals within the body and the mechanisms of defense of biochemical antioxidants. In living organisms, peroxidation of lipids in the wall of living cells is one of the main objectives of free radicals. In this situation, cell wall structure and its function are affected. Therefore, the high presence of free radicals, especially peroxides, plays a key role in the pathogenesis of a number of diseases, such as aging, cancer, cardiovascular disease, various degenerative diseases of the lungs, and also plays an important role in the pathogenesis and progression of diabetes (Ejtahed et al, 2012; Thanonkaew et al, 2008).

So, the use of antioxidants is important to increase the shelf-life and safety of foods (McCarthy et al, 2001; Sadighara & Barin, 2010). According to previous studies, some of the synthetic antioxidants used in the food industry as preservatives have side effects. Therefore, the addition of natural antioxidants in order to confidence the quality and safety of the food is essential (Sadighara & Barin, 2010).

Recently, the use of various natural flavors in yoghurt manufacturing has been searched increasingly. Spices are a new source of functional flavoring agents. There is now mounting scientific evidence of health benefits of plant, including antibacterial, antifungal, antioxidant, as well as anti-carcinogenic properties (Azhdarzadeh & Hojjati, 2016). Sumac (*Rhus coriaria L.*) is used in the Mediterranean region and Middle East as a spice. The fruits have been reported to possess antimicrobial and antioxidant properties (Kossah et al, 2009).

Literature review implies that sumac powder was nor used for improvement of antioxidant and antimicrobial activity in yoghurt so far. The aim of this research was to manufacture functionally prebiotic low fat yoghurt containing sumac powder with increased antioxidant and antimicrobial activity.

2. Materials and methods

2.1. Preparation of sumac powder

Aerial parts of *Rhus coriaria L.* were planted in full flowering state of East Azarbaijan in summer (Kalībar, Iran) and confirmed by the Herbarium of the Faculty of Pharmacy of Tabriz University scientifically. Separate parts of the plant were cleaned and dried at room temperature for one week. The dried plant was then turned into powder well using the mill. In order to prepare the sumac powder, the dried parts of the sumac were completely milled and separated using a mesh No 335 nm evaluated.

Then they were stored in dark glass containers and refrigerated until to evaluation.

Preparation of yoghurt containing sumac powder

In order to produce low-fat yoghurt containing sumac powder, fresh cow milk is used accordance with Fig (1).

Bacterial and fungal strains

The *E. coli* (ATCC® 25922™) and one *p. nutatum* (ATCC® 9179™) were obtained from the Persian type culture collection (PTCC). They were recognize using conventional morphological as well as biochemical tests. Stock cultures of the bacteria were kept in 20% glycerol PBS (phosphate buffered saline) at -70°C. Active cultures were generated by inoculating 100 µl of the thawed microbial stock suspensions into 5 ml of nutrient broth (Merck, Germany), followed by overnight incubation at 37°C. The mold was cultured overnight at 35°C in Sabouraud Dextrose Agar (SDA) (Merck, Germany). Each microorganism was suspended in sterile saline and diluted at ca. 10^7 colony-forming unit (CFU/ml).

Preparation of McFarland solution

In this study, McFarland solution was used to be 0.5 and 1 respectively concentrations to 1.5×10^8 cfu/g and 3×10^8cfu/g, to prepare the 0.5 McFarland solution, first, 9.95 mm of acid sulfur 1% with 0.05 mm barium chloride% 1 and mixed with vortex was mixed (Mahon et al, 2011), Also to prepare one McFarland solution First, 9.9 ml of sulfuric acid 1% with 1.1 barium chloride 1% Mix and mix well with vortex.

2.2. Analysis of pH and titratable acidity

The pH of homogenized yoghurt was determined using a digital pH meter. Titratable acidity (TA) was determined by titration with 0.1N NaOH. Yoghurt sample (3 mL) was transferred into an Erlenmeyer flask containing 27 mL of dH2O. Three to five drops of 0.1% phenolphthalein as pH indicator were added. The yoghurt mixture was then titrated with 0.1N NaOH with continuous stirring until a stable pink color was achieved. The amount of acid produced during fermentation was calculated as follows:

TA (% Lactic acid)=Dilution factor×V NaOH×0.1N×0.009×100% (Eq.1)

Where VNaOH was the volume of NaOH required to neutralize the acid. A dilution factor of 10 was used.

2.3. Antimicrobial activity

The pathogens used for antimicrobial activity were provided by the persian type culture collection (PTCC).The pathogens cultured in EMB (Eosin methylene blue) and YGC (Yeast Extract Glucose Chloramphenicol Agar). Then 100 µl of each sample was spread by pasteurized pipette to each plate. The plates were incubated at 37℃. The experiment was repeated three times.

2.4. Determination of antioxidant activity using 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) inhibition assay

The antioxidant activity of different percentage of sumac powder in prebiotic low fat yoghurt was evaluated by monitoring their ability to quench the stable free radical DPPH (2, 2 diphenyl-1-picrylhydrazyl) using the method, described by Choi et al, (2002) with brief modifications.

Briefly, various concentrations of the samples (12.5, 250, 500, 625, 1250, 2500 µg/mL) were mixed with 6 ml of methanolic solution of DPPH (0.2mM). Absorbance was determined at 517 nm after 30 min of reaction time at room temperature. The various concentrations of sumac powder in prebiotic low fat yoghurt providing 50% inhibition (IC50) was calculated from the graph-plotting inhibition effect. Inhibition of DPPH oxidation (%) was calculated as conforms (Apostolidis et al, 2007):

% Inhibition= (Acontrol – Aextract) /Acontrol×100

Where A was absorbance at 517 nm

2.5. Sensory analysis

Sensory analyses were carried out by 8 trained no smoking panels of aged 24 and 30

years. Samples were coded with three-digit numbers and randomly served at 7 to 10℃ in plastic cups (9 mL). Assessors completed a test assessment form to compare four sensory attributes (texcher, color, taste, and overall acceptability) using a five-point hedonic scale (1, extremely poor; 2, poor; 3, fair; 4, good; 5, excellent). Sensory evaluation was accomplished at 1, 7, 14, 21 and 28 day of refrigerated storage at 4C˚.

2.6. Statistical analysis

The results were analyzed mean ± SD and Parameters were compared among groups by one way analysis of variance (ANOVA) followed by Tukey post hoc test. All statistical analyses were performed using the SPSS version 25 and Minitab 18 .$P<0.05$ were considered statistically significant.

3.Results and discussions

3.1. Changes in pH, titratable acidity

The pH and TTA of yoghurt prepared with (0%,1%, 1.5%,5%) sumac powder are shown in Fig. 2A and B, respectively. Yoghurt with 5% sumac powder showed a slightly lower pH than yoghurt with 0% sumac powder (Fig. 2A). These results were in agreement with that obtained by mahmoudi et al. (2013), who reported that the TA increased and pH decreased gradually during cold storage period of yoghurt treated with different concentration of the T. polium EO(Mahmoudi et al, 2014).Finally, reported that the composition of concentration of sumac powder, fermentation temperature, storage duration, contamination, could influence the overall level of acidity and pH of stored yoghurt samples(Singh et al, 2011).The constantly higher TTA in yoghurt added with (0%,1%, 1.5%,5%) sumac powder (Fig. 2B) could be attributed to higher acid production due to the addition of sumac powder to yoghurt. Yoghurt added with 5% showed TTA contents in the range of 3.68. TTA is generally high depending on the decrease of pH. However, the addition of sumac powder showed a different tendency with some unknown reason.

3.2. Antimicrobial activity

3.2.1. E.coli

The antibacterial activity of *R*. coriaria was the most effective against bacteria and this could be linked to the chemical constitutes of the plant including the phytochemical components and the rate of these substances in screened extracts, where most of these groups have the antibacterial properties. Plants have formed the natural products make excellent lead for new drug development. The World Health Organization (WHO) is encouraging, promoting and facilitating the effective use of herbal medicine in developing countries for health programs (AL-Mizraqch et al, 2010).

Some studies claim that the phenolic compounds present in spices and herbs might also play a major role in their antimicrobial effects (Hara-Kudo et al, 2004). *R. coriaria* contains phenols, tannins, and as in many research explained the action of hydrophobic property of phenolic compounds (Seyyednejad et al, 2008).

The results show that, inhibitory effect was affected by increasing of sumac powder concentration to 5 present. In many reports, growth or survival of *E. coli* in yoghurt (pH 4.0 to 4.5) even the 30 to 40 days has been stated (Massa et al, 1997; Simsek et al, 2007). In this research, *E. coli* survived during 1days of cold storage in control sample. According to our results, Sean et al; (1998) reported that *E. coli* has been suggested not to survive during fermentation process of yoghurt also presence of this organism in ready to eat yoghurt would showed the post processing contamination (Dineen et al, 1998). The population of *E.coli* inoculated in plain live yoghurt constantly decreased from an initial inoculums level of 9.6 $\times 10^7$ cfu/g (Control sample) to 0 cfu/g (Sample containing 5% sumac powder) after storage for 1 day at 4°C. Farrag (1992) reported that population of the pathogenic microorganisms in yoghurt samples decreased at various levels during the cold storage (Farag et al, 1992).

3.2.2. P.notatoum

Fungal spoilage of food and feed is a common and global phenomenon. It has been

estimated that 5–10% of the world's food production is lost as a result of fungal spoilage (Alves et al, 2000). In addition to the negative financial consequences, fungal spoilage of food and feed also poses a serious health concern. Fungal growth on foodstuffs can result in the production of mycotoxins which are known to be toxic to humans and animals (Sweeney & Dobson, 1998). The ability of fungi to grow in food and feed depends on a variety of factors including water activity (a_w), pH and nutrient availability. In addition, storage conditions as well as the presence of other microbes dictate which types of fungi will grow in a given food system (Montville & Matthews, 1997).Moulds have the ability to grow in a wide variety of foods, with different genera showing affinity for particular food types.

The occurrence of genera as Penicillium is a serious and frequent problem in the dairy industry, because such species can grow satisfactorily at the yoghurt/air interface (Ndagijimana et al, 2008). Sources of microbial contamination during yoghurt production include contaminated starters, poorly cleaned filters, contaminated cups and lids, overall hygiene in the manufacturing process, contaminated flavoring materials and air quality in packaging areas (Vedamuthu, 1991). The significance of fungal contamination in foodstuffs does not only refer to the potential of fungi as spoilers but also to the ability of many of them to produce a great variety of mycotoxins to which humans are susceptible (Lopez et al, 1998).

The results of the antimicrobial effect of different concentration of sumac powder (0%, 1%, 1.5% and 5%) after 28 days showed that there was a significant difference between the samples ($P< 0.05$) (Table 1). Maximum antimicrobial effect of sumac powder against *P. notatum* related to sample yoghurt containing 5% sumac powder. Interaction between storage time and sumac concentration showed in Fig3.

3.3. The antioxidant activity during the storage of yoghurt

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, and ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS. These free radicals are the major points in lipid peroxidation. The antioxidants may mediate their effect by directly reacting with Reactive oxygen species (ROS), quenching them and/or chelating the catalytic metal ions. Several synthetic antioxidants, e.g., butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available but are quite unsafe and their toxicity is a problem of concern (Rashid et al, 2012). Natural antioxidants, especially phenolics and flavonoids, are safe and also bioactive which are capable of absorb and neutralize free radicals, quenching singlet and triplet oxygen or decomposing peroxides. Recently focus has been concentrated on identification of plants components with antioxidant ability that may be used for human diet (Rashid et al, 2012).

In order to evaluate the antioxidant effect of sumac in (0%, 1%, 1.5% and 5%) during 1, 7, 14, 21 and 28 days. According to the results, there is a significant difference between the different concentrations of sumac powder ($P<0.05$). Analysis of variance in Table (2).

The level of antioxidant activity in yoghurt samples containing different percentages of sumac powder was significantly higher than that of control samples ($P<0.05$). The sample containing 5% of the sumac powder had the higher antioxidant activity. At 1% concentration, it showed the lowest antioxidant activity in all times (Fig 4).

3.4. Sensory evaluation

The sensory properties of the yoghurt prepared with sumac powder at concentrations of 0%-5% were evaluated by 8 trained no smoking panels of aged 24 and 30 years, and the results are summarized in Table 3. The flavor score of yoghurt containing sumac powder ranged from 2.475to 3.675. The color value of

yoghurt containing sumac powder ranged from 3.625to 4.275.The texture value of yoghurt containing sumac powder ranged from 3.750 to 3.625. The mean acceptance scores ranged from 2.750 to 3.650 (Table3).

The overall acceptability increased with increasing amounts of added sumac powder. High scores was received by yoghurt with 5% sumac powder (Fig 5). Therefore, the addition of sumac powder in yoghurt improved the functional properties such as antioxidant activity as well as the sensory characteristics of yoghurt. These results indicate that the addition of sumac powder at a concentration of 5% would be good for the production of acceptable color and functionally enriched yoghurt fortified with antioxidant, anti-obesity, and anti-inflammatory components. In conclusion, the addition of sumac powder to yoghurt resulted in increased titratable acidity and water activity (aw). In sensory evaluation, yoghurt containing 5% sumac powder received higher scores for, flavor, color, overall acceptability.

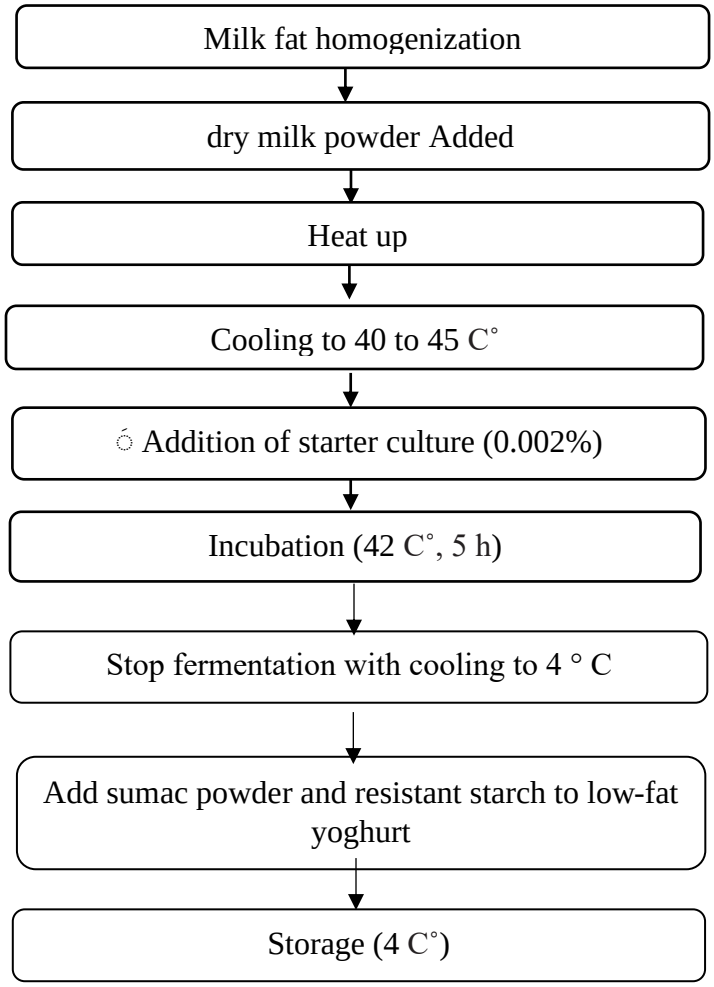

Figure 1. Procedure for the manufacture of low fat yoghurt with added sumac powder

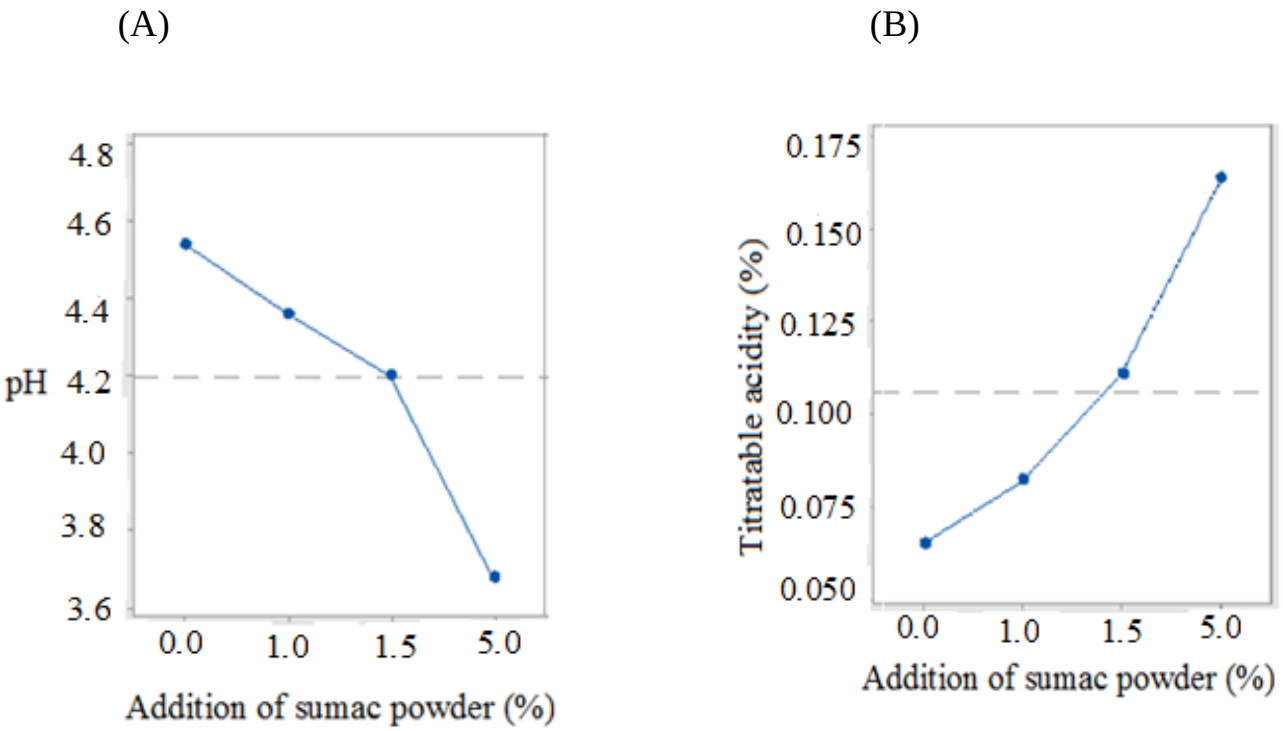

Figure 2. Changes in pH and titratable acidity (A) pH; (B) Titratable acidity

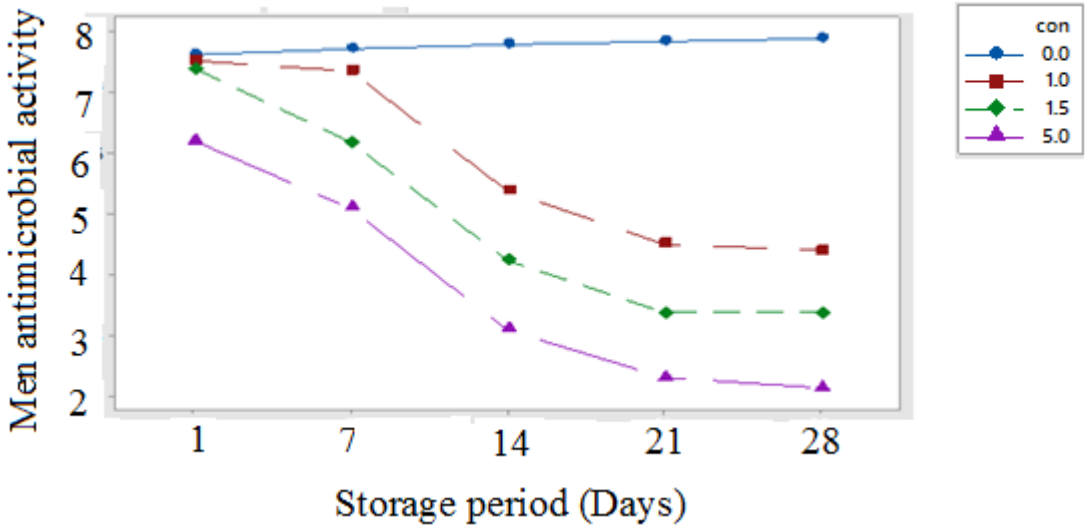

Figure 3. Change in antimicrobial activity versus storage time and sumac concentration

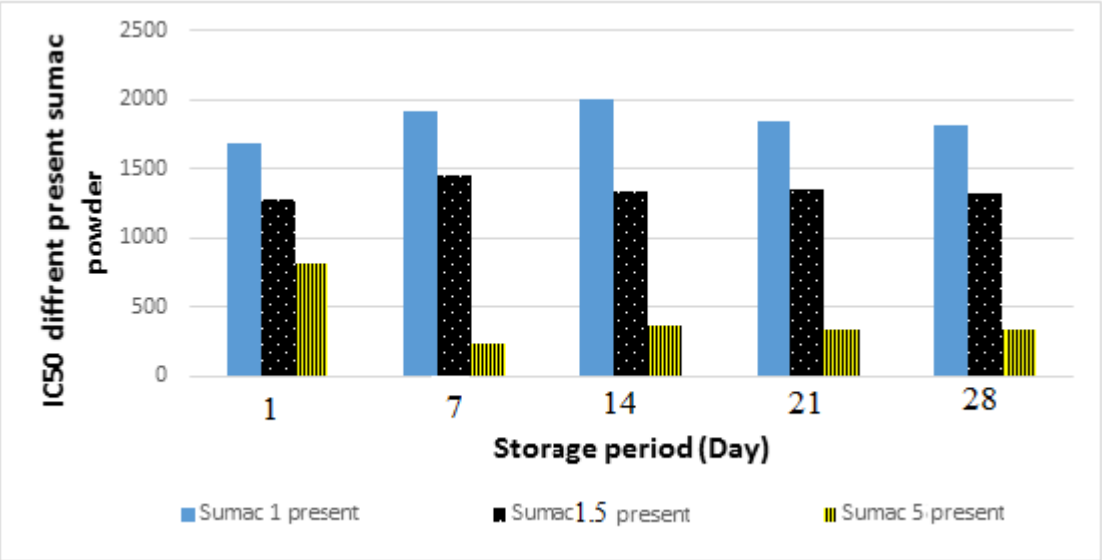

Figure 4. Antioxidant activity of different present sumac powder

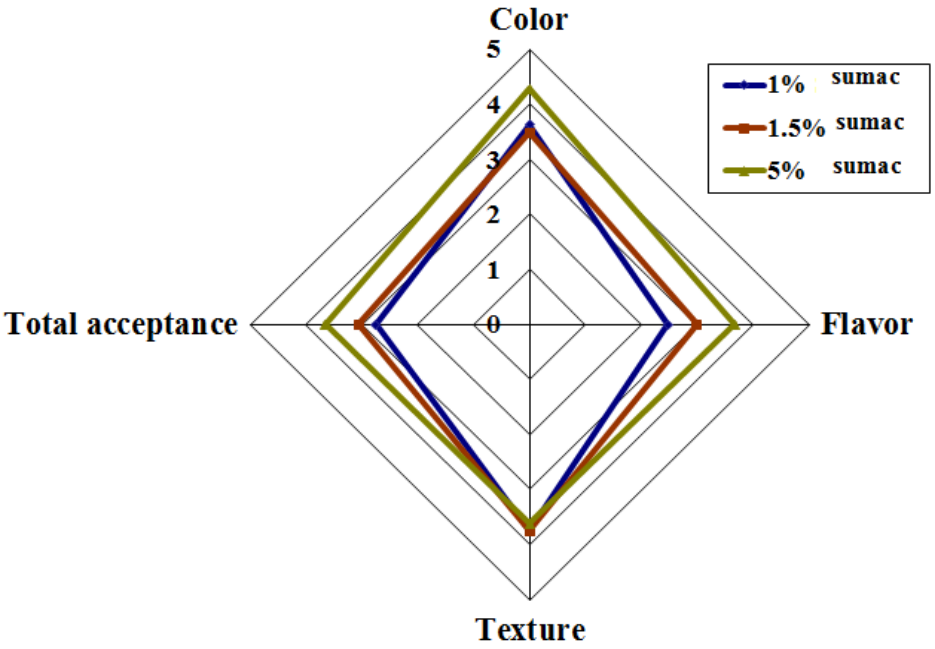

Figure 5. Spider chart representing sensory properties of yoghurt with sumac powder.

Table 1.Variation trend in the viable cell counts of *Penicillin notatum* during 28 days of refrigerated storage period, at 5-day intervals (log CFU/mL)

Day	0%	1%	1.5%	5%	P-Value
1	$4.3\times10^{5}\pm1\times10^{6\ cb}$	$3.4\times10^{5}\pm2\times10^{6\ cb}$	$2.5\times10^{5}\pm2.6\times10^{5\ d}$	$1.6\times10^{4}\pm2\times10^{5\ e}$	<0.001
7	$3.7\times10^{5}\pm2.7\times10^{6\ ab}$	$2.3\times10^{5}\pm2\times10^{6\ d}$	$1.5\times10^{5}\pm2.5\times10^{4\ e}$	$1.2\times10^{4}\pm1.5\times10^{3\ g}$	<0.001
14	$6.3\times10^{5}\pm 1.5\times10^{5\ ab}$	$2.6\times10^{3}\pm1\times10^{4\ f}$	$1.7\times10^{3}\pm1.5\times10^{2\ i}$	$1.2\times10^{2}\pm1.5\times10^{\ k}$	<0.001
21	$7.6\times10^{5}\pm1.5\times10^{5\ a}$	$2.6\times10^{3}\pm4.5\times10^{4\ h}$	$1.4\times10^{3}\pm2.5\times10^{2\ j}$	$2.3\times10\pm2\times10^{\ l}$	<0.001
28	$5.2\times10^{6}\pm4.5\times10^{5\ a}$	$2.7\times10^{4}\pm4.6\times10^{4\ ih}$	$2.4\times10\pm10^{2\ j}$	$1.3\times10\pm2.5\times10^{l}$	<0.001

The means shown with different letters are significantly different ($P < 0.05$).

Table 2.Variation trend antioxidant activity during 28 days of refrigerated storage period, at 5-day intervals

Day	0%	1%	1.5%	5%	P-Value
1	$7.633^{\ cb}\pm0.14$	$7.5309^{\ cb}\pm0.23$	$7.3963^{\ d}\pm0.29$	$6.2018^{\ e}\pm0.28$	0.000
7	$7.724^{\ ab}\pm0.11$	$7.3606^{\ d}\pm0.02$	$6.1817^{\ e}\pm0.25$	$5.889^{\ g}\pm0.01$	0.000
14	$7.8038^{\ ab}\pm0.24$	$5.3891^{\ f}\pm0.03$	$4.2460^{\ i}\pm0.27$	$3.0889^{\ k}\pm0.2$	0.000
21	$7.8491^{\ a}\pm0.53$	$4.5111^{\ h}\pm0.87$	$3.3792^{\ j}\pm0.28$	$2.30644^{\ l}\pm0.22$	0.000
28	$7.8994^{\ a}\pm0.01$	$4.394^{\ ih}\pm0.22$	$3.379^{\ j}\pm0.28$	$2.1305^{\ l}\pm0.20$	0.000

The means shown with different letters are significantly different ($P < 0.05$).

Table3. Sensory properties of yoghurt with sumac powder

Attributes		Addition of sumac powder		
	1%	1.5%	5%	P-Value
color	3.625±0.806	3.475±0.678	4.275±0.784	0.01
Flavor	2.475±1.280	3±1.339	3.675±1.071	0.04
Texture	3.750±1.103	3.750±0.980	3.625±0.952	0.06
Overall acceptability	2.750±0.980	3.050±0.845	3.650±1.001	0.03

Data are means±SD.

4. Conclusions

The results of the present study indicate that addition of different percentage sumac powder in prebiotic low fat yoghurt, not only minimized lipid and protein oxidation but also increased the microbial safety of the product. Sumac powder high levels of bioactive phenolic compounds that can help control foodborne pathogens and prevent lipid and protein oxidation. So, it is suggested that sumac powder, as a natural additive, could be used to increase the shelf life of industrial yoghurt, providing the consumer with food containing natural additives, which might be seen more healthful than those of synthetic source. There was no limitation in this research. In this study functional prebiotic low fat yoghurt containing sumac powder was manufactured and antioxidant and antimicrobial activity were evaluated. However, more studies are needed to evaluate the clinical health effect of sumac containing yoghurt on metabolic and food born disease.

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BIOLOGICALLY ACTIVE COMPLEX FOR MULTIFACTORIAL SUPPORT OF THE CENTRAL NERVOUS SYSTEM: NEW COMPOSITION, EFFICACY

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ABSTRACT

A new tablet form of biologically active food supplement for the multifactorial support of the central nervous system has been developed. It is named Supplement The supplement production technology prevents ingredients oxidative processes due to insignificant moisture content and exclusion of the active oxygen exposure. The biologically active complex facilitates memory retention and information retrieval, accelerates thinking process, and boosts the level of the overall energy potential by improving the central nervous system activity. A balanced combination of active substances (schisandra chinensis, ginkgo biloba) and sedative herbal components (valeriana, motherwort) allows achieving "fine tuning" effect when the above mentioned components act in the best way. These ingredients improve nerve tissue trophism, brain vessels tone, venous outflow and prevent hypertension. Essential and dispensable amino acids in the complex, such as tryptophan, glycine, tyrosine and glutamic acid, make it possible to control neuromediators synthesis and the molecular basis of memory, optimize carbohydrate-fat metabolism, accelerate the process of impulse transmission along the nerve fiber, while avoiding overexcitement. Most of the amino acids in the food supplement have antidepressant effect. Paraaminobenzoic acid reduces mental and physical fatigue and prevents hypoxia in the central nervous system. Another complex ingredient, Inositol, is one of the main nutrient components of the nerve tissue which contributes to its structure restoration. Group B vitamins help replenish energy and ensure its maximum use by the nerve tissue. The paper provides regulated indicators of the nutritional value of a specialized medical product that determine its functional properties. Efficacy was confirmed by clinical trials on a group of patients with angioneurosis. The authors demonstrated hygienic security of the developed product, its shelf life and storage conditions. They also conducted its production testing.

1.Introduction

Brain diseases have always been a topical problem due to their high occurrence and serious health consequences [Avstrievskikh et *al*., 2005; Kondratiev et *al*., 2015]. The initial period of pathologies under consideration are characterized by emotional instability, irritation, increased fatigability [Lebedeva et *al*., 2010; Osipova et *al*., 2012].

Negative influence of different environmental factors, including increased influence of free radicals, lead to even more serious brain [Fenech et *al*., 2011; Lund *al*., 2011; Witte and Byrom, 2014]. Headaches, dizziness, sleep disorders become constant and thinking possesses slow down. Absence of timely preventive measures may result in a stroke and other diseases of nervous and cardio-vascular

systems even at a young age [Chelnakova et *al*., 2015; Mirmiran et *al*., 2014; Stovner et *al*., 2007]. A proper diet, including nutrition supplement intake, plays an important part in solving these problems [Poznyakovsky et *al*., 2017; Bokeria, 2012]. This is the fastest and most effective way of dealing with the problems, proven by numerous research of Russian and foreign scientists [Fenech et *al*., 2011; Fulgoni et *al*., 2011; Gemming et *al*., 2013; Mozaffarian and Ludwig, 2010; Shukla and Manju Lata Mishra, 2018].

2.Materials and methods

The new formula of food supplement "Supplement" includes the following ingredients (mg per tablet): schisandra chinensis extract – 25 mg, valeriana officinalis extract – 10 mg, gingko biloba extract – 20 mg, motherwort extract – 25 mg, 5-hydroxytryptophan – 75 mg, glycine – 75 mg, glutamic acid - 75 mg, para-aminobenzoic acid (tyrosine) – 150 mg, inositol - 75 mg, thiamine mononitrate - 0.85 mg, pyridoxine hydrochloride (vitamin B6) – 1 mg, nicotineamide – 10 mg, calcium panthothenate (vitamin B5) – 2.5 mg, sifrol-5 – 100 mg, tocopherol acetate (vitamin E) -5 mg, B-carotene – 1.75 mg, vitamin C – 12.5 mg, hesperedin – 20 mg, dihydroquercetin -5 mg, superoxide dismutase – 100 mg, coenzyme Q10 – 1.25 mg, hibiscus extract – 11.5 mg.

To determine the functional properties of the food supplement it is advisable to characterize its components and their acting substances. [Gerasimenko et *al*., 2016; Krishnasree et *al*., 2018; Priya Sugandhi,2018; Vekovtsev et *al*., 2017; Lafaille, 2012]. Ginkgo biloba (extract) - the activity is determined by its flavonoids. Ginkgo biloba improves blood circulation, increases oxygen supply to the heart, brain and other organs, has an antioxidant effect, reduces blood pressure, and inhibits thrombotic risks. Valeriana officinalis acts as a sedative for people with central nervous system functional disorders, neuroses, hysteria, agitation caused by psychic trauma, insomnia, migraine; with cardio neuroses, impaired chronic coronary circulation, and heart pains; with high blood pressure, tachycardia caused by neurotic condition. It also helps reduce angioneurosis. Motherwort also acts as a sedative for nervous tension, hypertension, neuralgia, heart weakness, improves sleep and overall health. Its properties are similar to those of valeriana officinalis. 5-hydroxytryptophan, tryptophan amino acid, is a serotonin precursor. Serotonin may be called "neurotransmitter of happiness", satisfaction and tranquility. That is why 5-hydroxytryptophan, which helps serotonin production, is the best natural remedy for depression and anxiety. Its effect is as strong as that of synthetic antidepressants. Glycine has a sedative, mild tranquilizing and weak antidepressant effect, reduces feelings of anxiety, fear, and psycho-emotional stress. It has some nootropic properties, improves memory and associative processes. Inositol is a vitamin-like substance, more precisely, 6-atom alcohol. It possesses membrane-protective, antiatherosclerotic, nootropic, antidepressant and sedative properties, improves sleep and metabolism and restores nerve tissue structure. Inositol improves neural signals transmission. It is effectively used in treating diabetic neuropathy (considering its effect on metabolism in general) and diseases characterized by neural hyposensitivity. It is absolutely necessary for spinal cord cells development and functioning. Glutamic acid makes metabolic processes better, promotes oxidative processes and neutralizes protein metabolism byproducts as it binds to ammonia and forms glutamine, thus promoting neutralization and elimination of ammonia from the body, as well as increases the body resistance to hypoxia. This process is especially important in the brain as its tissue is particularly sensitive to ammonia concentration increase. Glutamic acid promotes acetylcholine and ATP synthesis and potassium ions transfer. Tyrosine is a dispensable amino acid which is formed in the body from the essential amino acid, phenylalanine. Tyrosine improves one's mood. Its deficiency leads to brain norepinephrine deficiency, which, in turn, can result in

depression. PABA (p-aminobenzoic acid) is one of folic acid main components, which promotes pantothenic acid assimilation. Deficiency of PABC can be followed by depression, weakness, irritability, nervousness, and gastrointestinal disorders. PABC promotes the restoration of gray hair, if it is caused by stress.

A balanced combination of active substances (schisandra chinensis, ginkgo biloba) and sedative herbal components (valeriana, motherwort) allows achieving "fine tuning" effect when the above mentioned components act in the best way. These ingredients improve nerve tissue trophism, brain vessels tone, venous outflow and prevent hypertension. Essential and dispensable amino acids in the complex, such as tryptophan, glycine, tyrosine and glutamic acid, make it possible to control neuromediators synthesis and the molecular basis of memory, optimize carbohydrate-fat metabolism, accelerate the process of impulse transmission along the nerve fiber, while avoiding overexcitement. Most of the amino acids in the food supplement have antidepressant effect. Paraaminobenzoic acid reduces mental and physical fatigue and prevents hypoxia in the central nervous system. Another complex ingredient, Inositol, is one of the main nutrient components of the nerve tissue which contributes to its structure restoration. Group B vitamins help replenish energy and ensure its maximum use by the nerve tissue.

The production technology of the suplement tablet form ensures insignificant moisture content and zero active aeration, which prevents destructive oxidative processes of biologically active substances and their active principles.

Comprehensive research has been done to study the supplement properties. The trial results made it possible to determine the regulated (standardized) indicators of quality (table 1), safety (table 2) and nutritional value (table 3), which characterize the supplement functional properties.

Table 1. Organoleptic, physicochemical characteristics and nutritional values of food supplement Supplement.

Characteristic	**Description**
Organoleptic characteristics	
Appearance	Oval tablets with transparent coating, contain pellets
Color	Beige, with brown and green pellets
Physicochemical characteristics	
Disintegration time	no longer than 30 min
Tablet average weight, g	1,2 ± 0,12
Tensile strength, H, not less	90
Friability strength, %, not less	97

Table 2. Safety characteristics.

Characteristc	**Sample content, mg per kg, no more than**
Toxic elements:	
Lead	5,0
Cadmium	1,0
Arsenic	3,0
Mercury	1,0
Pesticides:	
Sum of isomers HCCH	0,1
Heptachlor	Prohibited
DDT and sum of its metabolites	0,1

Aldrin	Prohibited
Microbiological characteristics:	
Mesophilic aerobic and facultative anaerobic microorganisms, CFU per gram(cm^3) of product	$5•10^4$
Coliform bacteria	0,1
Pathogenic microorganisms, including Salmonella	10,0
E. coli	1,0
Yeasts and molds, CFU per gram	100
Radionuclides	
Cesium-137, Bq/kg	200
Strontium-90, Bq/kg	100

Table 3. Nutritional value of food supplement "Supplement".

Nutritional value, mg per tablet	mg	% of dietary reference intake
Coenzyme Q10	1.25± 0,125	8
Hesperidin	20 ± 2,0	20
Vitamin C (Ascorbic Acid)	12,5± 0,125	17,8
Vitamin B1 (Thiamine)	0,85± 0,125	50
Vitamin B6 (Pyridoxine)	1,0± 0,1	50
Nicotinamid	10± 1,0	50
Calcium Pantothenate	2,5±0,25	50
Vitamin E (Tocopherol)	5±0,5	50
B-Carotene	1,75±0,175	35
Dihydroquercetin	5±0,5	20
Glycine	75±7,5	-
Tyrosine	50±15	-
Glutamic Acid	65	-
Schizandrin	0,4	-
Ginkgo Flavone Glycosides	3,0	-

Sanitary-hygienic and sanitary-toxicological tests results showed all characteristics compliance with technical regulations requirements [Manufacturing formula TC 027, 2012] and allowed to establish terms and conditions of storage - 3 years in a dry, light-proof place, at a temperature of no higher than 25 ° C.

"Supplement" efficacy and functional properties were proved by clinical trials. Patients with angioneurosis and cerebrovascular diseases were selected for this trial.

Altogether 105 patients with angioneurosis were observed and tested. They were divided into 3 groups. The first (main) group included 35 patients who took food supplement "Supplement" one tablet a day with a meal. They also took Trental (50mg) once a day. The period of treatment was 4 weeks. The second (main) group included 35 patients who took "Supplement" once a day with a meal in combination with Trental and food supplement "Gutta Viva" (1 gram a day). Gutta Viva should be dissolved in a glass of water and taken 1 hour before a meal. The period of treatment was 4 weeks. The third (control) group included 35 patients who took only Trental once a day during 4 weeks. Participants were people aged 38-50. The average age was 42.2±5.3. Patients were randomly grouped in terms of their gender, age, disease duration and severity. All of them were diagnosed with angioneurosis of moderate to severe degree. All patients complained about general health deterioration, headaches,

dizziness, worse memory retention and information retrieval, poor attention and concentration. They also showed emotional-volitional problems. All patients were clinically checked. Their blood and urine were tested, biochemistry blood test analyzed, ECG and EEG studied. All participants were examined by a neurologist who diagnosed most of them with muscle hypotension; half of them with hypertension and all of them with emotional-volitional sphere disorders. All patients self-assessed their emotional-volitional sphere using Beck Depression Inventory, Tsung depression scale and Sheehan anxiety scale. 30 patients of the first (main) group, 29 patients of the second (main) and 30 patients of the third (control) group were diagnosed with anxiety-depressive disorders. Thus about 30 participants of each group showed mixed anxiety and depression disorders. Assessment by Beck Depression Inventory revealed that 29 patients in each group had depression. Patients felt sad, were not satisfied with their life, disappointed in themselves and needed additional conditions to get down to work. The average level of depression by Tsung scale was 62 - 63 points (average depression level).

All participants showed a high depression level (not more than 80 points) by Sheehan anxiety scale. Anxiety level of more than 30 points is considered abnormal. The aim of the treatment was to achieve less than 20 points score.

Antioxidant effect of food supplement "Supplement" in combination with food supplement "Gutta Viva" was assessed.

Free radical oxidation process is considered a universal mechanism of cell damage. Most patients (95%) showed a high level of lipid peroxidation antioxidant – malondialdehyde.

"Supplement" intake in combination with food supplement "Guta Viva" is supposed to result in angioneurosis symptoms lessening: improved memory, attention and concentration, reduced emotional-volitional disorders. To achieve this aim it is essential to improve brain nutrition by the supplement ingredients qualitative and quantitative composition.

Research was done by the Therapy Department of the Advanced Studies School of the Siberian State Medical University headed by E.I. Beloborodova, Doctor of Medical Sciences, Professor, Honored Medical Doctor of the Russian Federation.

3.Results and discussions

The treatment of patients suffering from angioneurosis resulted in objectively and subjectively positive dynamics of their condition. "Supplement" tolerance was satisfactory; no side effects on internal organs, nervous system and skin were observed (Table 4). Positive effect was also evident when food supplement Supplement was combined with another supplement "Gutta Viva".

Table 4. Dynamics of angioneurosis clinical manifestations in the main and control groups before and after treatment.

Clinical manifestations	First main group (35 patients)	Second main group (35 patients)	Third control group (35 patients)
Better general condition and well-being	30 (85%)	32 (90%)	20 (40%)
Headache abatement	28 (80 %)	35 (100 %)	25 (50 %)
Better memory retention and information retrieval	30 (85 %)	35 (100 %)	20 (40%)
Better attention and concentration	28 (80 %)	30 (85 %)	25 (50 %)
Reduced emotional-volitional disorders	25 (70 %)	30 (85 %)	30 (60 %)
Better muscular tone	25 (70 %)	32(90 %)	20 (40%)
Lower blood pressure	12 (35 %)	17 (50 %)	10 (20%)
Positive EEG dynamics	25 (70 %)	30 (85 %)	10 (20%)

As can be seen in Table 4, patients with angioneurosis of the first two main groups showed positive dynamics after treatment, especially in the 2nd main group (who took both "Supplement" and "Gutta Viva" supplements). The overwhelming majority (90%) noted a better general health condition. All participants (100%) pointed to abated headaches, 100% demonstrated better memory retention and information retrieval, 85% spoke of better attention, concentration and reduced emotional-volition disorders.

Twenty-five patients (70%) in the 1st main group and the majority of patients (85%) in the 2nd main group, showed positive dynamics on EEG. As for the patients from the control group, we can also observe some clinical improvement, but significantly lower than in the main groups. Only 25% had lower blood pressure, and only 20% demonstrated positive EEG dynamics, while the main groups' results were 3 times as good.

After 4-week of the supplement Supplement intake in combination with Trental (in the first main group), "Supplement" + Trental + "Gutta Viva"(in the second main group) and only Trental (in the control group) we can observe the decline in the level of malondialdehyde. However, only 17 patients (50%) in the first group demonstrated a significant reduction by 1.4 times ($p < 0.05$), while the majority of the patients in the second main group (90%) showed a more significant reduction of 1.9 times ($p < 0.01$). Only 20% of patients in the third control group showed the level decline, so we cannot speak of malondialdehyde level normalization (table 5, fig.1).

Table 5. Malondialdehyde level dynamics in patients with angioneurosis before and after 4 weeks' treatment with food supplement "Supplement".

Characteristic	Main group (1st group)		Main group (2nd group.)		Control group (3rd group)	
	before	after	before	after	before	after
MDA, mmol /l	6.5±0.55	4.7±0.18 **	7.6±0.42*	4.2±0.52	7.01±0.27	6.2±0.24

Note:* ($p<0.05$) ** ($p<0.01$)

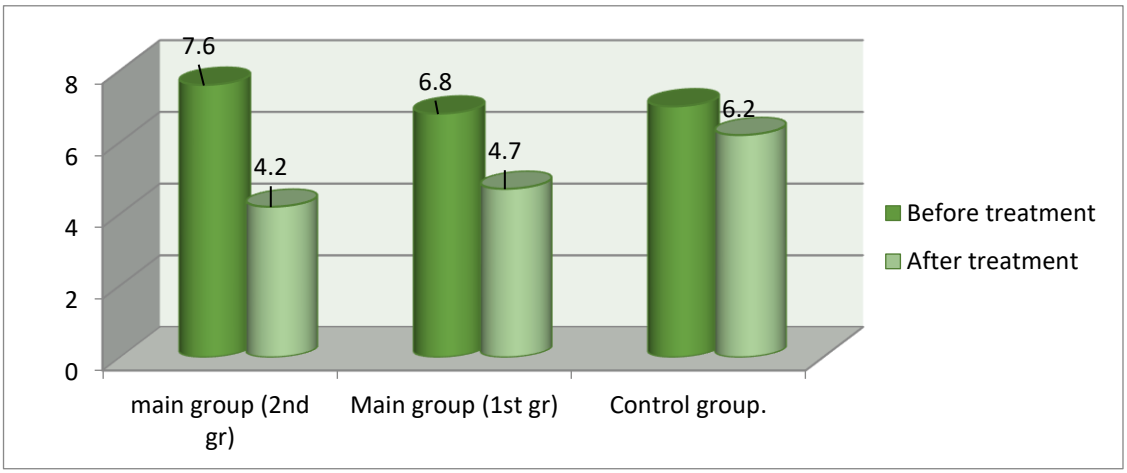

Figure 1. MDA level dynamics in patients with angioneurosis after 4 weeks' treatment.

Figure 1 shows dynamics in the 3 groups.The main substrate of LPO in liver microsomes is arachiodonic acid. Its microsome phospholipid decrease is followed by

peroxidation decline. By analyzing the obtained data, we can suppose that food supplement "Supplement" has an inhibiting effect on arachiodonic acid synthesis.

This effect becomes even more prominent if we combine "Supplement" with "Gutta Viva". It probably explains LPO inhibitory process and antioxidant protection activation in the main groups compared with the control group.

Statistical analysis shows significant decline ($p<0.05$) of depression and anxiety level (table 6).

Table 6. Dynamics of psychosomatic disorders symptoms in the groups after 4-week treatment with food supplement "Supplement" in combination with food supplement "Gutta Viva".

Treatment period	Groups	Beck scale (points)	Tsung scale (points)	Sheehan scale (points)
Before treatment	Main group (1st gr.)	28.0(27.0-29.0)	63.0 (62.0-64.0)	37.0 (34.5-39.0)
	Main group (2nd gr.)	26.0 (25.0-27.0)	63.0 (62.0-64.0)	36.0(35.0-37.0)
	Control group (3rd gr.)	28.0 (27.0-29.0)	62.0 (61.0-63.0)	37.0 (34.0-39.0)
After treatment	Main group (1st gr.)	20.0 (20.0-21.0)	45.0 (44.0-46.0)	19.0 (18.0-19.)
	Main group (2nd gr.)	16.0 (16.0-17.0)	40.0(39.0-41.0)	16.0 (15.0-16.0)
	Control group (3rd gr.)	28.0 (27.0-29.0)	60.0 (59.0-61.0)	27.0 (26.0-28.0)

All the patients of the first two groups demonstrated psychic health improvement: anxiety average level by Beck Depression Inventory, Tsung and Sheehan scales showed significant decrease ($p<0.05$). The third control group did not show any significant decrease (table 6).

Thus we can make a conclusion that food supplement "Supplement" intake improves patients' health conditions. We can speak of headache abatement, better memory retention and information retrieval, better attention and concentration, fewer emotional-volitional sphere disorders. Check-ups also showed lower blood pressure and positive EEG dynamics. Results in the first main group (only "Supplement" intake) were a bit worse than in the second group: 85% of participants of the first group observed improvement of their general health condition, 80% noted headache abatement and increased initiative, 85% showed better memory retention, 70% demonstrated fewer emotional-volitional sphere disorders and better muscle tone.

We can state that biologically active food supplement "Supplement" significantly improves cerebral blood flow, reduces blood viscosity, and protects cell membranes from damage.

Treatment with "Supplement" can improve brain metabolism which leads to better memory, concentration, increased initiative, weaker emotional-volitional disorders manifestation in patients suffering from angioneurosis.

Combination of food supplements "Supplement" and "Gutta Viva" (2nd main group) significantly influences disease manifestation. It improves antioxidant protection and stabilizes psycho- emotional state of patients with angioneurosis. The second main group also showed better results concerning clinical manifestations of angioneurosis: 100% observed abated headache, the majority noted better health condition, memory retention, and information retrieval, 85% demonstrated positive EEG dynamics.

MDA level dynamics (LPO index) in the main groups was characterized by a significant inhibition of lipid peroxidation process, which proves reduced cell damage.

Psycho-emotional sphere study noted psychological condition improvement in all patients of the two main groups ($p<0,05$). The best results were demonstrated by the second main group. The average level of anxiety by Sheehan scale was about 16 points.

4.Conclusions

The obtained results allow for the following conclusions:

- ✓ food supplement "Supplement" in combination with food supplement "Gutta Viva" facilitates cell membranes stabilization by reducing LPO processes, has significant antioxidation effect, improves patients' psycho-emotional condition and can be considered an effective treatment of patients with angioneurosis of moderate severity.
- ✓ food supplement "Supplement" in combination with vascular drug Trental contributes to early relief of the main angioneurosis symptoms. If we combine it with food supplement "Gutta Viva" it significantly improves the general health condition, attention, concentration, initiative, normalizes the psycho- emotional sphere, reduces lipid peroxidation, has a pronounced antioxidant effect.
- ✓ course treatment with in combination with Trental provides more pronounced therapeutic effect on disease progression, decreases LPO, improves psycho-emotional sphere.
- ✓ course treatment with in combination with Trental improves brain metabolic processes, attention, memory, normalizes blood pressure and muscle tone.
- ✓ the tested food supplement is characterized by good tolerability.
- ✓ "supplement" can be recommended as an additional antioxidant source.
- ✓ the developed product has the following competitive advantages:
- ✓ cifrol-5 provides 24 hours' persistent antioxiadant protection.
- ✓ scientifically valid food supplement formulation provides prolonged targeted effect;
- ✓ pelletized form of the ingredients makes it possible to release tablet active substances in the certain order;
- ✓ single intake of the complex (one tablet a day) makes its use convenient and affordable;
- ✓ physiological dosage of active substances prevents addiction and other side-effects.

Product "Supplement" was tested in production conditions at scientific-production association "Art-Life" enterprises in the city of Tomsk, certified in accordance with the requirements of the international standards series of the ISO 9000, 22000 and the GMP rules, which ensures quality consistency and competitiveness.

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EFFECTS OF SOAKING AND GERMINATION TIME ON THE ENGINEERING PROPERTIES OF FINGER MILLET (*ELEUSINE CORACANA*)

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ABSTRACT

The effect of germination time on malting loss and engineering properties namely: 1000 kernel weight, bulk density, true density, porosity, length, width, thickness, geometric mean diameter, arithmetic mean diameter, sphericity, surface area, sample volume and angle of repose were studied. Increase in germination time increased malting loss up to 35.27% after 96 hours of germination. Reduction in thousand kernel weight, bulk density and true density was 35.8%, 25.4% and 20.18%, respectively after 96 hours of germination. Porosity of grains was decreased while length increased with increase in germination time up to 72 hours of germination. Width and thickness showed an increase up to 36 hours and 24 hour of germination, respectively, followed by a linear decrease. A similar trend was observed in geometric and arithmetic mean diameter, sphericity, surface area and sample volume of grains. Angle of repose increased from 24.93° to 32.81° after 72 hours of germination followed by a linear decrease.

1.Introduction

Agriculture is facing various problems like deepening of water bed level and expansion in drylands (CGIAR, 2017). The changing climate conditions along with nutrient deficient soils are not favourable for cultivation of wheat and rice and worldwide agriculturists are searching for a suitable alternative to these cereals. In view of these constraints, millets have attracted the attention of agriculturists and food technologists, as these are rich in nutrients, can grow on soils with low fertility, require less irrigation and are resistant to insects and pests (Singh and Srivastava, 2006; Devi *et al.*, 2014). Owing to this, the year 2018 was declared as the International year of millets. In this connection, finger millet, one of the highly produced millet in India with excellent nutrient quality, projects a tremendous scope in designing foods for developed as well as developing world. It possesses good drought tolerance, has ability to tolerate salinity and can be grown in soils with pH range of 5.0 to 8.2 (Upadhyaya, 2011). Finger millet is most nutritious among the major cereal grains. It is rich in high quality protein (Admassu *et al.*, 2009) which contains all the essential amino acids (Mbithi-Mwikya *et al.*, 2000). The content of free sugars has been reported in the range of 0.47 to 1.5% (Nirmala *et al.*, 2000; Kumar, 2013) which along with high dietary fibre (12%) (Saleh *et al.*, 2013) make it a low glycemic food. It is also gluten free (Admassu *et al.*, 2009; Singh and Raghuvanshi, 2012; Devi *et al.*, 2014).The cholesterol lowering and cancer reducing studies of finger millet has also been reported (Burton and Froston, 1966;

Burton *et al.,* 1972). Owing to its health benefits worldwide researches are being conducted to develop value added health products from finger millet (Verma and Patel, 2013; Shukla and Srivastva, 2014). However, the high amount of phytates, phenols, tannins and enzymes in finger millet inhibits maximum absorption of its nutrients which decreases its food value. Many processing methods like soaking, germination, roasting, and fermentation have been recommended by various researchers to reduce the anti-nutrients of finger millet (Tatala *et al.,* 2007; Venkateswaran and Vijyalakshmi, 2010). Germination is believed to be the most promising methods to reduce anti-nutrients and change the functionality and digestibility of grains (Pawar *et al.,* 2007; Onyango *et al.,* 2013). The germinated grains can be dried to a safer moisture limit and can be stored for further processing.

Effects of soaking and germination on the chemical composition of finger millet has been studied by many researchers (Nirmala *et al.,* 2000; Tatala *et al.,* 2007; Venkateswaran and Vijyalakshmi, 2010) but the effect of germination time on the engineering properties of finger millet is still unexplored. Gravimetric properties like bulk density, true density and porosity is important to determine the drying rate and aeration properties as these properties affect the rate of air flow (Sobukola *et al.,* 2013). Knowledge of the dimensional properties like length, width, thickness, geometric mean diameter and arithmetic mean diameter are important in separation of undesirable materials like unwanted roots and shoots from malted grains. Surface and frictional properties like sphericity and angle of repose plays an important role in designing material handling equipments like hoppers, conveyor belts and storage structures like silos (Balasubramanian and Vishwanathan, 2010). Therefore, the present study was designed to study the effect of germination time on engineering properties of finger millet This information can help food researchers and processors to exploit the usage of malted finger millet grains in development of value added products.

2.Materials and methods

2.1. Procurement of raw material

Finger millet (*Eleusine corcana*) grains of variety VL Manduaa-315 were procured from Vivekananda Parvatiya Krishi Anusandhanshala Almora U.P. The grains were cleaned by winnowing to separate residual particles (husks, chaff and un-matured seeds). Cleaned grains were then collected, dried in a tray drier (Narang Scientific Works Limited) to the final moisture content of 8±0.5 %, and stored for further use. The moisture content of the samples was determined by oven drying method at 130 °C for 2 hours (AOAC, 2010).

2.2. Malting

Malting is a three step process consisting of soaking, germination and drying. In the present study clean grains were soaked overnight in potable water, excess water was removed, and were spread in thin layer in a seed germinator maintained at 25±2 ºC and 95% relative humidity. Germination was carried for a time interval ranging from 12 hours to 96 hours. After every 12 hours interval samples were removed, dried in a hot air oven (50±2°C) to moisture content of 8±0.5 %, de-vegetated and were stored for further analysis.

2.2.1 Malting loss

Malting loss is defined as the weight of grain lost during the malting process (Nirmala *et al.,* 2000). Malting loss was calculated by using the following formula

Total malting loss (%) = Weight of grains before malting - Weight of grains after malting × 100/ Weight of grains before malting (Eq.1)

2.3. Gravimetric properties

2.3.1. Thousand kernels weight

Thousand kernels weight was measured by selecting 1000 grains randomly from pre-cleaned grains. The selected kernels were weighed on a digital electronic balance. The test was performed five times and the mean value was calculated (Mariotti *et al.,* 2006).

2.3.2. Bulk density

Bulk density was measured using calibrated measuring cylinder of 1000 ml capacity. The cylinder was filled to appropriate height with the clean grains (Mariotti *et al.,* 2006). Bulk density was calculated by taking ratio of the sample weight and volume of the cylinder and was represented as Kg/m^3. Average of 5 replications was taken.

Bulk density = Sample weight/volume

2.3.3.True density

The true density was determined by the liquid displacement method. 10 grams of cleaned grains were immersed in a 50 ml measuring cylinder containing 20 ml of toulene. The amount of oil displacement was recorded and true density was calculated using the formula:

True density = Weight of grains/volume of displaced toluene

2.3.4.Porosity

Porosity is the measure of fraction in the bulk grain which is not occupied by the grain (Ramashia *et al.,* 2017). It is calculated by putting the values of true density and bulk density in the under given formula.

Porosity (ε) = [(Pt-Pb)/Pt]*100 (Eq.2)

Where, Pt = True density, Pb- Bulk density

2.4. Dimensional properties

The principal dimensions of length, width and thickness were measured with the help of a projector with a 10X scale by selecting 10 random grains for each treatment, drawing images on a white paper, measuring dimensions and finally reducing them to original size by dividing with 10. Further geometric mean diameter, arithmetic mean diameter, sphericity, surface area, and sample volume were calculated by using formulas given by various researchers (Sharma *et al.,* 1985; Sreenarayanan *et al.,* 1985; Jain and Bal, 1997; Ledbetter and Sisterson, 2010; Onyango *et al.,* 2013; Ramashia *et al.,* 2017).

Geometric mean diameter = $(LWT)^{1/3}$ (Eq.3)

Arithmetic mean diameter = $(L+W+T)^{1/3}$ (Eq.4)

Sphericity = $[(LWT)^{1/3/L}]100$ (Eq.5)

Surface area = $(\pi BL^2)/(2L-B)$ (Eq.6)

Sample volume = $(\pi B^2L^2)/[6(2L-B)]$ (Eq.7)

Where, L= Length, W= Width, T= Thickness, $B = (WT)^{0.5}$ (Eq.8)

2.5. Angle of repose

The angle of repose was determined on a plywood surface with the help of a cylinder opened at both the ends i.e. top and bottom end. The cylinder was kept on the plywood surface and was filled up to top with cleaned finger millet grains. The cylinder was then lifted up gradually from the surface until a conical heap was formed. Angle of repose was calculated from the height and base radius of the heap formed (Owolarafe *et al.,* 2007).

Angle of repose (θ) = $\tan^{-1}$ 2h/d (Eq.9)

3.Results and discussion

3.1. Malting loss and gravimetric properties

Malting loss has been defined as the loss of grain mass during germination. The effect of germination time on the malting loss and gravimetric properties has been shown in Table 1.

With increase in germination time malting loss increased gradually from 0 to 35.27 % after 96 hours of germination which might be due to the hydrolysis of stored complex carbohydrates into simple sugars (Handa *et al.,* 2017) and the utilization of these sugars in the embryonic growth (Vidal-Valverde *et al.,* 2002). Similar results have been reported by other researchers. Kumar (2013) reported a malting loss of 30 % in local finger millet variety of Himachal Pradesh while Nirmala *et al.,* (2000) reported a malting loss of 32 % in Indaf-15 variety. Malting loss had a direct significant ($P \leq 0.05$) relation with gravimetric properties (fig. 1) and an increase in malting loss resulted in decreased values for thousand kernel weight, bulk density and true density and same has been observed in correlation analysis of data (Table 4). Density is the measure of the mass per unit volume and is important to design the storage structures and handling equipments. Density is dependent on surface properties like surface area and sample volume and an increase in these parameters resulted in decrease in density. Porosity was reduced significantly ($P \leq 0.05$) in the initial 72 hours of

germination which might have been due to the migration of nutrients to surface and filling of pores during soaking. The reason for increase in porosity after 72 hours of germination might be due to the shrinking of grains and formation of cracks on the skin. A decrease in porosity has been also reported by Kumar and Prasad, on parboiling of rice (Kumar and Prasad, 2013). Porosity and density also affect the rate of moisture removal during drying (Bai *et al.*, 2012). The grains with low porosity have a greater resistance to the removal of moisture and hence require high power aeration fans.

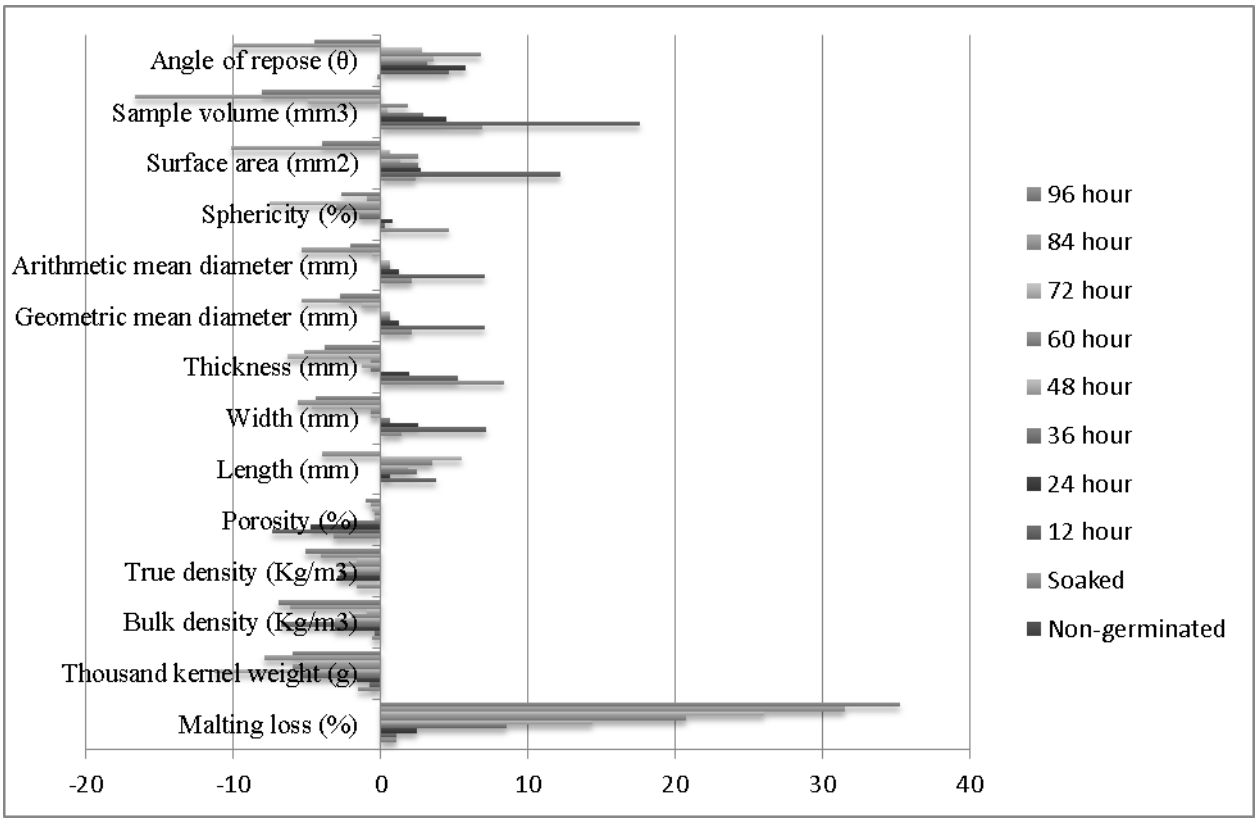

Figure 1. Effect of germination time on the malting loss and engineering properties in comparison to non-germinated finger millet grains (values for non-germinated parameters are taken as zero

Table 1. Effect of germination on malting loss and density related properties

Germination Time	Malting Loss (%)	Thousand Kernel Weight (g)	Bulk Density (Kg/m^3)	True Density (Kg/m^3)	Porosity (%)
Non-germinated	0.00^{i}	2.60±0.02^{a}	741.04±1.66^{a}	1250.00±1.1.5^{a}	36.61±0.26^{a}
Soaked	1.03±0.21^{h}	2.56±0.02^{b}	736.66±0.56^{b}	1230.33±1.76^{b}	35.47±0.18^{b}
12 hour	1.10±0.10^{h}	2.54±0.03^{b}	733.41±0.54^{c}	1229.00±0.58^{b}	33.03±0.11^{c}
24 hour	2.49±0.09^{g}	2.50±0.03^{c}	711.20±1.08^{d}	1194.33±2.60^{c}	31.54±0.37^{d}
36 hour	8.52±0.45^{f}	2.37±0.02^{d}	666.10±0.66^{e}	1162.03±2.30^{d}	30.86±0.59^{d}
48 hour	14.37±0.51^{e}	2.13±0.03^{e}	645.75±1.55^{f}	1142.73±1.76^{e}	30.74±0.28^{e}
60 hour	20.71±0.65^{d}	2.01±0.03^{f}	633.3±1.00^{g}	1109.33±2.33^{f}	30.70±0.19^{e}

72 hour	26.03±0.40^{c}	1.91±0.02^{g}	627.65±0.94^{h}	1091.73±0.88^{g}	29.93±0.04^{f}
84 hour	31.53±0.15^{b}	1.77±0.01^{h}	591.23±1.95^{i}	1049.03±1.00^{h}	30.92±0.07^{e}
96 hour	35.27±0.40^{a}	1.67±0.02^{i}	552.72±1.66^{i}	997.67±2.60^{i}	31.49±0.4^{d}

*Values are means ± SD of 5 replications. Different superscripts in a column indicate that they are significantly (P≤0.05) different to each other determined by Duncan's tests

Table 2. Effect of germination time on the dimensional properties of finger millet grains

Germination Time	Length (mm)	Width (mm)	Thickness (mm)	Geometric Mean Diameter (mm)	Arithmetic Mean Diameter (mm)
Non-germinated	1.52±0.03^{d}	1.40±0.06^{bc}	1.32±0.04^{cd}	1.41±0.04^{c}	1.41±0.04^{d}
Soaked	1.52±0.07^{d}	1.42±0.09^{bc}	1.44±0.13^{abc}	1.44±0.11^{bc}	1.44±0.11^{cd}
12 hour	1.58±0.09^{cd}	1.53±0.07^{a}	1.52±0.07^{ab}	1.55±0.07^{ab}	1.55±0.07^{abc}
24 hour	1.59±0.05^{cd}	1.57±0.04^{a}	1.55±0.04^{a}	1.57±0.04^{a}	1.57±0.04^{ab}
36 hour	1.63±0.03^{c}	1.58±0.05^{a}	1.54±0.05^{ab}	1.58±0.04^{a}	1.58±0.04^{a}
48 hour	1.66±0.07^{bc}	1.57±0.05^{a}	1.52±0.05^{ab}	1.59±0.06^{a}	1.59±0.06^{a}
60 hour	1.72±0.03^{b}	1.56±0.10^{a}	1.51±0.12^{ab}	1.59±0.08^{a}	1.59±0.08^{a}
72 hour	1.82±0.01^{a}	1.49±0.01^{ab}	1.42±0.01^{bc}	1.57±0.01^{a}	1.58±0.01^{a}
84 hour	1.75±0.01^{ab}	1.41±0.01^{bc}	1.35±0.02^{cd}	1.49±0.01^{abc}	1.50±0.01^{abcd}
96 hour	1.75±0.01^{ab}	1.35±0.01^{c}	1.30±0.01^{d}	1.45±0.01^{bc}	1.47±0.01^{bcd}

*Values are means ± SD of 5 replications. Different superscripts in a column indicate that they are significantly (P≤0.05) different to each other determined by Duncan's tests.

3.2. Dimensional properties

Grain dimensions like length, width, thickness, geometric mean diameter, arithmetic mean diameter are important in designing the sieves for separation of grains from undesirable materials. For the utilization of malted grains to develop value added products, it is important to remove vegetative growth and separate the sound grains from waste material. Maximum value for length (1.82) was obtained after 72 hours of germination while the value for width and thickness was highest at 36 hours and 24 hours of germination, respectively. The increase in dimensional properties like length, width and thickness of finger millet grains might have been due to the swelling of starch granules during soaking as water migrates to grains during soaking and leads to irreversible swelling (Mir and Bosco, 2013). One of the more reasons behind increase in length might be the adherence of dried epicotyl and hypocotyl to grain after drying as their complete removal is not possible. A prolonged germination resulted in decrease in dimensional properties which might have been due to the formation of pits and holes on the surface of germinated starch granules and formation of small size particles due to the breakdown of larger particles (Li *et al.*, 2017). A similar trend was observed in the geometric mean diameter and arithmetic mean diameter (Table 2).The diameter is dependent on length, width and thickness of grains and same has been observed in the correlation studies (Table 4). Width and thickness had a positive significant (P≤0.05) effect on geometric mean diameter

and arithmetic mean diameter (fig. 1) and an increase in width and thickness increased the diameter. The studies on the effect of germination time on the dimensional properties of grains are scarce. However; increase in moisture has been reported to increase the dimensional properties of grains (Bai *et al.*, 2012; Gely and Pagano, 2017).

3.3. Surface and frictional properties

Surface properties play an important role in drying whether sphericity and frictional properties like angle of repose affects the rate of sliding of grains on a surface. Frictional properties are important in designing hoppers and storage structures like silos. The frictional properties of grains determine the rate of flow of grains, feed rate and also help to determine the rate of emptying of storage structures (Balasubramanian and Viswanathan, 2010). Surface and frictional properties of non-germinated finger millet and malted grains has been presented in Table 3.

Table 3. Effect of germination on surface and frictional properties of finger millet grains

Germination Time	Sphericity (%)	Surface area (mm^2)	Sample volume (mm^3)	Angle of Repose (θ)
Non-germinated	93.1 ± 0.97^{cd}	6.49 ± 0.32^{d}	1.48 ± 0.12^{c}	24.99 ± 1.07^{g}
Soaked	97.6 ± 0.76^{ab}	6.65 ± 0.99^{cd}	1.59 ± 0.36^{bc}	24.93 ± 0.52^{g}
12 hour	97.9 ± 1.05^{a}	7.57 ± 0.76^{abc}	1.93 ± 0.28^{ab}	26.14 ± 0.21^{f}
24 hour	98.7 ± 0.58^{a}	7.78 ± 0.44^{ab}	2.02 ± 0.17^{a}	27.73 ± 0.53^{e}
36 hour	97.3 ± 1.31^{ab}	7.98 ± 0.37^{ab}	2.08 ± 0.17^{a}	28.64 ± 0.25^{d}
48 hour	95.4 ± 1.12^{bc}	8.09 ± 0.60^{ab}	2.09 ± 0.22^{a}	29.71 ± 0.48^{c}
60 hour	92.6 ± 3.5^{d}	8.30 ± 0.71^{ab}	2.13 ± 0.32^{a}	31.89 ± 0.38^{b}
72 hour	86.1 ± 0.28^{e}	8.35 ± 0.01^{a}	2.03 ± 0.00^{a}	32.81 ± 0.48^{a}
84 hour	85.3 ± 0.47^{ef}	7.58 ± 0.04^{abc}	1.74 ± 0.02^{abc}	29.81 ± 0.33^{c}
96 hour	83.1 ± 0.22^{f}	7.29 ± 0.06^{bcd}	1.61 ± 0.02^{bc}	28.52 ± 0.29^{de}

*Values are means ± SD of 5 replications. Different superscripts in a column indicate that they are significantly ($P < 0.05$) different to each other determined by Duncan's tests

Sphericity, surface area and surface volume of the non-germinated finger millet grains was 93.1, 6.49 mm^2 and 1.48 mm^3, respectively. Bai et al. (2012) reported sphericity ranging from 0.94 to 0.99% for non-germinated finger millet grains. Ramashia et al. (2017) reported sphericity in the range of 73.75 to 92.43% for the black, creamy and brown cultivars of finger millet. With increase in germination time from 0 to 24 hours, sphericity was increased from initial 93.1% to 98.7%. The increase in sphericity up to 24 hours might be due to the swelling of starch granules on soaking (Mir and Bosco, 2013). Germination beyond 24 hours resulted in linear decrease in sphericity i.e. from 98.7% to 83.1%. The reason for decrease in sphericity after 24 hours of germination might be due to more increase in length as compared to width and thickness. An increase in surface area (6.49 to 8.30 mm^2) and sample volume (1.48 to 2.13 mm^3) was observed which might have been due to the increase in dimensional parameters like length, width and thickness (Table 2) and same has been observed in correlation studies (Table 4).

Table 4. Correlation of the effect of germination time on various engineering parameters

Parameters	Malting Loss (%)	Thousand kernel Weight (g)	Bulk Density (Kg/m^3)	True Density (Kg/m^3)	Porosity (%)	Length (mm)	Width (mm)	Thickness (mm)	Geometric Mean Diameter (mm)	Arithmetic Mean Diameter (mm)	Sphericity (%)	Surface Area (mm^2)	Sample Volume (mm^3)	Angle of Repose (θ)
Malting loss (%)	1	-.997**	-.976**	-.984**	-.647*	.922**	-.349	-.472	.043	.131	-.907**	.398	.047	.714*
Thousand kernel weight (g)		1	.980**	.983**	.676*	-.926**	.300	.426	-.092	-.179	.882**	-.438	-.096	-.738*
Bulk density (Kg/m^3)			1	.992**	.707*	-.887**	.247	.373	-.122	-.208	.827**	-.448	-.127	-.704*
True density (Kg/m^3)				1	.692*	-.895**	.294	.402	-.086	-.176	.852**	-.421	-.090	-.689*
Porosity (%)					1	-.815**	-.457	-.328	-.764*	-.820**	.337	-.923**	-.760*	-.879**
Length (mm)						1	-.060	-.220	.345	.427	-.793**	.669*	.347	.900**
Width (mm)							1	.951**	.910**	.868**	.636*	.701*	.912**	.314
Thickness (mm)								1	.819**	.769**	.767**	.557	.818**	.128
Geometric mean diameter (mm)									1	.995**	.286	.927**	.998**	.639*
Arithmetic Mean Diameter (mm)										1	.200	.955**	.991**	.690*
Sphericity (%)											1	-.089	.285	-.500
Surface area(mm^2)												1	.929**	.870**
Sample volume (mm^3)													1	.653*
Angle of repose (θ)														1

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed)

An increase in surface area and sample volume has been also reported in maize, wheat and paddy on increase of moisture content (Adebowale *et al.*, 2012; Bai *et al.*, 2012; Gelly and Pagano, 2017). Soaking had a non-significant effect on angle of repose. However, a significant ($P \leq 0.05$) increase in angle of repose was found with increase in germination time. The reason for increase in angle of repose might be the adherence of dried epicotyl and hypocotyl to grain after drying which increased the overall friction and hence resulted in a high heap.

3.4. Cluster analysis

A dendrogram for different treatments (i.e. soaking time and germination time) on the engineering properties of finger millet grains is shown in Figure 2. Cluster analysis grouped the data in 2 major groups. The first group consists of soaked, 12 hour germinated grains, non-germinated grains and 24 hour germinated grains.

The second group consists of grains germinated for 36-96 hours. Group 2 was further subdivided into 2 subgroups and showed that the grains germinated for 60, 72, 36 and 48 hours were in same group and had the most similar engineering properties. While, the engineering properties of grains germinated for 84 hours and 96 hours were different from others.

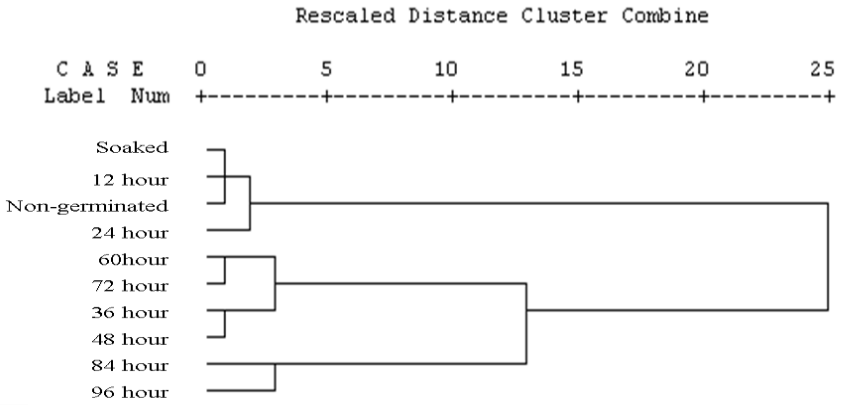

Figure 2. Dendrogram of the engineering properties of the finger millet grains as affected by the germination time

4.Conclusions

An increase in germination time of finger millet from 0 to 96 hours resulted in increased malting loss. Malting loss was directly proportional to thousand kernel weight, bulk density and true density and hence an increase in malting loss resulted in decreased values for these parameters. Initial germination time of 24 hours increased the dimensional and surface properties followed by a decrease on prolonged germination. Dendrogram grouped finger millet samples germinated for 0-24 hours in a single group and hence it can be concluded that existing handling equipments and storage structures can be used for finger millet germinated up to 24 hours with minimum changes. Correlation study suggests that malting loss had a significant effect on gravimetric and dimensional properties, which in turn affect the surface properties like surface area and sphericity. The change in sphericity had a significant effect on frictional properties like angle of repose. Therefore, in the light of results it is suggested that for extended germination time beyond 24 hours the material handling equipments and storage structures should be designed accordingly.

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THE CHEMICAL COMPOSITION, NUTRITION AND FRACTIONAL COMPOSITION OF WINTER RYE GRAIN PROTEINS AFTER VARIOUS METHODS OF EXPOSURE

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ABSTRACT
The choice of feed for cattle should be made by assessing the nutritional value of the feed, as well as the animal's nutritional needs. Winter rye is a major crop in northern latitudes and can be used as feed for livestock. Rye grain contains a sufficient amount of protein, but its quality may not be optimal for cattle to fully digest it. Nevertheless, there are a number of methods for processing the grain that can improve its nutritional properties, including by changing the fractional composition and solubility of its proteins. This study analyzed the influence of thermal (toasting, autoclaving, steaming, extrusion) and chemical (fermentation) methods for processing winter rye grain. Toasting and extrusion led to the greatest changes in its composition. After toasting the grain, the sugar content increased 5.81 times, while the amount of soluble fractions and protein degradability decreased by 17.92% and 11.65%, respectively, in comparison to untreated grain. Extrusion processing of winter rye led to an increase in sugar, crude protein, and metabolic energy by 49.82%, 32.24%, and 22.53%, respectively. The insoluble fraction and protein non-degradability also increased by 1.22 and 1.13 times, respectively. The enzymatic treatment of rye grains led to an increase in the insoluble fraction of proteins, as well as a significant increase in the sugar content (by 5.75 times) compared to the control of untreated raw materials. The results of this study can be used to develop optimal methods for processing winter rye grain for cattle feed, taking into account the impact on the nutritional properties of the grain, the subsequent effect on animal productivity and the economic feasibility of the method.

1.Introduction.

Feeding livestock should be organized appropriately to include an accurate assessment of the nutritional value of feed and consider an animal's nutritional needs. Protein is a necessary element of good nutrition in cattle, and for dairy breeds in particular.

The main source of protein for ruminants is the biomass of rumen microorganisms, which is formed during the fermentation of feed (Schwab et al, 1995). During fermentation, proteins break down to form ammonia, which is then used either for the formation of urea in the liver, or for the synthesis of amino acids by rumen microorganisms. Part of the microbial biomass

is removed from the rumen to the lower sections of the digestive tract, broken down in the abomasum and absorbed in the small intestine.

A number of publications indicate that the synthesis of essential amino acids by rumen microorganisms is not sufficient for animals with high productivity (Broderick, 2018). Therefore, when feeding animals, one should take into account not only the amount of protein, but also its breakdown in different parts of an animal's digestive tract. Using feeds with a higher fraction of protein that cannot be broken down in the rumen is an important strategy for increasing the productivity of cattle. This approach is more efficient and economical, has greater uniformity, and also takes into account the balance of essential amino acids in feed, their digestibility and taste (Schwab et al, 1995). There are a number of ways to increase the amount of protein that is not broken down in the rumen. These include thermal treatment (including the application of pressure), chemical treatment, fermentation, humidification, or a combination of these methods. Selecting the right method depends on the type of processed feed, its nutritional properties and the digestibility of the resulting product in the animal's intestines.

Rye, especially winter rye, is an important cereal crop in northern latitudes (in Canada, the northern regions of the USA, the European Union, and Russia), where it is highly adaptable and productive in various soil and climate conditions (Arendt & Zannini, 2013). Rye grain has robust nutritional properties, including proteins, which can make up 7.0-14.6% of the grain's nutrition, depending on where it was cultivated and the variety (Hansen et al, 2004). The majority of these proteins (70–80%) are prolamins and secalins, which are localized in the endosperm and perform a reserve function. The rest are globulins and albumin. This portion mainly consists of enzymes and structural proteins rich in essential amino acids such as lysine, histinin, arginine, and aspartic acid (Békés & Wrigley, 2016; Schalk et al, 2017). However, an excess of readily available proteins, such as globulins and albumin, can adversely affect protein metabolism in cattle, leading to excessive ammonia production during fermentation and to an increase in nitrogen excretion in urine and feces, thereby reducing the efficiency of nitrogen use in the body (Schwab & Broderick, 2017). In addition, winter rye grain contains a number of anti-nutritional substances, which include phytic acid, pentosans, pectins, β-glucans, tannins, trypsin and chymotrypsin inhibitors, and β-alkylresorcinol. They can reduce the efficiency of digestion, as well as the availability of nutrients, thereby affecting the growth of livestock (Lásztity, 1996).

Thus, given the prevalence of winter rye and its potential nutritional value, there is a need to search for technological methods to increase the nutritional value of winter rye for use as cattle feed. In this study, we evaluated the effects of various methods on the chemical and nutritional composition, as well as changes in protein fractions, in winter rye grain when preparing it as feed.

2.Materials and methods

This study used "Gift" grade winter rye (*Secale cereale*). This grade was developed by the "Federal Research Center Kazan Scientific Center of the Russian Academy of Sciences."

2.1.Grain Processing Methods

Toasting. The winter rye grain was pre-wetted with water until swelling. Next, the grain was poured in a thin layer onto an iron sheet and heated with constant stirring for 10–12 minutes at a temperature of 150°C until it was lightly browned (Isaichev et al, 2009).

Autoclaving. Before sterilization, the grain was mixed with water in a ratio of 1:1. The grain was then poured into heat-resistant containers and placed in an autoclave. Autoclaving was carried out under the following conditions: the vapor pressure in the sterilization chamber was 1.5–2 atmospheres, the operating temperature was 134° C, the sterilization time was 60 minutes, and the volume of the autoclaving container was 1 liter (Stamets, 2000).

2.2.Steaming.

The grain was ground, immersed in water, heated to a temperature of 95-100°C and infused for 120 minutes (Isaichev et al, 2009).

2.3.Extruding. A PE-1 press extruder (Penza, Russia) was used to process the grain. The crushed grain was placed in a press extruder and subjected to short-term but intense mechanical and barothermal action at high pressure (2.5–3.0 MPa) at a temperature of 110–135° C, as well as shearing forces in the mechanics of the apparatus. The finished product had a finely porous structure due to a sharp pressure drop when exiting the extruder (Morozkov & Sitnikov, 2013).

2.4.Fermentation. Crushed winter rye grain was poured into a container in a 3:1 ratio with water heated to a temperature of 80–90°C. Next, the NIST-2 polyenzyme complex (Russia), containing 550 units of alpha-amylase, gluconase (40 units), pectinase (150 units), xylanase (200 units) and protease (2 units), was added to the mixture in the amount of 0.1% of the grain mass and mixed thoroughly. The duration of this treatment was 2-3 hours.

2.5.Analytical research.

The mass fraction of crude protein was determined by the Kjeldahl method according to GOST 32044.1-2012 (ISO 5983-1:2005) (GOST 32044.1-2012, 2014); crude fiber - using intermediate filtration according to GOST 31675-2012 (GOST 31675-2012, 2014), crude fat – for skim residue according to GOST 13496.15-97 (GOST 13496.15-97, 2011). A fractional analysis of proteins in winter rye grains was done by determining the amount of protein in fractions taken after sequential extractions with water, weak solutions of neutral salts, alcohol and alkaline solutions. Each fraction was transferred to Kjeldahl flasks, burned with sulfuric acid, and the amount of nitrogen was determined according to Kjeldahl (GOST 13496.4-93 Feed, compound feed) (GOST 13496.4-93, 2011).

Protein digestibility was determined using a regression equation based on a direct relationship between protein digestibility and solubility in a buffer solution (Verite et al, 1979).

$$P = 0.65 \times a + 35 \quad \text{(Eq.1)}$$

where:

P – degradability of crude protein;

a − solubility (% of crude protein).

3.Results and discussions

3.1.The influence of physico-chemical exposure on the chemical composition and nutrition of winter rye

We compared the effects of various processing methods on the chemical composition (content of crude protein, fiber, fat and nitrogen-free extract [NFE]) and nutrition (energy exchange and sugar content) of winter rye grains (Table 1).

Table 1. The chemical composition and nutritional value of winter rye after various forms of physical and chemical exposure (in air-dry conditions

Type of grain processing	Chemical Composition, %				Nutrition in 1 Kg	
	Crude protein	Crude fiber	Crude fat	NFE	Metabolic energy, MJ	Sugar content, g
Unprocessed grain	8.87	2.35	1.79	78.09	13.98	56.00
Toasting	9.01	2.37	1.51	77.06	13.79	325.5
Autoclaving	9.57	3.05	1.87	76.51	13.92	64.00
Steaming	9.28	3.02	1.50	77.49	13.96	246.00
Extruding	10.73	4.08	1.07	77.52	14.13	83.90
Fermentation (NIST-2)	8.96	2.27	1.23	75.43	13.46	322.00

Heat treatment significantly improves the feed quality of grains, as it forms a large number of aromatic substances, increases enzymatic activity significantly, neutralizes toxins and kills toxin producers (Boyd et al, 2017). Toasting (conductive) gives the grain a pleasant taste and increases the digestibility of its starch. We analyzed the chemical composition of winter rye grains after toasting and found that this type of exposure contributed to a maximum increase in sugar content in winter rye up to 325.5 g/kg, which was 5.81 times higher than the control value (Table 1).

Autoclaving grains increases the availability of protein for digestion and the digestibility of dry matter. In addition, this method sterilizes the feed without reducing its nutritional properties 9McAllister et al, 1991). We found that autoclaving winter rye grains led to a significant increase in the proportion of crude fiber (by 34%), the sugar content (by 14%), and crude protein (by 7%) compared to the control.

Steaming winter rye grains causes a significant increase in the concentration of sugars and fiber by 4.39 and 1.35 times, respectively, compared with the control. Elevated fiber levels are apparently caused by structural changes in carbohydrates.

Extrusion processing is one of the most effective methods of processing grain. Extrusion does two things: the source material is mechanically deformed and a loose structure is formed in the finished product due to the large pressure difference between the extruder and the atmosphere. These mechanisms rearrange the structure of the grain, which deactivates digestive tract enzyme inhibitors, neutralizes toxic substances, sterilizes, improves palatability, and dextrinizes the starch and part of the fiber into glucose (Rathod & Annapure, 2016). Using extrusion on winter rye increases the content of not only sugar by 49.82% and crude protein by 32.24%, but also metabolic energy by 22.53%, compared with the control.

Fermenting feeds is another approach for increasing the availability of feed nutrients in which proteins, fats, and complex carbohydrates are broken down using enzymes. Fermentation can utilize microorganisms such as lactobacilli and yeast (Missotten et al, 2015; Skrede, 2007), or a mixture of hydrolase class enzymes (Rusakov & Kosolapov, 2012). We studied the effect of using the NIST-2 polyenzyme complex on the chemical composition and nutritional value of winter rye grain. The NIST-2 enzyme complex contains enzymes that hydrolyze complex carbohydrates (alpha-amylase, gluconase, pectinase, xylanase). When treating winter rye grains with the NIST-2 complex, a significant increase in the amount of sugars (5.75 times) was found in comparison with the control, untreated raw material.

All the methods for processing winter rye grain that we tested led to a decrease in the content of crude fat and the most significant decrease was observed in the extrusion of winter rye grains.

3.2.The fractional composition of the protein after various types of physico-chemical exposure

Along with increasing the overall nutritional value of feed for ruminants, it is very important to normalize the total level of protein, taking into account soluble fractions. The methods we studied for processing winter rye grain do not significantly affect the crude protein content in the processed grain, however, they can potentially affect its qualitative composition.

Among the proteins of cereal grains, including rye grains, water-soluble proteins, albumin, have the highest biological value. They contain all the essential amino acids in nearly optimal proportions, with only a slight deficiency in methionine content (Békés & Wrigley, 2016). Rye grain has a high content of readily soluble protein fractions (Lásztity, 1996). When comparing the impact of different methods for processing rye grain on the albumin fraction, we found a that it decreased by 9.40% - 10.16% after using barothermal methods (frying, autoclaving and extrusion) compared to

the control (Figure 1). Fermentation and steaming did not have a significant effect on the albumin fraction.

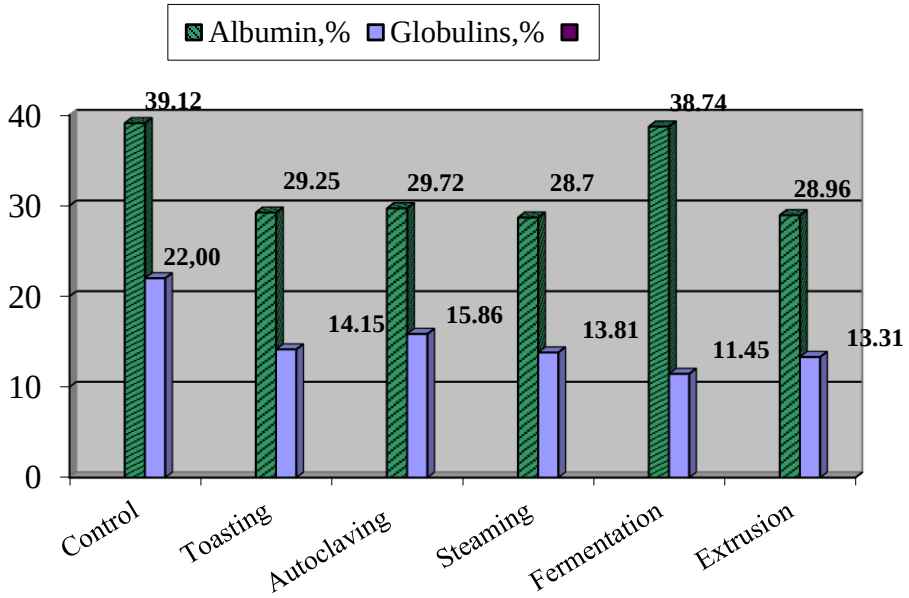

Figure 1. Changes in albumin and globulin protein fractions

The globulin protein fraction (salt-soluble grain proteins) is also characterized by a rather well-balanced amino acid composition, but has a reduced number of some essential amino acids (methionine, tryptophan, leucine) compared to albumin (Zilić et al, 2011). All the methods for processing rye grain studied had a similar effect with respect to decreasing the proportion of globulins. The maximum decrease in globulins was observed after high-temperature fermentation using NIST-2, which was 9.18% lower than the control. Autoclaving and toasting had a minimal impact (6.15–7.85% compared with the control, respectively).

Rye prolamins (secalins) are reserve proteins in grains and have an unbalanced amino acid composition. They have a high proportion of glutamine, proline and glycine, which can be 70-80% of all amino acid residues, as well as a reduced content of such essential amino acids as lysine, tryptophan, and methionine (Békés & Wrigley, 2016; Wieser, 2008). Toasting, steaming and fermentation caused a significant decrease in the proportion of prolamins in rye grain, while autoclaving and extrusion had a less pronounced effect on these proteins (Figure 2).

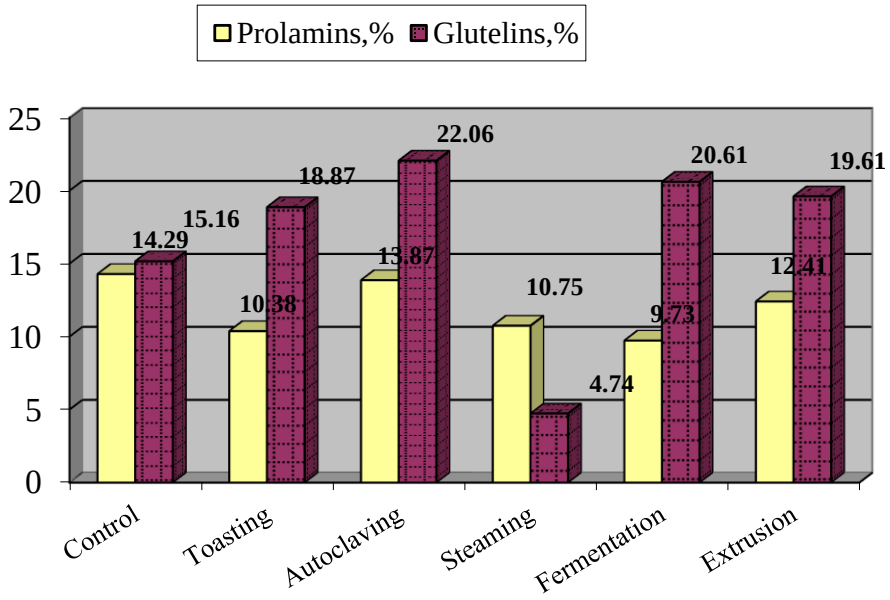

Figure 2. Changes in prolamine and glutelin protein fractions

Glutelins are also reserve proteins in rye grain and are characterized by a significant deficiency in lysine, tryptophan and methionine (Békés & Wrigley, 2016). Fermentation and autoclaving significantly increased the proportion of glutelins in winter rye grain (by 5.45% and 6.90% more than the initial value, respectively). After toasting and extrusion, the increase was in the range of 3.1%–4.74%, while steaming significantly reduced the content of glutelins (by more than three times).

A decrease in the amount of all soluble fractions is observed, compared to the control, after applying various methods for processing winter rye grain (Figure 3). It should be noted that the greatest decrease was found after extrusion and toasting (by 16.28% - 17.92%). After steaming, autoclaving, and fermentation, the decrease is in the range of 7.41% - 10.04% compared to untreated grain.

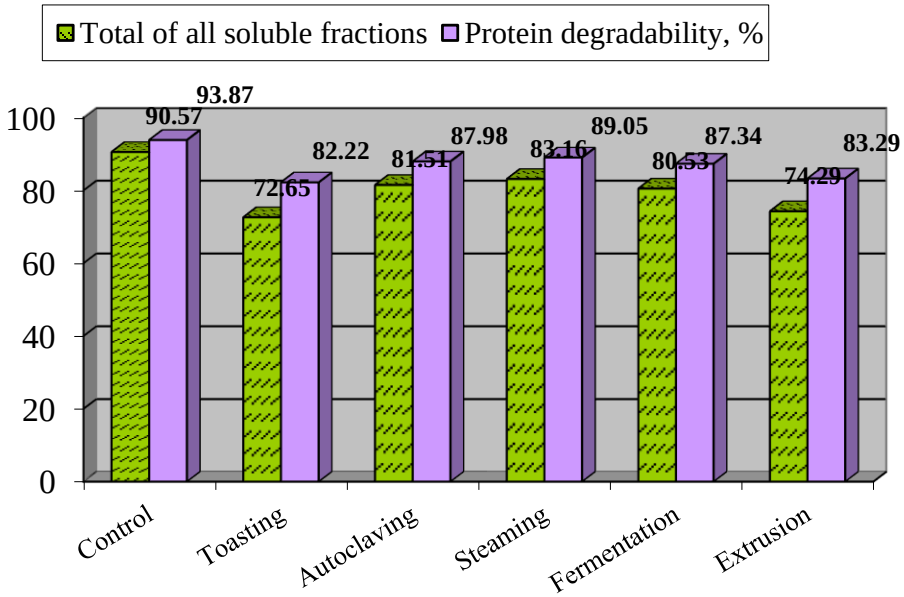

Figure 3. Changes in protein solubility and degradability

A similar result was observed with respect to the proportion of degradable protein. The greatest decrease in this indicator was observed after toasting and extruding winter rye grain (by 11.65% and 10.58%, respectively, compared with untreated grain). Other methods of grain processing had less of an impact on this indicator.

3.3.Discussions

This study is one of the first to compare various methods of physico-chemical exposure on winter rye grain for use as cattle feed. Winter rye is one of the most promising fodder plants in northern regions, as it is a frost-resistant crop that can grow in various types of soil. Both forage and grain feed made from rye have a high protein content, which can be a significant advantage of using rye as a feed crop. This factor should be taken into account when planning the diet of large and small cattle due to the unique features of their digestion. Using technologically simple and inexpensive methods for processing crude rye may be appropriate in this case.

Processing rye grain is done in global agricultural practice to improve its nutritional properties, its taste and to get rid of anti-nutritional substances. It has been shown that toasting increases the digestibility of feed consisting of 60% and 80% rye grain, mainly due to an increase in the digestibility of complex carbohydrates (Sharma et al, 1981). This is consistent with the increased sugar content observed after toasting.

In addition, preparing rye by flattening the grains and applying the MEC-SH-1(Sibbiopharm LLC PA, Novosybirsk, Russian Federatrion) enzymatic complex containing hydrolytic enzymes increased the digestibility of the feed *in vitro*, and most importantly, increased productivity in cattle on this feed (Rusakov & Kosolapov, 2012). Extruding rye grains also increases the sugar content of the feed and contributes to an increase in milk yield compared with animals from the control groups (Morozkov & Sitnikov, 2013). In our research, toasting, extrusion, and high-temperature fermentation were found to contribute to an increase in sugar content. However, only future livestock research can confirm the effectiveness of the grain processing methods from this study in real agricultural practice.

An important observation is that all the methods we studied for processing winter rye reduced the fat content of the raw grain, with extrusion having the most pronounced effect (-39.1%). This factor should be taken into account when using processed grain in cattle feed. Nevertheless, it should be noted that the methods for processing rye grain that we studied did not significantly reduce the value of total energy exchanged, which is promising for future use.

The development of feed for cattle should take into account the peculiarities of protein metabolism, including the proportion of proteins that are broken down and not broken down in the rumen. An increase in the fraction of proteins that are not digestible in the rumen in feed contributes to an increase in milk production in cows (summarized in (Boyd et al, 2017)). This is achieved via appropriate processing of the feed. We found that the methods we tested did not significantly affect the crude protein content in rye grain. However, they did affect the content of readily soluble protein fractions (albumin) (Figure 1), reduce the proportion of soluble protein fractions, and, to a lesser extent, protein breakdown (Figure 3), thereby increasing the amount of protein that is not broken down in the rumen.

Heat treatment of grain is the most common treatment method (Morozkov & Sitnikov, 2013) and, according to the results of our study, seems to be most effective for increasing the fraction of protein that is not broken down in the rumen. Extrusion has similar properties. Steaming grain also reduced the amount of soluble protein fractions, but it also affected the glutelin fraction, reducing it by more than three times. Interestingly, the most effective decrease in the globulin fraction was observed after processing grain with the protease-containing preparation NIST-2, but this preparation had practically no effect on the albumin fraction. It is possible that

using a higher concentration of proteases or selecting other enzymes could increase the efficiency of the enzymatic treatment of grain.

In addition to increasing the productivity of livestock, using various methods of grain processing to prepare feed for large and small cattle has other advantages. Studies on goats have shown that supplements containing protein that is not broken down in the rumen increase resistance in livestock to infection by gastrointestinal nematodes (Cériac et al, 2019). Using these supplements when feeding cattle, including dairy cows, can have the same effect.

In addition, much attention is currently being paid to the environmental impact of keeping livestock. Recently, Haro et al. showed that feeding sheep using feed with a high content of protein that is not digestible in the rumen reduces methane production, which is associated with global climate change processes (Haro et al, 2018). A number of other studies also confirm that processing feed reduces methane production in the rumen (Knapp et al, 2014). Thus, using locally sourced raw winter rye grains and processing them for livestock feed can contribute to reducing the environmental impact of animal husbandry.

4.Conclusions

Winter rye grain is appropriate feed for cattle, but physico-chemical treatment is recommended to increase its nutritional value. The methods for processing winter rye grain in this study influenced the qualitative and quantitative composition of nutrients in the grain and led to an increase in its sugar content, as well as an increase in its undigestible protein fraction, which is an essential component for feeding ruminants. This study can be used to further develop nutritious feed for cattle, thereby maximizing the productivity of livestock.

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PRODUCTION OF BREAD WITH EGGSHELL POWDER AND THE INCREASE OF CALCIUM CONTENT IN THE BODY

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ABSTRACT

Calcium is the most abundant mineral in the body. An inadequate intake of this mineral can cause problems in several vital processes. Eggshell powder is a rich source of calcium that has been shown to have positive effects on bone metabolism. However, the eggshell is classified as a waste material by the food industry, therefore it is not used despite having different properties, its calcium content being one of the most important. The aim of the present investigation was to produce bread using eggshell powder as the raw material in order to develop a bread product rich in calcium. Also, to determine the increase of the calcium content in the body by means of biological analysis. The investigation started with the production of eggshell powder (EP) and the determination of its calcium content by atomic absorption. Then it was replaced in 3 different percentages (10%, 15% and 20%) in addition to a blank sample (0%) to compare the results. The methodology included the conduct of the biological study, where the substitution at 10% obtained higher percentage of assimilation (9.19%) compared to 15% and 20% which were 6.87% and 4.35% respectively. Proximal, mineral (Ca) and microbiological evaluations (molds) of the bread were performed after obtaining the optimal substitution. The results were obtained: humidity (23.8%), ash (5.24%), fat (3.83%), proteins (9.25%), fiber (2.10%), carbohydrates (55.78%), kilocalories (295.79 KJ) and calcium (29.47 mg/g). Finally, the microbiological analysis showed harmlessness in bread.

1. Introduction

Calcium (Ca) is the most abundant divalent cation in the human body. It constitutes between 1.5 – 2.0% of the body's total weight. More than 99% of the body's calcium is found in the skeleton. It occupies a central place in biological systems and is responsible, together with other elements, for structural functions affecting the skeleton and soft tissues, and for regulatory functions such as neuromuscular transmission of chemical and electrical stimuli, the secretion of cells, blood clotting, oxygen transport, and enzymatic activity (Valdés, 2009).

Additionally, a hen's eggshell is an excellent natural source of calcium, consisting of 96% calcium carbonate (Alais *et al.*, 1990). However, the eggshell is commonly considered a waste. On the other hand, it is commonly consumed by people with scarce resources, those who, in some cases, say they prepare the powdered shell in different homemade solutions such as milk, orange juice or lemon, as well as in vinegar (5% acetic acid) or in soup (hot water). Therefore, a study that supports the use of the common eggshell as a source of calcium and as the use of a waste would be very useful and applicable (Gómez, 2011).

Roverský *et al.* (2003) also note that eggshell powder is a natural source of calcium with positive effects on bone metabolism. In addition, different studies have shown that eggshell powder has positive antirachitic effects in rats and humans.

To provide consumers with more opportunities to increase their calcium intake without resorting to supplementation, manufacturers in the United States are recommended to fortify foods and beverages with calcium if certain criteria are met: the food should be commonly consumed. The additional ingredient compatible with the product must be added, and the technology to strengthen the food must be simple, safe and cost effective (Nollet and Toldrá, 2012). In addition, fortification with calcium has been shown to be an economical way to obtain additional calcium (Keller *et al.*, 2002). Moreover, the human body cannot produce adequate amounts of calcium without external support. Additionally, calcium is daily lost through hair, skin, nails, sweat, urine, and feces. This loss of calcium must be replaced, or the body will take calcium from the bones to perform other functions (Kessenich, 2008).

It is important to highlight that bread is one of the staple foods in many countries of the world. Currently, the consumption of bread and bakery products made from refined wheat flour is often the usual trend. However, this flour is characterized by a limited nutritional value (Isserliyska *et al.*, 2001).

In the present, different food products for children are being developed, which are mainly characterized as fortified and nutritious products. Within them we can find bakery products made by replacing wheat flour with flour of tubers, of cereals or of native grains, which increases their nutritional value (Obrego *et al.*, 2013).

Therefore, the present study aimed to investigate the increase of calcium content in the body through biological tests with mice.

2. Materials and methods

2.1. Obtaining eggshell powder

Eggshells were collected from different sources such as restaurants, dealerships, bakeries, food trucks , etc. They were washed and disinfected with a 100 ppm sodium hypochlorite concentration for 5 min, according to tests carried out for validation by a HACCP system in a Food Dealer audited by SMC Slovensko (The Mediterranean Certification Society) in 2018. Next they were dried at 120°C for 60 min based by preliminary tests. Then the shells were ground and passed through a mesh sieve for 10 min with an opening of 106 μm based by preliminary tests. The calcium content was then determined by atomic absorption.

2.2. Bread making

The method used for the production of the product is the direct method. The loaves were prepared according to the standardized formulation with a slight modification of Mesas and Alegre (2002). The wheat flour was replaced by eggshell powder (EP) in 3 different percentages which were 10% (F1), 15% (F2), and 20% (F3), these were compared with a blank sample with 0% (F0) EP substitution. The kneading lasted 5 min. and 50 gr were weighed for each bread. The bread was baked at 180°C for 15 minutes. The loaves were then removed, cooled and stored for further study.

2.3. The Biological Analysis

2.3.1. Animals

Fifteen (15) albino male mice (Mus musculus) of Balb/c/CNPB strain of experimentation were selected for the investigation at an age of 35 to 38 days old, with a weight in the range of 19 to 22 g. They were divided into five groups (samples in triplicate) distributed as follows:

One standard group (GP)

One white group (GB)

Three experimental groups (GE10, GE15 and GE20)

After two weeks of adaptation, in which the mice were fed a standard maintenance feed for rodents (1152.09 mg Ca/100g), the standard

group was analyzed to know the initial conditions of calcium in their bodies. It should be noted that each rodent was in a cage with its own drinking trough and feeder, thus avoiding aggression and injuries between them.

2.3.2. Diet

The white group (GB) and the three experimental groups (GE10, GE15 and GE20) received food orally "ad libitum" for 28 days, the difference was the calcium content in the food supplied. The white group's food was bread with a conventional formulation (F0) containing 0.32mg Ca/g of bread, while the different formulations: Fp1, Fp2, Fp3 contained 29.94 mg, 44.75 mg and 59.55 mg of calcium per gram of bread respectively.

2.3.3. Environmental conditions

The environmental conditions in which the animals were kept throughout the experiment were constant, 12 light hours and 12 dark hours in a temperature range between 20°C to 25°C (Fuentes *et al*., 2008).

2.3.4. Cleaning up

The cages were cleaned and disinfected 3 times a week. The cages were washed using detergent and a brush, then they were disinfected with 0.5% sodium hypochlorite. In the same way the plates containing rodent feces were cleaned. They were left to breathe for 5 min so that the chemical used was volatilized.

2.3.5 Collection of data and samples

During the time of the study the amount of food ingested was weighed daily and the variation in the animals' body weight was measured weekly. A precision scale was used for these measures.

2.3.6. The slaughter

The experimental protocol to which the animals were subjected was designed according to the guidelines proposed in the "Guide for the management and care of laboratory animals: Mouse", which exists in Peru approved by the Resolution N°309-2008-J-OPE/INS.

After 28 days of experimentation, rodents were killed by inhalation of chloroform. The animals were placed in a glass bell containing the chemical, with the lid closed. After 30 to 50 seconds the animals were immobilized. To ensure the animal's death you should wait at least 30 seconds after its last breath. (Servicio de Experimentación Animal, 2005)**.**

2.3.7. Determination of Calcium

Calcium was determined by the atomic absorption method.

2.4. Optimal product analysis

2.4.1. Proximate composition

The proximal composition of the optimal product was determined. Humidity by the NTP 209.085 method, ash by the 2.173 method of AOAC , fat by the 209.093 method, proteins by the 2.057 method of AOAC, fiber by the NTP 209.074 method and carbohydrates by the 31.043 method of the AOAC.

2.4.2. Microbiological analysis

Mould count: ICMSF Volume I, 2nd. Edition, Part II, Method I, p. 166-167.

2.4.3. Calcium content of the optimal product

Calcium content was determined by atomic absorption.

2.5. The Statistical Analysis

The collected data were statistically evaluated by a variance analysis (ANOVA) and the Tukey test with a reliability level of 95%, using the statistical software Minitab 18.

3.Results and discussions

3.1. Calcium content in eggshell powder

The calcium content in the eggshell powder turned out to be 394.58 mg/g, this high calcium content was due to the calcium carbonate content present in the composition of the flour studied.

Brun *et al.* (2013) found that a hen's eggshell has a high calcium content of 381±89 mg Ca/g of eggshell, content similar to that written by Bartter *et al.* (2018) who indicate that the eggshell contains 380 mg Ca/g of calcium. Likewise, Schaafsma *et al.* (2000) found 385 to 401 mg/g Ca in the eggshell, depending on its

origin. These values are similar to those obtained in this work.

3.2. Biological Analysis

3.2.1. Food intake

The food intake for the white group (GB) and the experimental groups (GE10, GE15 and GE20) was 44.98 ± 2.48 g / week.

The intake was constant and equivalent throughout the duration of the treatment, as shown in table 1.

Table 1. Data on food intake during the treatment period

Groups	Weeks				Average (g)
	1	2	3	4	
GB	35.45	40.22	49.89	44.42	44.49
GE10	42.77	48.77	48.46	37.96	47.46
GE15	42.07	48.13	50.70	48.93	43.04
GE20	37.61	43.39	50.17	40.99	42.50

Note: GB = White group, GE10 = Experimental group of 10% substitution, GE15 = Experimental group of 15% substitution, GE20 = Experimental group of 20% substitution.

According to Fuentes *et al.* (2008), laboratory mice had a daily consumption of 3 to 6 grams of food, which means that they had a consumption between 21 and 42 g / week.

3.2.2. Total Body Weight

The variation of the animals' body weight for each experimental group and during the 4 weeks of treatment is shown in table 2.

Table 2. Variation of the body weight of the mice during the experimental time

Groups	Weeks (g)				
	0	1	2	3	4
GB	26.07	29.53	31.13	33.23	33.40
GE10	30.85	30.85	32.23	33.50	31.83
GE15	27.70	28.80	31.67	32.50	31.14
GE20	24.03	24.03	24.67	26.80	26.90

The white group (GB) has a greater increase in body weight. In addition, a slight weight gain is observed in the experimental groups with calcium-rich diets (GE10, GE15 and GE20). However, these values are not significantly different ($p > 0.05$).

In the same way as indicated by different authors, such as Gunther *et al.* (2005), after subjecting three groups of subjects to a treatment with different calcium content (800, 1000, and 1300 mg Ca / day respectively), no significant differences in the body weight of the individuals were found. Lappe *et al.* (2004), after administering to groups of people a diet with different calcium content (900 vs 1500 mg Ca / day), did not notice any difference in the weight of the subjects neither. Snijder *et al.* (2007), after reviewing the data from the HOORM´S study, also found no association between body weight and calcium intake.

However, other publications show inconsistent results. Zemel *et al*. (2000), after conducting a study in rats, concluded that high calcium diets reduced weight gain. Metz *et al*. (1988) pointed out that, after subjecting Wistar Kyoto rats to a normocaloric treatment with high calcium content (4 g / kg feed) for eleven weeks, they also found an association between the high level of calcium in the diet and the reduction in the animals' body weight. González *et al*. (2013) worked with adults from 20 to 59 years of age, where they concluded that there is an inverse association between dietary calcium intake and high body mass index.

3.2.3. Calcium content in rodents

According to the results obtained from the variance analysis regarding the calcium content, there was a significant difference ($p < 0.05$). This shows that not all the averages of the set out groups are equal.

When there was a significant difference, the respective Tukey significance tests for treatments were performed, which indicate us the similarities and differences and help us select the best treatment statistically.

Table 3. Calcium content of experimental groups

Experimental group	Calcium content (g/100 g)
GB	1119.02[b]
GE10	1251.53[a]
GE15	1224.95[a]
GE 20	1195.99[ab]

Values are average of triplicate analysis. The different letters in the same row indicate significant differences P <0.05 between the variants

The best results are shown by GE10 with a diet of 29.94 mg Ca / g of the F1 product (bread with 10% replacement). It shows a significantly higher calcium content compared to the white group (GB) that had a diet based on a bakery product with traditional formulation (F0).

3.2.4. Calcium Assimilation

After 2 weeks of adaptation and before starting the experiment, the analysis of calcium content in the standard group (GP) was performed giving an average value of 1146.15 mg Ca / 100 g. This value shows us the initial conditions of the experimental units, that way we can know the mineral assimilation for each group.

The assimilation of calcium from the different formulations for making the bread with partial replacement of wheat flour with eggshell powder (F1, F2 and F3) and a traditional formulation (F0), was determined according to the content of initial calcium (GP).

Table 4. Calcium Assimilation

Groups	Calcium content (mg/100g)		Assimilation %
	Initial	Final	
GB	1146.15	1119.02	- 2.37
GE10	1146.15	1251.53	9.19
GE15	1146.15	1224.95	6.87
GE20	1146.15	1195.99	4.35

In table 4 it is shown that the experimental groups that had a diet based on bread with eggshell powder increased the calcium content, obtaining an assimilation of 9.19%, 6.87% and 4.35% for groups GE10, GE15 and GE20 respectively. However, the opposite is true regarding the white group (GB) where the assimilation was negative (-2.37%).

The white group (GB) presents a loss of calcium with a decrease of 2.37%. This is due to the change of diet of 11.52 mg Ca / g of the feed to a diet based on bread with traditional formulation with a calcium content of 0.33 mg Ca / 100 g, causing bone resorption.

In addition, GE10 has a greater assimilation of calcium after having changed from a feed diet to a diet with 29.94 mg Ca / g of bread with partial substitution of wheat flour with eggshell powder (10%). Nevertheless, GE15 and GE20 have lower assimilations despite having a higher calcium content in the diets provided.

Malm (1958), Heaney *et al*. (1975), Oguido and De Angelis (1995) showed that the fraction of calcium absorbed by the diet is inversely proportional to the amount ingested. This fact could be due to a delay in the disintegration of the mineral during intestinal transit (Milbradt *et al.* 2017).

Bao *et al*. (1998) studied the possibility of rats absorbing and using calcium from the fortified diet with different sources of calcium, within which eggshell powder was mentioned and then was revealed to have a very high absorption and utilization capacity.

In addition, Dutch and Japanese researchers reported that eggshell calcium has positive effects (Ali and Badawy, 2017).

In the same way, Schaafsma *et al.* (2002); Rovenský *et al.* (2003); Brun *et al.* (2013) and Fina *et al.* (2016) showed in humans that a calcium supplementation from eggshell powder has a positive effect with an adequate intake of this mineral.

3.3. Optimal product analysis

3.3.1 Proximate Composition

In table 5, bread with partial substitution of wheat flour with eggshell powder in 10% (F1) has 23.80% moisture. This value is within the parameters indicated in the Sanitary Standard for Manufacture, Processing and Expenditure of Bakery, Biscuit and Pastry Products RM No. 1020-2010 / MINSA, which indicates that common or tilled bread must contain a moisture of 23% (min.) - 35% (max.).

On the other hand, the ash content is 5.24%. This is due to the amount of minerals that eggshell powder contains. Ray *et al.* (2017) made chocolate cake using eggshell powder, in which they found that a substitution of 9% contained 5.5% of ashes.

Table 5. Proximate Composition of bread with 10% substitution

Analysis	Unity	Results
Moisture	%	23.80
Ash	%	5.24
Fat	%	3.83
Protein	%	9.25
Fiber	%	2.10
Carbohydrates	%	55.78
Energy	KJ	295.79

According to the National Institute of Health (INS, 2009), tilled bread contains 9.60% proteins, 0.3% total fat, 71.8% total carbohydrates, 1.2% crude fiber and 328 KJ energy. According to the results shown in table 5, the Proximate Composition of the product is greater regarding the fat and fiber content. However, it is lower regarding the protein, carbohydrate and energy content.

3.3.2. Microbiological analysis

Table 6 shows that the mold count is less than 10 in CFU / g. This parameter is permissible according to the Sanitary Standard for the Manufacture, Processing and Expenditure of Bakery, Biscuit and Pastry Products R.M. N ° 1020-2010 / MINSA.

Table 6. Microbiological analysis of bread with 10% substitution

Determination	Results	Units
Molds	<10	UFC/g

Note: UFC = Colony forming units

3.3.3. Calcium content in bread

Table 7 shows the calcium content in the optimal product, resulting in 29.47 mg / g. This result is in line with Ali and Badawy (2017), who mentioned that increasing the content of eggshell powder in bread strips, increases the calcium content in comparison to a control sample. In the substitution at 10% they found a calcium content of 30.78 mg / g of bread.

Table 7. Calcium content of bread with 10% substitution

Analysis	Results	Unity
Calcium	29.47	mg/g

Each bread weighs around 40 grams. Therefore the calcium content is 1178.95 mg / unit of bread.

However, INS (2009), in the Peruvian Food Composition Tables, indicate that the calcium content of tilled bread is 0.4 mg / g of the food. The content of this mineral in the final product has a high value compared to that written by the National Institute of Health, this is because of the high ash content in eggshell powder which mostly comes from calcium carbonate (CaCO3).

Piscoya C. (2002) mentions that calcium carbonate is a food additive that has greater advantages over others, such as citrate and acetate, since carbonate has a higher percentage of elemental calcium (40.04%), has low cost and Food Codex indicates its conditions of use.

Most studies agree on the need to maintain a high calcium intake in adolescence, as a dairy diet, fortified foods or medicated supplements, because the only modifiable determinants of

bone mass peak are exogenous (Sánchez *et al.*, 2003).

4. Conclusions

According to the results, the formulation of bread with partial substitution of wheat flour (HT) with eggshell powder (HCH) at 10% (F1) has the highest calcium assimilation compared to bread with conventional formulation (F0). It is worth mentioning that this product rich in calcium and generated from an organic residue would contribute to solve calcium deficit in the body.

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UVB EXPOSURE INDUCED ACCUMULATION OF PHENOLICS AND RESVERATROL AND ENHANCED ANTIOXIDANT ACTIVITIES IN PEANUT SPROUTS

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UVB.

ABSTRACT

Resveratrol is a phenolic compound which naturally presents in peanuts (*Arachis hypogaea* L.) but with low amount. Resveratrol and phenolic compounds are induced in plants by UVB exposure. This study aimed to investigate the accumulation of phenolics and resveratrol and antioxidants in sprouts of three peanut cultivars, Lac sen, L14 and L27. Peanut seeds were germinated under control and UVB exposure (one hour every day at 9 am). Sprouts were harvested at three stages: 1, 3 and 5 days (D1, D3 and D5). Total phenolic (TP) and resveratrol contents and antioxidant activity were measured using Folin-Ciocalteu, HLPC coupled with UV detector and DPPH methods, respectively. The study found both germination time and UVB exposure induced the accumulation of phenolics and resveratrol and increased the antioxidant activity. Compared to D1 sprouts, D5 sprouts had significant increases in TP and resveratrol contents and the antioxidant activity by 42.31%, 508.75% and 77.91%, respectively, while UVB exposure enhanced TP and resveratrol contents and the antioxidant activity by 11.11%, 62.81% and 26.17%. Resveratrol content ranged from 5.57 µg/g DW in L27 sprouts at D1 under control conditions to 110.16 µg/g DW in Lac sen sprouts at D5 under UVB exposure. UVB exposure induced the accumulation of phenolics, particularly resveratrol in peanut sprouts, suggesting this is a potential approach to produce functional foods from peanut sprouts.

1.Introduction

Peanut (*Arachis hypogaea* L.) is one of the most important species belonging to Leguminosae family, which is the third largest family of higher plants. It is an industrial crop with high economic value in agricultural sector not only because of its oil but also of its sources of proteins, minerals and vitamins (Krishna et al., 2015). Furthermore, peanut is a source of diverse bioactive substances such as stilbene, flavonoid, phenolic acid, and phytosterols which are beneficial for human health. They can function as antioxidants, and can be involved in activating liver detoxification enzymes, blocking bacterial activity or virus toxins, inhibiting cholesterol absorption and reducing platelet aggregation (Pennington, 2002).

Resveratrol, a phenolic compound belonging to the stilbene group, has been a bioactive substance of interest over the past decade due to its potential benefits for human health. Resveratrol was first identified in the roots of white hellebore (*Veratrum grandiflorum* O Loes) in 1940 (Aggarwal et al., 2004). This substance was then found in grapes, red wine and peanuts (King et al., 2006). Studies show that resveratrol reduces the risk of cardiovascular disease, Alzheimer's disease, inhibits cancer growth and slows the aging process (Sales and Resurreccion, 2014; Hasan et al., 2013). Recently, Ha et al. (2015) revealed supplementary of peanut sprout extracts reduced abdominal fat and enhanced health indices in obese women.

Resveratrol naturally exists in peanut seeds but its content is low; therefore, the investigation of methods to increase resveratrol content in peanuts has recently been of interest. Germination is considered an approach to increase the content of bioactive substances such as phenolics in some legumes (Tang et al., 2014; Lin and Lai, 2006) and GABA in rice (Zhang et al., 2014). Some studies on peanuts found that germination enhanced resveratrol contents in peanut sprouts (Limmongkon et al., 2017; Wang et al., 2005). Soaking temperature, germination temperature and germination time also have effects on resveratrol content; a study by Yu et al. (2016) indicated that peanut seeds soaked at 35^{o}C for 6 hours resulted in the highest resveratrol content and the best germination temperature is 32^{o}C and the resveratrol content increased with germination time.

UV light is ultraviolet light and is divided into three types: UVA (320-400 nm), UVB (280-320 nm), UVC (200-280 nm) (Surjadinata et al., 2017). UVB is the ultraviolet source that affects organisms at a moderate level and recent studies showed that UVB light induced the biosynthesis of phenolic compounds in mung bean sprouts (Wang et al., 2017), *vigna mungo* sprouts (Shaukat et al., 2013) and wheat and pea sprouts (Alexieva et al., 2001). To my knowledge, no study has investigated the effect of UVB on bioactive compounds in peanut sprouts. Thus, this study aims to test the hypothesis that UVB exposure induces the accumulation of total phenolics and resveratrol and increase antioxidant activity of peanut sprouts.

2.Materials and methods

2.1.Materials

Peanut seeds

Three peanut cultivars used for this study were Lac sen, L14 and L27 (grown in Bac Giang and Bac Ninh provinces and harvested in June 2018).

Peanut sprouts

Firstly, peanut seeds were washed three times with water and treated with 70% ethanol for 30 seconds. The seeds were then washed with water and soaked in a water bath (GFL, Germany) at 30^{o}C for six hours. After that, the seeds were germinated in plastic cups (dimension: 9.5 cm depth x 8.5 cm top diameter x 5.5 cm base diameter). The seeds were germinated under dark conditions in a box which was placed in a 30^{o}C-room. The experiment was designed with two formulas: UVB exposure and control (nonUVB exposure). For UVB exposure, sprouts were treated with UVB for one hour every day at 9 am and the UVB lamp was placed in a distance of 20 cm above the sprouts. UVB lamp was 45 cm in length, 15W, 10% UVB (Repti Glo 10.0, China).

The germinated seeds were collected on days 1, 3 and 5. The experiment was repeated three times. The collected sprouts were then kept at -22°C, then freeze-dried for three days by ModulyoD Freeze Dryer (Thermo electron corporation, USA), ground and stored at -22°C for the analysis of phenolic compounds and resveratrol contents and antioxidant activity.

2.2.Chemicals and reagents

All chemicals were analytical-graded. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and Trolox were purchased from Sigma (USA). Gallic acid

and Folin–Ciocalteu reagent were purchased from Merk (Germany). Acetonitrile and methanol were obtained from Samchun (Spain). Other chemicals were from China.

2.3.Determination of germination rate and sprout length

The germination rate and the sprout length were measured at harvest time. The germination rate was calculated by the percentage of germinated seeds. The sprout length was measured on 15 randomly selected germinated seeds by a ruler.

2.4.Determination of total phenolic (TP) content

Seed and sprout extracts were prepared using the method by Yu et al. (2016) with some modifications. 500 mg of milled seeds or milled sprouts were weighed into a 15-mL Falcon tube and 10 mL of ethanol 80% was added. The sample was extracted in 45 minutes at 80^{o}C and shaken by using a vortex every 10 minutes. The extract was then centrifuged at 6000 rpm for 20 minutes and the supernatant was collected and stored at -22^{o}C.

TP content was measured using the Folin-Ciocalteu method described by Fu et al. (2011). In brief, 0.5 mL of the diluted sample was transferred into a test tube and 2.5 mL of 1:10 diluted Folin-Ciocalteu reagent was then added and mixed well. After 4 minutes, 2 mL of 7.5% Na_2CO_3 was added. The reaction was incubated at room temperature in the dark for 2 h and the absorbance of the mixture was then measured at 760 nm using a UV–visible spectrophotometer (UV-Vis 1800, Shimadzu, Japan). A calibration curve was established based on working-standard solutions of 0.02, 0.04, 0.06, 0.08 and 0.1 mg/mL for the calculation of TP content. The results were expressed in gallic acid equivalents per gram dry weight (mg GAE/g DW).

TP content was calculated by the following formulas:

$$TPC = \frac{A*V*dF}{a*m} \quad \text{(Eq.1)}$$

In which

TPC: Total phenolic content (mg GAE/g DW)

A: Absorbance at 760 nm

V: Volume of the extract (mL)

dF: Dilution factor

a: Slope factor of gallic acid standard curve

m: Sample weight (g)

2.5.Antioxidant capacity measurement

The extracts of total phenolics were used for antioxidant activity determination. Antioxidant capacity was determined using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay described by Thaipong et al. (2006) with some modifications. The stock solution of DPPH was made by dissolving 24 mg of DPPH with 100 mL of methanol and then kept at -23^{o}C until needed. The working solution of DPPH was prepared by diluting 10 mL of the stock solution with 45 mL of methanol. The reaction was carried out by mixing 150 µL of diluted sprout extract with 2850 µL of the DPPH solution for 30 minutes. A control was also prepared by using 150 µL of methanol instead of sprout extract. The absorbance was then measured at 515 nm using a spectrophotometer (UV-Vis 1800, Shimadzu, Japan). The results were calculated based on a standard curve established from a set of Trolox standard solutions of 50, 100, 250, 500, 750 and 1000 µM. The results were expressed in Trolox equivalents (µM TE/g DW).

Antioxidant activity was calculated by the following formulas:

$$AA = \frac{AA\%*V*dF}{a*m*1000} \quad \text{(Eq.2)}$$

In which

AA: Antioxidant activity (µM TE/g DW)

AA%: % inhibition calculated by this equation

$$AA\% = \frac{(Acontrol - Asample)*100}{Acontrol} \quad \text{(Eq.3)}$$

V: Volume of the extract (mL)

dF: Dilution factor

a: Slope factor of Trolox standard curve equation

m: Sample weight (g)

1000: Conversion factor from mL to L (of the extract).

2.6.Determination of resveratrol content

Samples were extracted as described for the total phenolic extraction. Resveratrol content was determined using the Agilent 1260 Series HPLC system equipped with a UV detector (Agilent Technologies) and LC - Solution software. The column used was Kinetex 5 µm EVO C18 100 Å, 150 x 4.6 µM. The wavelength and column temperature were set at 306 nm and 30°C, respectively. The injection volume and the flow rate were 40 µL and 1 mL/minute, respectively. The mobile phase was a gradient (as described in Table 1) of deionized water mixed with formic acid (0.1%) (phase A) and acetonitrile mixed with formic acid (0.1%) (phase B). The results were calculated based on a standard curve established from a set of resveratrol standard solutions: 0.76, 1.56, 3.125, 6.25 and 12.5 µg/mL and their peak areas. The results were expressed in µg/g DW.

Resveratrol content was calculated as the following equation:

$$\text{Resveratrol content} = \frac{S*V*dF}{a*m} \quad \text{(Eq.4)}$$

In which

Resveratrol content (µg/g DW)

S: Peak area

V: Volume of the extract (mL)

dF: Dilution factor

a: Slope factor of resveratrol standard curve

m: Sample weight (g)

Table 1. The gradient of mobile phase

Time (minutes)	Phase B (%)
0	0
5	15
20	20
30	100
35	100
40	0
42	0
42.01	0

2.7.Statistical analysis

Data and comparisons were analyzed using R version 3.5.0. Sprout variables were analysed by three-way independent ANOVA (Cultivar x Germination time x UVB). Significant differences between the means were analysed by Duncan's test ($P<0.05$). The particular set of variables (TP content and 100-seed weight) was subjected to Pearson's correlation analysis (Field, 2013).

3.Results and discussions

3.1.Hundred-seed weight, TP and resveratrol contents and antioxidant activities of seeds

Significant ($P<0.05$) variations in the TP content and antioxidant activity of seeds were observed between the three peanut cultivars (Table 2). Lac sen seeds had the highest TP content (4.73 ± 0.19 mg GAE/g DW), while L14 seeds had the lowest TP content (2.97 ± 0.20 mg GAE/g DW). The trend of antioxidant activity was different from that of TP content in the three cultivars, in which Lac sen seeds had the highest antioxidant activity (3.46 ± 0.07 µM TE/g DW), and the antioxidant activity was the lowest for L27 seed (2.40 ± 0.17 µM TE/g DW). The higher seed TP content might be due to the lower 100-seed weight (Table 2) because the TP content of seeds had significantly negative correlation with 100-seed weight (r^2=-0.92,

P<0.001). Phenolic compounds mainly exist in peanut skins (Khaopha et al., 2012) and smaller seeds have more skins than larger seeds; thus, smaller seeds result in higher TP contents than larger seeds.

Table 2. Hundred-seed weight, TP and resveratrol content and antioxidant activity of seeds of three peanut cultivars. Data represent means ± standard deviations (n=3, except n=1 for resveratrol content).

Cultivar	100-seed weight (g)	TP content (mg GAE/g DW)	Resveratrol content (μg/g DW)	Antioxidant activity (μM TE/g DW)
Lac sen	46.83 ± 0.76^{b}	4.73 ± 0.19^{a}	4.20	3.46 ± 0.07^{a}
L14	65.30 ± 5.79^{a}	2.97 ± 0.20^{b}	7.41	2.89 ± 0.13^{b}
L27	59.65 ± 1.13^{a}	3.89 ± 0.16^{c}	5.92	2.40 ± 0.17^{c}

Different letters show significant differences within each column (*P<0.05*).

Previous studies showed TP contents ranged from about 0.10-2.30 mg GAE/g fresh weight among 20 peanut cultivars (Chukwumah et al., 2009), 0.15-3.04 mg GAE/g DW among 15 genotypes (Khaopha et al., 2012) and 0.15-0.53 mg GAE/g DW among 5 genotypes (Adhikari et al., 2018). Khaopha et al. (2012) reported that TP contents mainly present in peanut skins and the color of peanut skin had strong correlation with the TP content of peanut kernels (Chukwumah et al., 2009) and peanut skins with pink color contained significantly higher TP contents than those with gray and yellow Khaopha et al. (2012). Peanut cultivars in our study had pink color and had slightly higher TP contents (2.97 ± 0.20 - 4.73 ± 0.19 mg GAE/g DW) compared to those with pink color (1.83-3.04 mg GAE/g DW) reported by Khaopha et al. (2012). Similar to our study, Craft et al. (2010) showed antioxidant activities also varied (3.02-11.9 μM TE/g DW) among 8 peanut cultivars.

Seed resveratrol contents varied (0.13-3.4 μg/g DW) depending on cultivars (Adhikari et al., 2018; Yu et al., 2016; Wang and Pittman, 2009). In this study, the resveratrol content of seeds ranged from 4.20 to 7.41 μg/g DW, in which Lac sen seeds had the lowest resveratrol content, whereas that of L14 was the highest (Table 2). In our study, the peanuts cultivars had higher resveratrol contents compared to previous reports (Adhikari et al., 2018; Yu et al., 2016; Wang and Pittman, 2009). This might be attributed to genotypes or agronomic conditions.

3.2.Germination rate and sprout length

Table 3. Effect of cultivar, germination time and UVB on germination rate, sprout length, TP and resveratrol contents and antioxidant activity. Germination rate is measured in percentage; sprout length is measured in cm; TP content is measured in mg GAE/g DW; resveratrol content is measured in μg resveratrol/g DW; antioxidant activity is measured in μM TE/g DW. *, ** and *** significant at P<0.05, P<0.01 and P<0.001, respectively.

	Germination rate	Sprout length	TP content	Resveratrol content	Antioxidant activity
Cultivar (C)					
Lac sen	88.9	4.88	4.02	40.44	1.00

L14	83.9	5.28	3.81	25.84	1.29
L27	95.2	5.52	3.51	26.30	1.35
Germination time (GT)					
D1	83.1	1.82	3.12	10.17	0.86
D3	93.5	4.59	3.77	20.50	1.25
D5	91.2	9.26	4.44	61.91	1.53
UVB					
Control	89.4	5.4	3.6	23.34	1.07
UVB exposure	89.3	5.1	4.0	38.37	1.35
F *ratio*					
Cultivar (C)	11.30^{***}	4.58^{*}	11.95^{***}	27.70^{***}	42.47^{***}
Germination time (GT)	10.52^{***}	620.35^{**}	77.67^{***}	93.37^{***}	462.18^{***}
UVB	0.00	3.27	16.22^{***}	46.50^{***}	104.49^{***}
C × GT	9.21^{***}	5.24^{**}	3.95^{**}	6.52^{***}	25.54^{***}
C × UVB	1.24	1.35	0.50	0.31	5.95^{**}
GT × UVB	0.31	2.60	0.05	2.43	34.15^{***}
C × GT × UVB	1.40	0.36	1.14	0.24	3.44^{*}

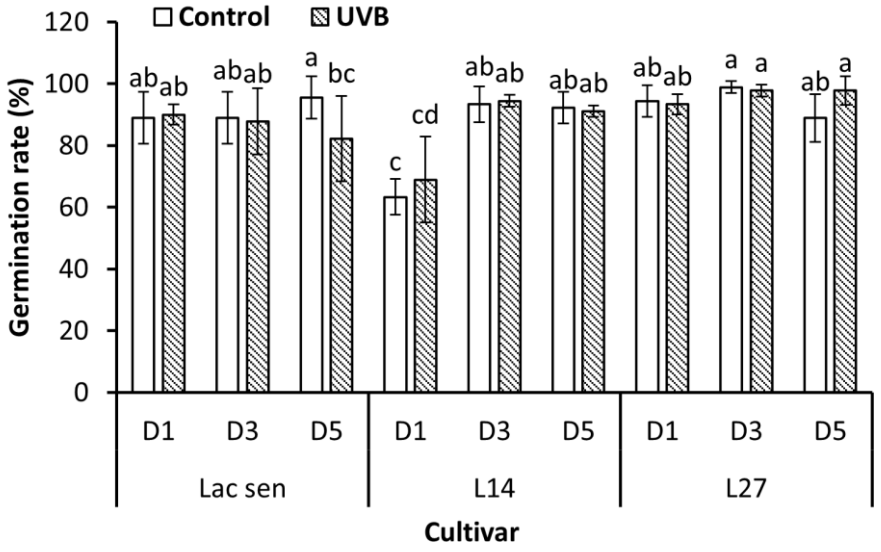

Figure 1. Effect of germination time and UVB exposure on germination rates of three peanut cultivars. Data represent means ± standard deviations (n=3). Different letters show significant differences (*P<0.05*).

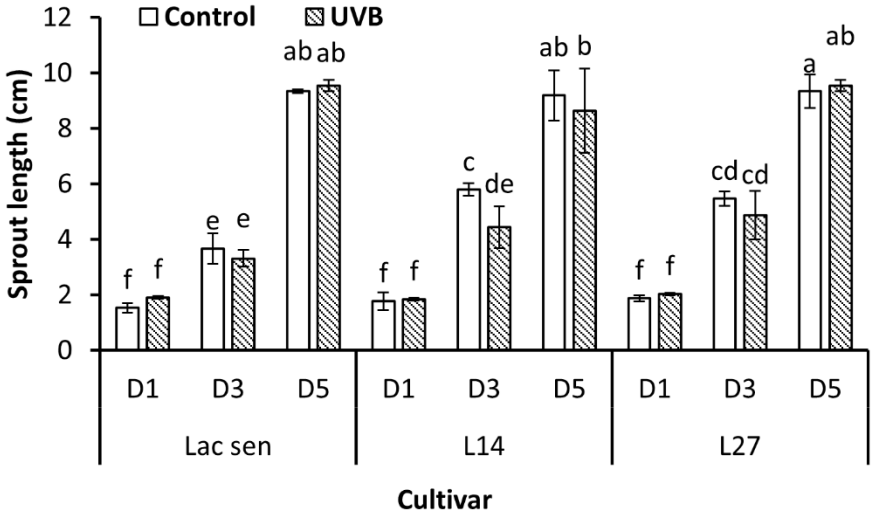

Figure 2. Effect of germination time and UVB exposure on sprout length of three peanut cultivars. Data represent means ± standard deviations (n=3). Different letters show significant differences ($P<0.05$).

UVB exposure had no effects on germination rate and sprout length, while the cultivar (C) and germination time (GT) significantly affected these two parameters (Table 3). Significant interaction between cultivar and germination time was observed. This means cultivars behave differently in germination rate and sprout length during germination. Indeed, although the germination rates of two cultivars Lac sen and L27 were not different between germination periods, averagely about 90%, this variable of L14 was significantly lower at day 1 (D1) (about 65%) compared to day 3 (D3) and day 5 (D5) (Figure 1). Sprout length increased dramatically during kernel germination, ranging from 2 cm at D1 to about 9 cm at D5.

There were no significant differences in sprout length between cultivars at D1 and D5, but at D3, this variable of Lac sen was lower than that of the other two cultivars (Figure 2).

3.3.TP and resveratrol contents and antioxidant activities

All three factors: cultivar, germination time and UVB exposure significantly affected the TP content, the resveratrol content and the antioxidant activity (Table 2). No significant C x UVB, GT x UVB and C x GT x UVB interactions occurred for TP content and resveratrol content, but C x GT interactions were found for these two variables. Significant C x GT, C x UVB, GT x UVB and C x GT x UVB interactions were observed for antioxidant activity.

TP contents of all cultivars significantly increased through D1 to D5 (Table 3, Table 4). On average, the TP content enhanced 42.31% at D5 compared to D1 (Table 3), but the changes varied between cultivars and by UVB treatments. Under control conditions, the TP content of L27 increased by 27.3% from D1 (3.00 mg GAE/g DW) to D5, while these increases were higher with 54.8% and 57.7% for Lac sen and L14, respectively. Under UVB exposure, an increase in TP content from D1 (3.60 ± 0.48 mg GAE/g DW) to D5 was 35.3% for Lac sen, lower than the increases in L27 and L14 with 40.6% and 41.6%, respectively. Increases in TP content in 13 different germinated seeds have been reported (Cevallos-Casals and Cisneros-Zevallos, 2010). However, Limmongkon et al. (2017) indicated that phenolic accumulation of five peanut cultivars

during germination did not show any consistent trend through D1 to D4. For example, TP content of Tainan increased from D1 to D4, while that of Kalasin1 decreased from D1 to D3 then increased. The TP contents of sprout cotyledon and sprout without cotyledon also varied among six peanut cultivars through D5 to D9 (Adhikari et al., 2018).

Abiotic stresses including UVB exposure stimulate the biosynthesis of phenolic compounds (Surjadinata et al., 2017). In our study, UVB exposure led to significantly increasing the TP content by 11.11% (Table 3). Cultivars did not show consistent trend in TP contents through D1 to D5 (Table 4). For Lac sen, UVB exposure resulted in significant increases in TP contents of 18.0% and 16.8% at D1 and D3, respectively, but showed no effect at D5. However, UVB exposure had no significant impact on TP contents of L14 sprouts at any days. UVB exposure did not affect TP contents of L27 sprouts at D1 and D3, but significantly led to a rise of 17.0% at D5. The accumulation of phenolics is a protective mechanism of plants to respond to UVB exposure (Escobar-Bravo et al., 2017). UVB exposure also led to enhancements of TP contents in mung bean sprouts (Wang et al., 2017), *vigna mungo* sprouts (Shaukat et al., 2013) and wheat and pea sprouts (Alexieva et al., 2001). Previous studies found UVB exposure resulted in the increases of kaempferol (2.1-folds) and quercetin (1.5-folds) (flavonoids) in broccoli sprouts (Mewis et al., 2012), and an increase of anthocyanins, about 3-folds and 2-folds in wheat and pea sprouts, respectively (Alexieva et al., 2001). Therefore, UVB exposure could be a potential approach to increase beneficial phenolics in seed sprouts.

Table 4. Effect of germination time and UVB exposure on TP content of three peanut cultivars. Data represent means ± standard deviations (n=3). Different capital letters show significant differences within each column (*P<0.05*); different small letters show significant differences in each row (*P<0.05*).

Cultivar	UVB exposure	TP content (mg GAE/g DW)		
		D1	D3	D5
Lac sen	UVB	3.60 ± 0.48^{Ac}	4.25 ± 0.10^{Ab}	4.87 ± 0.05^{Aa}
	Control	3.05 ± 0.12^{Bc}	3.64 ± 0.24^{BCDb}	4.72 ± 0.34^{Aa}
L14	UVB	3.15 ± 0.16^{ABb}	4.14 ± 0.33^{ABa}	4.46 ± 0.33^{ABa}
	Control	2.72 ± 0.12^{Bb}	4.04 ± 0.07^{ABCa}	4.29 ± 0.63^{ABa}
L27	UVB	3.18 ± 0.25^{ABb}	3.42 ± 0.15^{CDb}	4.47 ± 0.26^{Aa}
	Control	3.00 ± 0.21^{Bb}	3.13 ± 0.65^{Dab}	3.82 ± 0.34^{Ba}

Resveratrol is a phenolic naturally found in peanut seeds, but its content is low (Sales and Resurreccion, 2014). Studies revealed the content of this compound can be enhanced by seed germination (Hasan and Bae, 2017; Wang et al., 2005). Our study found that the resveratrol content increased 508.75% at D5 compared to that at D1 (Table 3). The increases varied between cultivars and by UVB treatments (Table 5). Under both UVB exposure and control conditions, resveratrol contents of all three cultivars increased dramatically from D1 through D5. Under nonUVB exposure, resveratrol content of Lac sen sprouts at D5 had an enhancement of 6.2-fold compared to that of D1 (17.59 μg/g DW). L27 sprouts also showed a 7.0-fold increase in resveratrol content at D5 compared to D1 (5.57 μg/g DW), while the increase in this variable for L14 sprouts at D5

compared to D1 (8.37 μg/g DW) was 4.4-fold. Under UVB exposure, resveratrol contents also increased 5.9-, 6.9- and 6.1-fold at D5 compared to D1 for Lac sen, L14 and L27 sprouts, respectively; resveratrol contents of these cultivars at D1 were 17.59, 9.50 and 9.57 μg/g DW respectively. The enhancements of resveratrol content in our study are similar to findings in three peanut cultivars in the study by Wang et al. (2005), in which cultivar Tainan 11 showed an increase from 3.7 μg/g DW at D0 to 17.7 μg/g DW at D6. Another research reported peanut sprouts at D5 reached resveratrol content of 32.87 μg/g DW, which was about 9.8-fold higher than that in non-germinated peanuts (Yu et al., 2016). Similarly, resveratrol contents in sprouts of six peanut genotypes at D5 were higher than that in seeds (Adhikari et al., 2018). However, Limmongkon et al. (2017) found two of five peanut cultivars had downward trends in resveratrol content from D1 to D4 of germination, while the other three cultivars did not show any consistent trend. These differences could be due to differences in genotypes.

Resveratrol is synthesized in plants in response to abiotic stresses (Hasan and Bae, 2017), but to our knowledge, no study has focused on the effect of UVB light on resveratrol response in peanut sprouts. Our study showed that UVB exposure significantly enhanced the accumulation of resveratrol by 62.81% on average (Table 3). The enhancements differed at different germination time and between cultivars. At D1, UVB exposure resulted in significant increases in resveratrol contents in Lac sen and L27, but not in L14. The increases by UVB treatment occurred in all cultivars at D3 and D5. At D5, UVB exposure led to the highest increase of resveratrol content with 78.2% in Lac sen, followed by L14 with 77.0% and L27 with 49.2%. The accumulation of resveratrol in peanut seedlings and peanut leaves increased 196-fold and 200-fold, respectively in response to UV light (Tang et al., 2010; Chung et al., 2003). Another study showed UV exposure on sliced peanuts induced the synthesis of resveratrol (Potrebko and Resurreccion, 2009). Post-harvest treatment with UVB enhanced resveratrol in grapes two-fold (Cantos et al., 2000). According to Tang et al. (2010), resveratrol is considered to be associated with plant defense responses because it is induced by to stresses. Accumulation of resveratrol could be to a factor to alleviate reactive oxygen species (ROS) induced by UVB exposure, thus preventing plant tissues from damages.

Table 5. Effect of germination time and UVB exposure on resveratrol content of three peanut cultivars. Data represent means ± standard deviations (n=3). Different capital letters show significant differences within each column ($P<0.05$); different small letters show significant differences in each row ($P<0.05$).

Cultivar	UVB exposure	Resveratrol content (μg resveratrol/g DW)		
		D1	D3	D5
Lac sen	UVB	17.59 ± 2.70^{Ab}	26.84 ± 2.84^{Ab}	110.16 ± 18.6^{Aa}
	Control	10.43 ± 1.33^{Bb}	15.76 ± 0.90^{Bb}	61.82 ± 6.54^{Ba}
L14	UVB	9.50 ± 0.86^{Bc}	19.51 ± 3.05^{Bb}	65.24 ± 4.25^{Ba}
	Control	8.37 ± 0.61^{Bc}	15.52 ± 0.14^{Bb}	36.85 ± 2.16^{Ca}
L27	UVB	9.57 ± 0.32^{Bc}	28.62 ± 3.12^{Ab}	58.29 ± 6.32^{Ba}
	Control	5.57 ± 0.44^{Cc}	16.69 ± 2.40^{Bb}	39.06 ± 5.47^{Ca}

Antioxidants are considered to be radical scavenger which reacts readily with radicals or ROS and the present of these substances is usually measured by antioxidant activity (Sindhi et al., 2013). Accumulation of antioxidants is a protective mechanism of plants in response to stresses (Das and Roychoudhury, 2014). In our current study, UVB exposure was found to increase antioxidant activity by 26.17% on average (Table 3); however, the effect of UVB was different among cultivars (Table 6). UVB exposure led to a significant increase of antioxidant activity in Lac sen sprouts at D3 from 0.92 ± 0.11 to 1.32 ± 0.22 μM TE/g DW, while no significant enhancement occurred at D1 and D5. UVB treatment increased antioxidant activity of L14 sprouts by 34.6% at D1, whereas this variable was not significantly affected at D3 and D5. An enhancement of 18.7% was observed in L27 sprouts at D5 by UVB exposure, but this did not occur at D1 and D3. To our knowledge, no information has been reported on the effect of UVB exposure on the antioxidant activity in seed sprouts, but previous studies found the antioxidant activity of tobacco leaves enhanced after the plants were treated with UVB (Shen et al., 2017) and the antioxidant activity in the plants, *Deschampsia antarctica* Desv. enhanced after 3 h of exposure to UVB (Köhler et al., 2017). Post-harvest UVB exposure also increased antioxidant activities of whole, sliced and peeled carrots (Avena-Bustillos et al., 2012; Surjadinata et al., 2017). The enhancements of antioxidant activity by UVB treatments in the peanuts sprouts as well as in the plant tissues might be associated with the increases of antioxidants, such as phenolics. Enhanced antioxidant activities could also be a way of plants to alleviate ROS when exposed to UVB.

Similar to TP and resveratrol contents, antioxidant activities also increased during the germination of peanut cultivars; this variable enhanced at D5 by 77.91% compared to D1 (Table 3). Lac sen showed greater increase in antioxidant activities at D5 than the other cultivars under both treatment conditions (Table 6). The values of this variable in Lac sen sprouts significantly increased by 207.1% and 166.1% at D5 compared to D1 under control conditions and UVB exposure, respectively; followed by L14 sprouts with 83.3% and 56.2%, while the enhancements in L27 sprouts were the lowest with 40.2% and 44.7%. The results from this study are in agreement with a report by Wang et al. (2005) where antioxidant activities of three peanut cultivars enhanced with an increase of germination time. In another study, antioxidant activities of all seven peanut cultivars at D10 of germination were significantly higher than those at D0 (soaked peanuts) (Yang et al., 2019). A study on 13 other seeds also showed significantly increased antioxidant activities in all sprouts after seven days of germination compared to soaked and original seeds (Cevallos-Casals and Cisneros-Zevallos, 2010). The increase of antioxidant activity could be related to the enhancement of antioxidants such as phenolic compounds, and therefore peanut sprouts can be potential sources for functional food production.

Table 6. Effect of germination time and UVB exposure on resveratrol content of three peanut cultivars. Data represent means ± standard deviations (n=3). Different capital letters show significant differences within each column (*P<0.05*); different small letters show significant differences in each row (*P<0.05*).

Cultivar	UVB exposure	Antioxidant activity (μM TE/g DW)		
		D1	D3	D5
Lac sen	UVB	0.56 ± 0.06^{Db}	1.32 ± 0.22^{ABa}	1.49 ± 0.22^{BCa}

	Control	0.42 ± 0.15^{Dc}	0.92 ± 0.11^{Cb}	1.29 ± 0.07^{Ca}
L14	UVB	1.05 ± 0.05^{Bb}	1.63 ± 0.22^{BCa}	1.64 ± 0.06^{ABa}
	Control	0.78 ± 0.15^{Cb}	1.16 ± 0.18^{Ba}	1.43 ± 0.24^{BCa}
L27	UVB	1.23 ± 0.06^{Ab}	1.40 ± 0.23^{ABb}	1.78 ± 0.11^{Aa}
	Control	1.07 ± 0.05^{ABb}	1.07 ± 0.10^{BCb}	$1.50 \quad 0.07^{BCa}$

4.Conclusions

This study revealed both germination time and UVB exposure increased TP and resveratrol contents and antioxidant activities. TP and resveratrol contents and the antioxidant activity at D5 increased 42.31%, 508.75% and 77.91% on average compared to D1, respectively, while UVB exposure enhanced TP and resveratrol contents and the antioxidant activity by 11.11%, 62.81% and 26.17% on average. Peanut cultivars responded differently to germination time and UVB exposure. UVB exposure significantly enhanced TP content of L27 sprouts at D5 by 17.0% but this effect did not occur in the other cultivars. UVB exposure significantly induced the accumulation of resveratrol in D5 sprouts of all cultivars, in which Lac sen sprouts at D5 showed the highest content with 110.16 μg/g DW. UVB exposure also significantly enhanced the antioxidant activity of L27 at D5, but the effect was not found in the other two cultivars. UVB exposure induced the accumulation of phenolics, particularly resveratrol in peanut sprouts, suggesting that peanut sprouts can be potential sources for the production functional foods.

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EFFICIENCY OF SPICE EXTRACTS IN SOYBEAN OIL STABILITY DURING HEATING

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ABSTRACT

The aim of the present study was to evaluate the efficiency of spice extracts in soybean oil stability regarding oxidative deterioration during heating. The treatments i) soybean oil (Control), ii) soybean oil with thyme extract (TE), iii) soybean oil with basil and thyme extracts (Mixture 1), iv) soybean oil with oregano and thyme extracts (Mixture 2), and v) soybean oil with tert-butyl-hydroquinone (TBHQ) were subjected to 180 °C for 30 h; the samples were taken at the times 0, 5, 10, 15, 20, 25, and 30 h and analyzed as to peroxide value, conjugated dienoic acids, oxidative stability, total polar compounds, fatty acid and triacylglycerol composition. The spice extracts improved soybean oil resistance against thermal deterioration and, therefore, they may be recommended as sources of powerful antioxidant for stabilization of unsaturated vegetable oils.

1.Introduction

Lipid oxidation has been recognized as the main problem that affects edible oils, causing changes in their chemical, sensory, and nutritional properties (Velasco and Dobarganes, 2002). It is known that the products from lipid oxidation are associated with several risks to health, such as aging, heart diseases and cancer. The addition of antioxidants is effective at delaying lipid oxidation (Suja et al., 2004). However, there is concern related to the possible adverse effects that synthetic antioxidants, obtained by several diet components, may cause to health. Therefore, it is important to indentify new natural components in foods that present antioxidant activity (Hemalatha and Ghafoorunissa, 2007).

From the point of view of the current consumer, aspects such as safety, health, and quality become pertinent on agriculture and food production. In those circumstances, research and development of safe natural antioxidants in order to replace synthetic antioxidants have been performed, by the use of sources such as citric fruit seeds (Luzia and Jorge, 2013), spices (Hossain et al., 2010), and medicinal plants (Krishnaiah et al., 2011).

Spices are natural products that have been used not only as flavoring and coloring agents, but also as food additives, as well as in popular medicine, and all over the world for thousands of years. Many spices have also been recognized due to their digestive, carminative, antibacterial, anti-inflammatory, antimutagenic actions, their anticancer potential, etc (Ceylan and Fung, 2004; Srinivasan, 2005). Spices are important antioxidant sources in daily intake, especially in food cultures in which they are regularly used in the form of fresh, dried, or dehydrated herbs (Carlsen et al., 2010).

The antioxidant potential of spices is due to the presence of certain phytochemicals, generically named as phenolic compounds,

such as rosmarinic and caffeic acids in rosemary, salvia, oregano and thyme (Pukalskas et al., 2005; Shan et al., 2005; Hashemi and Haghighi, 2018).

Basil (*Ocimum basilicum* L.), which is originated from Southeastern Asia and Central Africa, is an aromatic herb that is traditionally used in the treatment of headaches, diarrhea, constipation, moles, worms, and renal disorders (Simon et al., 1999). It is also source of aroma and essential oils which contain biologically active compounds with antimicrobial properties (Wannissorn et al., 2005). Oregano (*Origanum vulgare* L.) is one of the most popular seasonings in the world, with highly favorable flavor for consumers; it is widely used for its antimicrobial and antioxidant properties. Thyme (*Thymus vulgaris* L.), which has its main center of dispersion in the Mediterranean region, is a plant that is distributed all over the world. It has commonly been used as spice in cooking, in order to add flavor to dishes (Yanishlieva et al., 2006).

Although literature shows antioxidant properties of spice extracts, there is no information that describes the extract efficiency in soybean oil oxidative stability. Thus, the aim of this study was to evaluate the spice extract efficiency in soybean oil stability regarding oxidative deterioration during heating.

2. Materials and methods

2.1. Materials

2.1.1. Spices

In order to perform the experiments, basil (*Ocimum basilicum* L.), oregano (*Origanum vulgare* L.), and thyme (*Thymus vulgaris* L.), were purchased *in natura*, in the market of São José do Rio Preto–SP, Brazil; damaged leaves, stems, and roots were removed. Then, the spices were subjected to drying in an oven, at 40 °C, for 24 h. After dehydration, the materials were ground and passed in tyler sieve 3.5, with 5.66 mm opening, for obtainment of the extracts.

2.1.2. Oil

In order to perform the experiment, refined soybean oil with no addition of synthetic antioxidants (TBHQ and citric acid) was used. The soybean oil was processed by the company Cargill Agrícola S/A, Uberlândia-MG, Brazil, and came in packages of 900 mL.

2.1.3. Antioxidants

The synthetic antioxidant used was tert-butyl-hydroquinone (50 mg/kg) (TBHQ), presented in the form of powder, provided by the company Danisco S/A, Cotia-SP, Brazil. In order to obtain the thyme, basil, and oregano ethanolic extracts, 25 g of spices were added to 75 mL of ethanol, mixed, under intense agitation, for 30 min. Then, they were filtered in vacuum pump, and the supernatants were separated from the precipitates. The removal of the solvent used for extract obtainment was performed in rotary evaporator, under reduced pressure, at 40 °C, with the purpose of determining, by direct weighing, the extraction yields. Each extract was applied at a concentration of 2,000 mg/kg in soybean oil.

2.1.4. Experimental Essay

Five treatments were subjected to thermoxidation and conducted in two repetitions: i) soybean oil (Control), ii) soybean oil with thyme extract (TE), iii) soybean oil with basil and thyme extracts (Mixture 1), iv) soybean oil with oregano and thyme extracts (Mixture 2), and v) soybean oil with terc-butyl-hydroquinone (TBHQ). In this essay, the treatments were conducted in a heated plate, using 50 mL beakers containing 40 mL of sample, with surface/volume relation of 0.4/cm. This value corresponds to what is normally used in deep frying. The temperature used was 180 °C, which is normally employed in immersion frying. The essay was conducted discontinuously, with 10 h of heating/day; the samples were taken at different periods of time (0, 5, 10, 15, 20, 25, and 30 h). The temperature was controlled with the assistance of a thermometer. All samples, at different time intervals, were collected in amber glass in the presence of inert gas (N_2) and stored at approximate temperature of –18 °C until the moment of analyses.

2.2. Methods

2.2.1. Peroxide Value

Determined in potentiometric titrator (Metrohm Ltd., Herisau, Switzerland), model 793, according to the method Cd 8b–90 (AOCS 2009), expressed as meq O_2/kg.

2.2.2. Conjugated Dienoic Acids

Determined in spectrophotometer (Shimadzu, Chiyoda-ku, Tokyo, Japan), model Uv-Vis mini 1240, according to the method Ti 1A–64 (AOCS, 2009), expressed as %.

2.2.3. Oxidative Stability

Determined in Rancimat (Metrohm Ltd., Herisau, Switzerland), model 743, according to the methodology Cd 12b–92 (AOCS, 2009). Oil samples (3 g) were transferred to reaction tubes and subjected to oxidation at temperature of 100 °C, with 20 L/h airflow. The curve of electrical conductivity *versus* time was automatically registered during the oxidation reaction and the induction period was expressed in h.

2.2.4. Total Polar Compounds

Determined by the chromatographic method proposed by Dobarganes et al. (2000). The level of polar compounds, polar fraction, was calculated regarding the unaltered triacylglycerols, considering that the retained polar compounds were included in the polar fraction. The results, obtained through column chromatography, were expressed as percentage.

2.2.5. Fatty Acids

Determined by chromatography in gaseous phase, with previous esterification of the samples, according to the procedure described by Ce 2–66 (AOCS, 2009). For fatty acid determination, the method Ce 1–62 (AOCS, 2009) was used, with adaptations. The analysis was performed in CG (Varian Inc., Walnut Creek, CA, USA), model 3900, with flame ionization detector, *split* injector, and automatic sampler. Analysis conditions: fused silica capillary column (CP–Sil 88, Microsorb, Varian Inc., Walnut Creek, CA, USA) of 60 m length, with 0.25 mm internal diameter and 0.20 μm film thickness. The programming of the column temperature was initiated at 90 °C, for 4 min, heated at 10C/min until 195 °C, and kept isothermal for 16 min. The temperatures used in the injector and detector were 230 and 250 °C, respectively. The carrier gas was hydrogen with 30 mL/min linear speed. The fatty acids were identified by comparison of retention times of fatty acid methyl ester pure standards with separated sample components and the quantification was performed by area normalization. A mixture compound of 37 fatty acid methyl esters was used as standard (Supelco, Bellefonte, USA) from C4:0 to C24:1, with purity between 99.1 and 99.9%.

2.2.6. Triacylglycerol Composition

Determined by using the computer program developed by Antoniosi Filho and co-workers (1995), which considers the fatty acid composition of the samples.

2.3. Experimental Delineation

The experiment was performed in factorial scheme, in completely randomized delineation. Variance analysis and Tukey test at 5% were obtained by the program ESTAT – System for Statistical Analyses, version 2.0.

3.Results and discussions

The evaluation of oxidation parameters represents the difference between formation and decomposition of peroxides, primary oxidation products, colorless and odorless (Silva and Jorge, 2014).

In Table 1, during heating, an oscillation of peroxides is observed in the treatments, due to their formation and degradation in lipid oxidation secondary compounds. In the final heating time, it is observed that the antioxidants did not present significant difference concerning the peroxide formation, when compared with Control. According to Dysseler and Dieffenbacher (2000), since peroxides decompose at 150 °C, it is difficult to follow their accumulation during the heating times.

When fatty acids of polyunsaturated oils are

oxidized, especially linoleic and linolenic acids, conjugated dienes are formed. This conjugated structure highly absorbs ultraviolet light in the wavelength between 232 and 234 nm (Kulas and Ackman, 2001).

In Table 1, it can be noticed, concerning heating times for each treatment, that the levels of conjugated dienoic acids increased gradually, during the thermoxidation process, from the beginning to the end of heating, from 0.3 to 3.3% in Control, from 0.3 to 2.5% in TE, from 0.3 to 2.6% in Mixture 1, from 0.3 to 2.6% in Mixture 2, and from 0.3 to 2.9% in TBHQ.

In the treatments inside each heating time, it is verified that, initially, there was no significant difference of diene levels in all the treatments. However, after heating was initiated, the natural antioxidants presented higher antioxidant action, differing significantly from Control and from TBHQ, which presented, in the time of 30 h, reduction of 24.1%, 19.8%, and 21.0% in the formation of conjugated dienes, respectively, in TE, Mixture 1, and Mixture 2.

The oxidative stability is defined as the time to reach detectable rancidity level or change in the oxidation rate and is directly influenced by the presence of antioxidant which, in its turn, suffers the action of high temperatures. It is observed, in Table 1, that during heating, soybean oil had severe decrease in the induction period, which was expected, since it is free from antioxidant addition and since it only contains antioxidants that are naturally present, such as tocopherols. Nevertheless, soybean oil added TBHQ also presented high oxidative stability loss, while the spice extracts, especially thyme, were more efficient in the protection of oil against oxidation.

Table 1. Influence of antioxidants on the analytical measures performed in soybean oil during heating at 180ºC.

Treatments	Heating times (h)						
	0	5	10	15	20	25	30
Peroxide value (meq/kg)							
Control	0.7 ± 0.0^{dB}	4.1 ± 0.4^{bC}	2.0 ± 0.0^{cdC}	6.2 ± 0.0^{aA}	2.9 ± 0.3^{bcA}	3.9 ± 0.0^{bcA}	2.5 ± 0.0^{bcdA}
TE	1.6 ± 0.2^{cAB}	10.5 ± 0.6^{aA}	3.3 ± 0.5^{bcBC}	3.9 ± 0.5^{bBC}	3.7 ± 0.3^{bA}	4.1 ± 0.0^{bA}	3.6 ± 0.0^{bcA}
Mixture 1	2.1 ± 0.0^{dAB}	7.3 ± 0.4^{aB}	5.5 ± 0.3^{abA}	5.0 ± 0.9^{bcAB}	3.4 ± 0.2^{cdA}	4.0 ± 0.1^{bcdA}	3.7 ± 0.2^{bcdA}
Mixture 2	2.7 ± 0.0^{cA}	7.1 ± 0.1^{aB}	4.3 ± 0.5^{bcAB}	6.1 ± 0.1^{abA}	4.1 ± 0.1^{cA}	4.5 ± 0.0^{bcA}	4.1 ± 0.2^{cA}
TBHQ	0.8 ± 0.0^{cB}	6.1 ± 0.3^{aB}	1.8 ± 0.0^{bcC}	3.2 ± 0.0^{bC}	2.6 ± 0.9^{bcA}	1.9 ± 0.0^{bcB}	2.5 ± 0.0^{bcA}
Conjugated dienoic acids (%)							
Control	0.3 ± 0.0^{gA}	1.7 ± 0.0^{fA}	2.3 ± 0.0^{eA}	2.5 ± 0.0^{dA}	2.8 ± 0.0^{cA}	2.9 ± 0.1^{bA}	3.3 ± 0.0^{aA}
TE	0.3 ± 0.0^{eA}	0.9 ± 0.0^{dD}	1.7 ± 0.0^{cB}	2.2 ± 0.0^{bB}	2.3 ± 0.0^{bBC}	2.4 ± 0.0^{abD}	2.5 ± 0.0^{aC}
Mixture 1	0.3 ± 0.0^{fA}	1.1 ± 0.0^{eC}	1.4 ± 0.0^{dC}	2.1 ± 0.0^{cC}	2.2 ± 0.0^{bC}	$2.0 \pm 0,0^{aBC}$	2.6 ± 0.0^{aC}
Mixture 2	0.3 ± 0.0^{eA}	1.0 ± 0.0^{dCD}	1.2 ± 0.0^{dD}	2.2 ± 0.0^{cBC}	2.4 ± 0.0^{bB}	2.5 ± 0.0^{abCD}	2.6 ± 0.0^{aC}
TBHQ	0.3 ± 0.0^{eA}	1.6 ± 0.0^{dB}	2.3 ± 0.0^{cA}	2.5 ± 0.0^{bA}	2.6 ± 0.0^{bA}	2.7 ± 0.0^{bB}	2.9 ± 0.0^{aB}
Oxidative stability (h)							
Control	15.6 ± 0.0^{aB}	8.1 ± 0.0^{bB}	7.4 ± 0.0^{bcB}	6.8 ± 0.4^{cBC}	3.0 ± 0.0^{dC}	2.5 ± 0.0^{dC}	2.3 ± 0.1^{dD}
TE	16.4 ± 0.0^{aB}	10.1 ± 0.0^{bA}	9.3 ± 0.0^{bA}	9.0 ± 0.5^{bA}	7.3 ± 0.2^{cA}	6.0 ± 0.2^{dA}	5.7 ± 0.1^{dA}
Mixture 1	16.5 ± 0.2^{aB}	10.3 ± 0.1^{bA}	10.0 ± 0.0^{bA}	6.4 ± 0.2^{cC}	6.6 ± 0.2^{cdAB}	5.3 ± 0.1^{dAB}	3.9 ± 0.1^{bE}
Mixture 2	16.3 ± 0.1^{aB}	10.5 ± 0.2^{bA}	10.0 ± 0.0^{bA}	7.8 ± 0.0^{cB}	6.1 ± 0.2^{dB}	4.8 ± 0.1^{eB}	3.7 ± 0.4^{eBC}
TBHQ	22.6 ± 0.0^{aA}	8.3 ± 0.0^{bB}	7.7 ± 0.1^{bB}	3.8 ± 0.0^{cD}	3.4 ± 0.0^{cC}	3.0 ± 0.0^{cC}	2.6 ± 0.0^{cCD}
Total polar compounds (%)							
Control	4.4 ± 0.0^{gA}	18.9 ± 0.0^{fA}	23.3 ± 0.0^{eA}	38.1 ± 0.1^{dA}	40.7 ± 0.4^{cA}	45.1 ± 0.3^{bA}	57.3 ± 0.0^{aA}
TE	4.2 ± 0.0^{gA}	10.7 ± 0.6^{fC}	14.4 ± 0.4^{eC}	24.1 ± 0.0^{dE}	26.1 ± 0.1^{cE}	27.8 ± 0.0^{bE}	30.8 ± 0.2^{aE}
Mixture 1	5.4 ± 0.1^{gA}	14.8 ± 0.6^{fB}	19.3 ± 0.0^{eB}	32.3 ± 0.2^{dC}	35.3 ± 0.1^{cC}	39.3 ± 0.1^{bC}	42.8 ± 0.2^{aC}
Mixture 2	5.2 ± 0.0^{gA}	9.6 ± 0.0^{fC}	11.3 ± 0.1^{eD}	29.3 ± 0.6^{dD}	32.1 ± 0.0^{cD}	35.5 ± 0.4^{bD}	39.0 ± 0.3^{aD}
TBHQ	5.4 ± 0.1^{gA}	16.1 ± 0.1^{fB}	20.2 ± 0.0^{eB}	34.5 ± 0.2^{dB}	37.0 ± 0.2^{cB}	40.7 ± 0.3^{bB}	52.9 ± 0.2^{aB}

Control: soybean oil; TE: soybean oil with thyme extract; Mixture 1: soybean oil with thyme and basil extracts; Mixture 2: soybean oil with thyme and oregano extracts; TBHQ: soybean oil with tert-butyl-hydroquinone.
a, b...(line) and A, B…(column): means followed by the same letter do not differ by Tukey test ($p > 0,05$).

Until 10 h, it can be seen in Table 1 that, TE and Mixtures 1 and 2 did not present significant difference and, from 15 h on, TE showed higher stability until final time, thus, not presenting synergism among the spice extracts.

In a study performed by Ramalho and Jorge (2008), the addition of rosemary extract to soybean oil, under heating at 180 °C, for 10 h, presented higher stability to oil when compared with the addition of synthetic antioxidants. In the present study, the results obtained regarding the oxidative stability indicated that the antioxidant capacity of spice extracts was higher, when compared with the synthetic antioxidant TBHQ after the beginning of heating.

The determination of the total quantity of alteration products, originated as a consequence of high temperature processes, constitutes the base for oil use limitation in some countries, established around 24–27% of polar compounds (Firestone et al., 1991). Table 1 shows that there was an increase in the value of total polar compounds, in all treatments, with the increase of heating times. Control ranged from 4.4 to 57.3%, TE from 4.2 to 30.8%, Mixture 1 from 5.4 to 42.8%, Mixture 2 from 5.2 to 39.0%, and TBHQ from 5.4 to 52.9%.

In the treatments inside each heating time, it is verified that, at initial time, there was no significant difference in the levels of polar compounds in all treatments. It is noticed that, in the final time, at 30 h of heating, TE (30.8%) presented higher protection to soybean oil, retarding the formation of polar compounds in 46.2% and differing significantly from all the other treatments.

In Brazil, there is no legislation that establishes a maximum limit for polar compound value in oils heated in high temperatures. After 20 h, at 180 °C, all treatments exceeded 25%, maximum limit recommended by International Legislation.

It can be observed in Table 2 that the values of fatty acid composition, in the initial oils found in this study, are close to those reported by Gunstone (1996), who establishes values of 15, 22, and 61% of saturated, C18:1, and C18:2 + C18:3 fatty acids, respectively, for soybean oil.

When evaluating the means of fatty acid composition in the treatments during the heating process, a significant difference was verified ($p < 0,05$), with an increase in the percentage of saturated and C18:1 fatty acid, and a decrease in the quantity of C18:2 + C18:3, which are considered essential fatty acids.

Table 2. Means of saturated and C18:2 + C18:3 fatty acids in soybean oil during heating at 180°C.

Treatments	Heating times (h)		
	0	15	30
Saturated (%)			
Control	13.6^{cE}	16.3^{bB}	17.3^{aB}
TE	14.1^{cA}	16.2^{bC}	16.4^{aD}
Mixture 1	13.9^{cB}	16.2^{bB}	17.3^{aB}
Mixture 2	13.8^{cC}	16.0^{bD}	16.9^{aC}
TBHQ	13.7^{cD}	16.9^{bA}	19.0^{aA}
C18:1 (%)			
Control	27.1^{cAB}	29.0^{bD}	30.1^{aC}
TE	26.9^{cC}	28.8^{bE}	29.3^{aE}
Mixture 1	27.2^{cA}	29.3^{bB}	30.5^{aB}
Mixture 2	27.0^{cC}	29.4^{bA}	29.8^{aD}
TBHQ	27.1^{cB}	29.2^{bC}	31.9^{aA}
C18:2 + C18:3 (%)			
Control	58.3^{aA}	54.1^{bB}	51.7^{cC}
TE	57.8^{aC}	54.4^{bA}	53.7^{cA}
Mixture 1	57.8^{aC}	53.9^{bD}	51.3^{cD}
Mixture 2	58.2^{aB}	54.0^{bC}	52.6^{cB}
TBHQ	58.2^{aB}	53.1^{bE}	47.8^{cE}

Control: soybean oil; TE: soybean oil with thyme extract; Mixture 1: soybean oil with thyme and basil extracts; Mixture 2: soybean oil with thyme and oregano extracts; TBHQ: soybean oil with tert-butyl-hydroquinone.
a, b...(line) and A, B…(column): means followed by the same letter do not differ by Tukey test ($p > 0,05$).

Thus, Table 2 shows that, in the end of the heating process, the percentages of saturated fatty acids increased in 27.7, 16.3, 24.5, 23.0, and 39.2% in Control, TE, Mixture 1, Mixture 2, and TBHQ, respectively. The same behavior was observed in C18:1 fatty acid, resulting in an 11.0% increase in Control, 8.8% in TE, 12.2% in Mixture 1, 10.5% in Mixture 2, and

18.0% in TBHQ. Concerning essential fatty acids, there was a decrease of linoleic and linolenic acids in higher percentages in TBHQ (17.7%), followed by Mixture 1 (11.3%), Control (11.3%), Mixture 2 (9.5%), and TE (7.2%).

According to Pantzaris (1998), the decrease in linoleic and linolenic acids content during the heating process is due to their destruction by oxidation, polymerization, among other factors, and should, thus, be an important quality test for oils.

With the results, it is possible to consider that, regardless of the treatments studied, there was an increase of the saturated and monounsaturated fatty acids.

Oils and fats are considered as complex samples, due to the great number of different triacylglycerols that form them. Therefore, triacylglycerol identification is a difficult process, in which the number of possible structural forms is high, compared with the number of present fatty acids.

Table 3 shows the main individual triacylglycerols composition, classified according to the number of carbons.

Table 3. Main triglycerides composition (%) in soybean oil added thyme extract before and after heating at 180 °C.

Triglycerides	Heating times (h)	
	0	30
C50		
POP	1.1	1.5
PLP	2.2	2.6
C52		
PLS	1.2	1.4
POO	2.6	3.5
PLO	10.5	12.1
PLL	10.5	10.5
PLaL	1.6	1.2
C54		
SLO	2.8	3.2
OOO	2.1	2.7
SLL	2.8	2.8
OLO	12.7	14.3
OLL	25.4	24.8
OLaL	3.8	2.8
LLL	17.0	14.3
LLaL	3.8	2.5

P: palmitic acid; S: stearic; O: oleic; L: linoleic; La: α-linolenic acid.

For the determination of this composition, the main triacylglycerols represent the components of higher concentrations in the isomer, in which the number of carbons ranged from 50 to 54. Due to the high level of soybean oil unsaturation, the main species of each group are the ones that have two or three unsaturated acids as major. Therefore, a significant number of unsaturated fatty acids is found in the composition of the main triacylglycerols.

4. Conclusions

The results show that the application of natural antioxidants avoids lipid oxidation during heating processes, making possible the effect on the results found of synergism or antagonism among the antioxidants added to soybean oil. Thyme and mixtures extract in the 2,000 mg/kg concentration are efficient, when compared with the synthetic antioxidant, TBHQ, stabilization, in the 50 mg/kg concentration, used by vegetable oil industries. The extracts improve soybean oil resistance against the deteriorative thermal alterations and, therefore, the spice extracts may be recommended as sources of powerful antioxidants for stabilization of food systems, especially in unsaturated vegetable oils.

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IDENTIFICATION PARAMETERS FOR COMPARISON OF NATURALLY AND COMMERCIALLY LIME JUICE

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ABSTRACT

Public consumption of lemon and lime juice and its best-selling market induces jobber to carry out numerous adulterations in order to reduce production costs and thereby endangering people's health and create numerous problems for the relevant regulatory authorities. It seems the previous proposed parameters are not suitable and reliable for detection of lime juice adulteration. In this regards, several important parameters as cations, anions, citric acid and isocitric acid were studied for distinguish between naturally and commercially Persian lime juices. For this purposed, various cations (Li^+, Na^+,$NH4^+$, K^+,Ca^{2+} and Mg^{2+}) and anion (F^-, Cl^-, NO_2, Br^-, NO_3^-, $PO4^{3-}$ and SO_3^{2-}) were investigated in naturally and commercially lime juice using Ion chromatography system equipped with Suppressed conductivity detector. Citric acid and isocitric acid were determined and quantified using HPLC–UV detection. After optimization the method, linear calibration curves were plotted. The average recoveries of the analytes were higher than 72%. Our results showed significant difference in cations (Li^+, Na^+ and K^+) and anion (Cl^-) between natural and commercial lime juice samples. The citric acid: isocitric acid ratio was found with a mean of 280 ±86 in natural products, while this ratio was found with a mean of 503 ±149 in commercial products using HPLC method. This result shows that this method would be useful for determining of routine adulteration in food control laboratories. This is the first study for showing of real difference between natural and commercial difference in lime juice consumed by Iranian population.

1. Introduction

Lime is an evergreen hybrid fruit plant that belongs to the citrus family and contains acidic juice vesicles(Ikeda and Spitler 1964). There are various species of citrus trees whose fruits are called limes. Kaffir lime, West Indian lime, Persian lime, desert lime and sweet lime are some of the most common types of lime that vary in size, color and flavor(Liu *et al.* 2012; Saeidi *et al.* 2011). Among citrus fruits, limes are excellent sources of important nutrients including vitamin C and folate(Scherer *et al.* 2012).They also contain dietary fiber, and other bioactive components such as carotenoids and flavonoids, organic acid (e.g. citric acid) and mineral elements (e.g. potassium) involved in numerous health promoting properties(Silalahi 2002). Inorganic elements are vital and essential compounds to public health similar to vitamins and amino acids(Giacomo *et al.* 1974). As

minerals need for regulating and building the body cells, transfer of chemical substances in and out of the cells, controlling in the composition of body fluid and cells(Lorente *et al.* 2014; Silalahi 2002). In Iran, limes are used, occasionally as a fresh fruit; generally, lime wedges and lime juice are served as a garnish to salads, or Iranian dishes (Saeidi *et al.* 2011).

Lime juice is one of the most popular flavors in Iran(Saeidi *et al.* 2011). Although most Iranians tended to use homemade lime juice in the past, but nowadays, commercially lime juice consumption is on the rise(Lorente *et al.* 2014). Popularity and the cost of the lime juice make it as target for adulteration(áJohn Dennis 1998). Food adulteration is committed in different ways including combining food with cheaper ingredients, concealing the quality, selling rotten food, replacing authentic ingredients with other ingredients and adding chemicals. Some forms of food adulteration are harmful like using industrial coloring and chemicals which put human health at risk such as tooth enamel damage and gastrointestinal symptoms(áJohn Dennis 1998; Álvarez *et al.* 2014; Lorente *et al.* 2014; Scherer *et al.* 2012; Uçan *et al.* 2016; Yamamoto *et al.* 2008).

Food adulteration in different parts of the world varies depend on geographic region, economic condition and social culture such as the other fruit juice, incidence of lime juice adulteration can be achieved easily through the addition of water, citric acid, colorants or other cheaper fruit juices or ingredients to the original fruit juice (Lorente *et al.* 2014; Muntean 2010; Scherer *et al.* 2012; Uçan *et al.* 2016).

It is known that the chemical composition of fruits such as lemon juice is variable according to many factors such as the variety of fruit, ripening stage, geographical location and horticultural practices. It is also known that in industry, the juices are commonly submitted to many legal processing practices such as thermal treatment, adding organic acids (generally citric acid) or sugar to adjust the flavor of the final product. The previous industrial practices can also change the chemical composition of the final product. Thus, we cannot consider all the types of difference in the chemical composition of the sample as an adulteration(Cautela *et al.* 2008; Fügel *et al.* 2005).

It was recently reported that the quantification and characterization of organic acids appear to be useful in estimating the amount of fruit and controlling the fruit authenticity. However, since organic acids are essential technological ingredients of most recipes, this analytical tool is not evidently applicable to jams and fruit preparations. Furthermore, depending on cultivar and degree of ripeness, organic acid contents are subject to considerable variations, which limits their applicability as a quantitative marker in fruit juices too (Fügel *et al.* 2005).

Unfortunately, sometimes the type of lime juice adulterations in Iran is different from developed countries such as full synthetic products which are harmful for popular health such as adding cheap fillers or additives, hiding food quality, misleading consumers by providing wrong information about the product or mislabeling, selling rotten food, (improper substitution) sometimes substitute harmful material instead of real ingredients(Saeidi *et al.* 2011).

Among regulatory systems, International Fruit Juice Union (IFU) has been specially developed and provided collection of chemical and microbiological methods for several fruit juices authenticity including lime(áJohn Dennis 1998; Cautela *et al.* 2008; Nuncio-Jáuregui *et al.* 2014; Zhang *et al.* 2009). Identification of lime juice adulteration is a complex issue which can be changed by adding a series of frauds via analytical methods, which can often be time-consuming and cost effect(Álvarez *et al.* 2014; Cautela *et al.* 2008; Lorente *et al.* 2014; Muntean 2010; Nuncio-Jáuregui *et al.* 2014; Penniston *et al.* 2008; Robards *et al.* 1997; Saeidi *et al.* 2011; Scherer *et al.* 2012; Uçan *et al.* 2016). Since there is not robustness methods for detection of natural lime juice from commercial products in adulteration, we tested whether (a) is there reliable and suitable inorganic compound to able distinguish between natural and commercial lime juice (b) is the

citric acid: isocitric acid ratio as satisfying and preferable factor between natural and commercial lemon juice difference (c) the acidification of lime juice can be help in comparison of natural from commercial lime juice.

Therefore, it investigated the concentration of mineral element and organic acid in commercial and natural citrus lime juice to find new markers for identification of natural from commercial lime juice products using by Ion Chromatography and High performance liquid chromatography (HPLC) methods.

2. Materials and methods

2.1. Materials

All solutions were prepared using analytical grade reagents and deionized water. Anions (Fluoride, Chloride, Bromide, Nitrite, Nitrate, Phosphate, Sulphite) and also cations (Lithium, Sodium, Potassium, Magnesium, Ammonium, Calcium and other chemicals or solvents were purchased from Merck (Darmstadt, Germany). All sample containers and glassware were thoroughly cleaned with 0.1M HCl solution and then finally with deionized water.

2.2.1. Samples

The appropriate experimental plan in present study had to contain three categories of lemon juice samples: 1) natural sample which prepared from lemon juice samples from dilution of samples with deionized water (1:50 v/v) and filtered (0.45µm) prior to analysis.; 2) Commercial sample which prepared from market collected samples; 3) In-laboratory adulterated lemon juice samples which can be prepared from fruit and different adulteration materials. Unfortunately, due to the lack of information on the preparation of adulteration materials which used in commercial lemon juice and following of persons which informed the percentage of fruit and different adulteration materials, it had not possible to investigate the third category of samples and omitted the difference in the chemical composition found between natural and commercial samples is related to the adulteration practices. Therefore, we decided to investigate only 2 categories of lemon juice samples in our investigation. The twenty samples (10 naturally lime juice compared to 10 commercial lime juices) were collected randomly from local supermarkets and convenient stores in different provinces of Iran (Dezphol, Astara, Jahrom, Lar, Bandarabas, Shiraz, Mazandaran, Zanjan, Tehran and etc). All the samples were stored at 4ºC until analysis.

2.2.2. Apparatus:

The 881 Compact Ion chromatography system (Metrohm, Switzerland) equipped with Suppressed conductivity detector with Metrohm suppressor Module (MSM, 50 mmol/L H_2SO_4). The MagIC net (version 2.3) software was used for monitoring system and data analysis. The ultrasonic bath was provided by Powersonic 405 from Daihan Lab Tech CO (Nanyangiu, Korea). Anion column metrosep A Supp 4 (size 250×4.0, pressure 8 M Pa, cond 2 µs/cm) and cationic column metrosep C 4 (150×4.0) , pressure7/5 M Pa ,cond 900 µs/cm) was used for measurement of anions and cations involved in our samples.

A high-performance liquid chromatography system equipped with auto sampler (Waters 717), binary HPLC pump (Waters 1525), and a dual λ absorbance UV detector (Waters 2487) was used for the analysis. The reverse-phase column was a Waters Capital 40 form (20+20) cm×4.6 µm, 4 µm particle size (Waters, Milford, MA, USA) at 30 °C. The mobile phase was composed of the KH_2PO_4 buffer (0.01M) that was filtered through a 0.45 mm membrane and degassed by a sonication process before use. The flow rate was 0.6 ml min^{-1}. The UV detector operated at wavelength of 270 nm.

The injection volume for both standards and samples was 100 µl. The run time and retention times for citric acid and isocitric acid were 23.83±0.16 min and 15.52±0.02 min, respectively. To evaluate the reliability of the results, in addition to applying the common validation assessment to the developed method, internal quality control experiments were also performed. Each working day, a blank and a spiked sample were also analyzed. Samples were spiked with citric acid and isocitric acid

concentrations at desired levels and according to the recovery values, the results of citric acid and isocitric acid in the real samples were corrected.

2.2.3. Standard, sample and solvent preparation

Ion stock standard solutions were prepared in deionized water weekly and then these standards were used to prepare mixed standard for analysis. Working standard solutions were prepared daily by diluting the mix standard with deionized water. As sample was clear, no extraction and cleanup was needed .The samples were diluted 1:50 with deionized water to obtain a final volume of 50 ml and filtered (0.45µm) prior to analysis. Mobile phase for determination of anions was made up of sodium hydrogen carbonate (1.7 mmol.L^{-1}), sodium carbonate (1.8 mmol.L^{-1}) and sulfuric acid (1.8 mmol.L^{-1}) and sulfuric acid (50 mmol.L^{-1}) with a flow rate of 1 ml/min. Selective mobile phase for cations was made up of nitric acid (1.7 mmol.L^{-1}) and Dipicolinic acid (0.7 mmol.L^{-1}) with a flow rate of 0.9 ml.$min^{-1.}$

Citric acid and isocitric acid were determined and quantified using high-performance liquid chromatography chromatography–UV detection. This method has already been validated by Faroogh Life Sciences Research Laboratory as a part of National Research Project under Institute of Standards & Industrial Research of Iran and in due course after successful validation by Inter-laboratory Comparison mean shall be published as Iranian National Standard. Briefly, Sample preparation was done with adding of 50 µl lime juice and 50µl internal standard (formic acid and acetic acid was used an internal standard (2 mg.ml^{-1}). and diluted with water (3ml) and finally filtered (0.45 µm) and degassed by a sonication process prior to analysis. Mobile phase for determination of citric acid and isocitric acid were KH_2PO_4 (0.01 M) with a flow rate of 0.6 ml/min at 30°Cwith UV detector at wavelength of 270 nm.

2.2.4. Validation Procedure

The validation process was done in suggested parameters: *1. System suitability:* The relative standard deviation (RSD) ≤3% from 5 consecutive injections of standard samples in different days. *2. Linearity:* Employing five concentration levels for establishing of standard calibration curves with high value of the regression coefficient (R^2>0.99).*3.Precision and accuracy*: based on three consecutive injections of ions at five desired concentration levels (intra-day and inter-day) via determining of recovery. *4. Limits of detection (LOD) and quantification (LOQ):* The LOD and LOQ were estimated by signal-to-noise ratio, 3:1 and 10:1, respectively in samples.

2.3. Statistical Analysis

All statistical analyses were performed using the SPSS software (Window version 18) and Excel 2007 software. Results were presented as means ± SD. Assays were performed in triplicate and the probability. Mann-Whitney test was applied for determining of significance in comparisons of two groups. The confidence level required for significance was set at P<0.05.

3. Results and discussions

Adulteration of fruit juice is widespread issue which is become from 20-30 years ago due to no policing and economic reasons(áJohn Dennis 1998; Zhang *et al.* 2009). Besides, detection of fruit juice adulteration is very complicated; application of powerful method is needed. Unfortunately, there were no precise ingredient percentages of lemon juice for determination of natural products from adulterated samples due to relation of adulteration practices to geographical and biological effects(Fügel *et al.* 2005; Liu *et al.* 2012). On the other hands, we couldn't find any informed person which provide any data about the percentage and composition of lemon juice adulteration in present study or similar investigation.

Also, the chemical composition of limes affected by the variety of lime, ripening stage, geographical location and horticultural

practices. Together, the limes in industry tolerated thermal treatment and adding of organic acids (especially citric acid) or sugar to adjust the flavor of the final product which changes the chemical composition (Ikeda and Spitler 1964; Lorente *et al.* 2014). Due to higher amount of citric acid in lime or fruit juice, determining of this organic acid singly is not suitable marker in determination of adulteration. But, the recent data suggested that isocitric acid which can be found in low concentration in lime juice produced by conversion of citric acid by aconitase and isocitrate dehydrogenase enzyme. Therefore, the ratio citric/isocitric acid could be serves as a reference index of authenticity using application of powerful method.

Therefore, it decided usage of ion chromatography as an effective tool in identifying the type of cations and anions that are readily available in common products and HPLC methods for identification of citric acid and isocitric acid as another confirmation criterion in distinguish of natural lime juice from commercially products. Our study showed type and concentration of specific minerals and organic acids found in fruit juice products are important profiles in identifying of lime juice adulteration in Iran which continue to be discussed.

Table 1. Determination of calibration curve range, LOQ, LOD and retention time of cations and anions parameters in lime juices

Ions	Concentration range(ppm)	LOD	LOQ	RT (min)	Recovery for LL[a]±RSD%	Recovery for HL[b]±RSD%
Cations						
Li	0.0625-1	0.02	0.0625	1.52	99±0.8	90±7.3
Na	0.3125-5	0.1	0.3125	2.4	99±6.4	83±3.4
NH_4	0.3125-5	0.1	0.3125	2.9	103±4	89±10
K	1.25-20	0.4	1.25	4.35	99±1.7	102±6.04
Ca	0.125-2	0.2	0.125	11.18	100±0.1	100±9.1
Mg	0.625-10	0.2	0.625	12.76	91±1.1	74±0.09
Anions						
F	1-16	0.3	1	3.88	99±0.2	102+3.8
Cl	1-24	0.5	1.5	4.95	98±1.08	91±3.2
NO_2	1-24	0.5	1.75	5.53	102±0.09	73±1.2
Br	1.25-20	0.4	1.25	6.48	91±5.8	92±5.2
NO_3	2.5-40	0.8	2.5	7.07	101±0.2	102±4.02
PO_4	5-80	2	5	10.19	87±3.5	103±5.8
SO_3	1.25-20	0.7	2	11.64	96±2.5	99±1.9

[a] LL-Lowest validation level ;[b]HL-highest validation level

3.1. Analytical method performance in IC

An external calibration curve was constructed using 5 standards for each of cations (Li^+, Na^+,$NH4^+$, K^+ ,Ca^{2+} and Mg^{2+}) and anion (F^-, Cl^-, NO2, Br^-, $NO3^-$, $PO4^{3-}$and $SO3^{2-}$) in the satisfied range. The calibration curve was linear in determined concentration range with a correlation coefficient (R^2) greater than 0.99. The relative standard deviation (RSD) for each concentration examined was less than 5% in standard samples. The LOD and LOQ based on the signal-to-noise ratio were achieved and shown in Table.1. The recovery experiments were performed by spiking the blank samples at five determined levels based on standard concentration. This experiment was carried out

with three replicates at each level. The results of the recovery experiments were between 90% in many of spiked samples except Na and Mg in spiked levels (0.3125 and 0.625 ppm) (Table.2). The RSD range of recoveries was less than 12% (Table.3). The chromatogram of mixed standard solution for anions and cations were shown in Figure.1A and Figure.1B. Retention time of lithium, sodium, ammonium, potassium, calcium and magnesium in the spiked samples were at 1.52, 2.40, 2.90, 4.35, 11.18 and 12.76 min, respectively. Retention time for anions including fluoride, chloride, nitrite, bromide, nitrate, phosphate and solphite were 3.88, 4.95, 5.53, 6.48, 7.07, 10.19 and 11.64 min, respectively. The chromatograms of standards (cations and anions), naturally and commercially lime juice (blank or real sample) and spiked sample in naturally samples are shown in Figure.1 (A to G).

Table.2. Level of cations and anions concentration in real and commercial lime juice Samples

Ions amount (ppm) in real samples (Mean ±SD)			
Cations	Natural samples	Commercially samples	P value-significant
Li	4.43±2.6 ***	ND	**P<0.001**
Na	46.5±43.2**	701.28±625.8	P=0.004; **P<0.01**
NH_4	143.6±63.6	272.6±267.8	P=0.156; P>0.05
K	191±54.1*	103.2±83	P=0.012; **P<0.05**
Ca	42.7±28.6	25.8±24.6	P=0.174; P>0.05
Mg	6±4.8	6±6.2	P=1; P>0.05
Anions			
F	79.4±41.5	55.2±40.11	P=0.201; P>0.05
Cl	104±41*	322.7±254.7	P=0.015; **P<0.05**
NO_2	2.3±2.03*	ND	P=0.027; **P<0.05**
Br	13.8±23.4	5.8±14.7	P=0.372; P>0.05
NO_3	60.5±50.1	38.3±25.5	P=0.228; P>0.05
PO_4	1153.5±1026.2*	298.7±347.8	P=0.023; **P<0.05**
SO_3	133.7±240.4	611.5±968.3	P=0.147; P>0.05

n=10 * Compared with between groups ND: Non Detected

Table 3. Determination of calibration curve range, LOQ, LOD and retention time of citric acid, isocitric acid, formic acid & acetic acid in lime juices

Organic acid	**Concentration range ($mg.ml^{-1}$)**	**LOQ**	**LOD**	**Retention time (Mean±SD)**
Citric acid	0.125-1.5	40	12.5	23.83±0.16
Isocitric acid	0.00125-0.05	0.5	0.017	15.52±0.02
Formic acid	2	---	---	13.19±0.01
Acetic acid	2	---	---	18.79±0.01

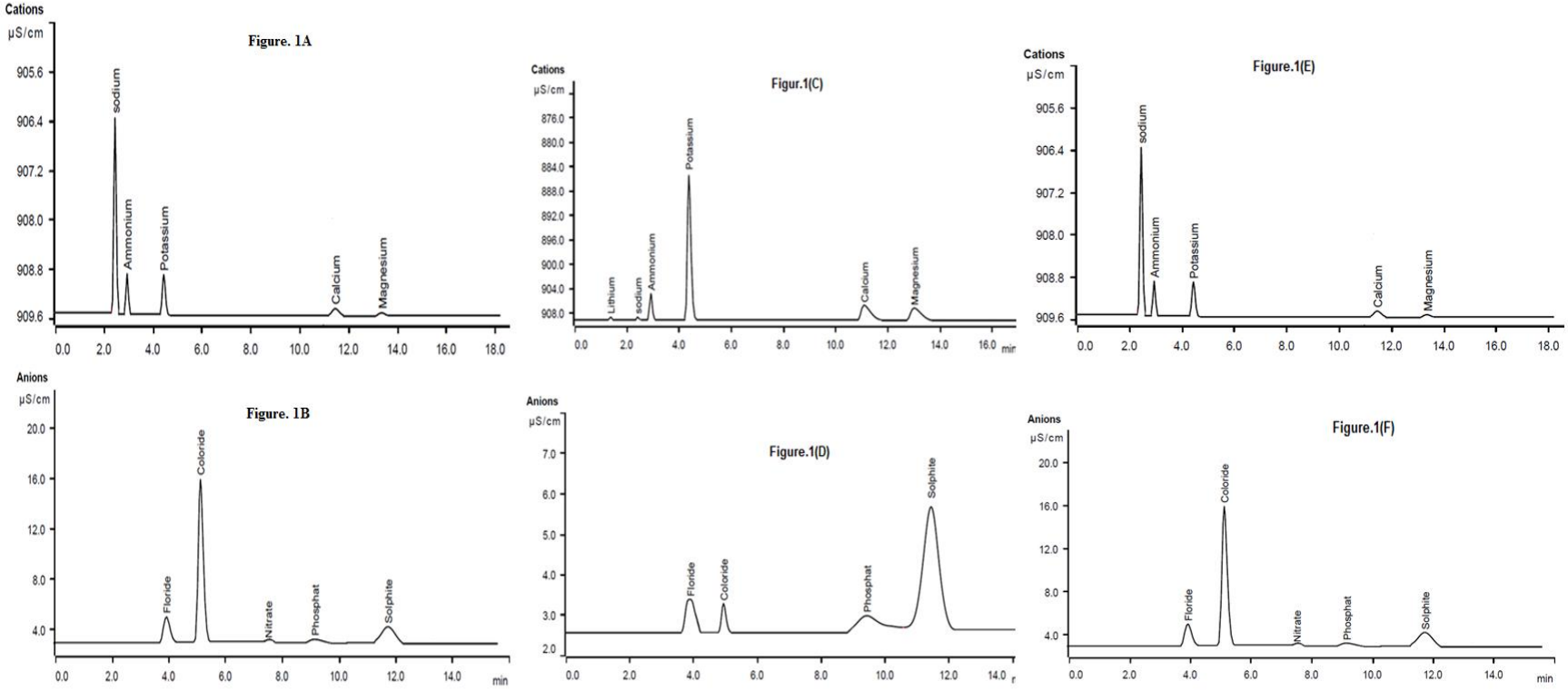
Table.4. Level of Citric acid, Isocitric acid concentration and Citric: isocitric acid ratio in real and commercial lime juice samples

Lemon juice Samples	**Citric acid(mg/ml)**	**Isocitric acid(mg/ml)**	**Citric/isocitric acid**
Natural	69.7±4.09	0.273±0.09	279.93±85.85
Commercial	67.02±5.52	0.143±0.03***	503.3±148.55***

*** Compared with between groups

Figure.1. IC Chromatograms of a:

A. Cation Standard including Li (0.5 ppm), Na (2.5 ppm), NH4 (2.5 ppm), K(10 ppm), Ca(1 ppm) and Mg(5 ppm)
B. Anion Standard including F (8 ppm), Cl (12 ppm), NO_2 (14 ppm), Br (10 ppm), NO_3 (20ppm), PO_4(40 ppm) and Mg(10 ppm)
C. Cations in real or blank sample in natural lime Juice
D. Anions in real or blank sample in natural lime Juice
E. Cations in real or blank sample in Commercially lime juice
F. Anions in real or blank sample in Commercially lime juice
G. Spiked Sample



3.2. Amount of ions in lime juice

The obtained results of ions (cations and anions) concentrations in naturally fresh and commercially lime juice are shown in Table 4. Our finding indicated that the investigated anions (Li, Na, $NH4^+$, Ca, K, Mg) and cations (F, Cl, NO_2, Br, NO_3, PO_4, SO_3) were detected in all of the naturally fresh lime juice samples. The t-test analysis showed that there was significant difference ($p<0.05$) between mean levels of the lithium, sodium, potassium and chloride ions. Besides, our results confirmed that lithium and nitrite were found in all of natural samples without no observation in all commercial lime juice samples ($P<0.05$) as the specific difference between two groups of lime juice samples (natural and commercially).

The level of lithium in all commercial lime juice samples was below the LOD which was found in all of naturally lime juice products in the range of 4.43±2.6 ppm. The lowest and highest level of Lithium in naturally products was observed in the samples collected from Bandarabas (1.41ppm) and Mazandaran (9.99 ppm), (Data not shown). It seems that level of lithium in lime juice can be used as identical marker between naturally and commercial lime juice in Iran.

Based on our results, sodium was detected in all of the analyzed samples (naturally and commercially lime juice. The mean concentration level of sodium in naturally and commercially limes juice samples were 47±43 and 701±625 ppm, respectively (Table.2). The P value less than 0.05 showed the significant difference between means of sodium level in lime juice is another identifiable marker similar to lithium in natural and commercial lime juice adulteration in Iran.

The highest level of sodium in natural and commercial products were observed in the samples collected from Bandarabas (123ppm) and Semnan (1915 ppm), respectively (Data not shown). Also, the highest level of potassium in natural and commercial lime juice were observed in the samples collected from Mazandaran (236ppm) and Esfahan (215ppm) respectively. The highest levels of Chloride in natural and commercial products were observed in the samples collected from Bandarabas (176ppm) and Esfahan (662 ppm) respectively.

Figure.2.(A)

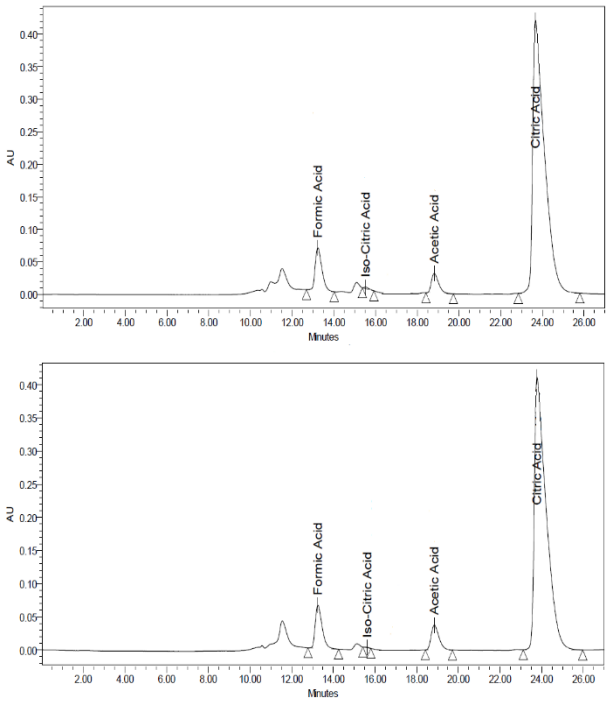


Figure.2.(B)

Figure.2. HPLC Chromatograms of a: Organic acids (citric acid and isocitric acid) in natural lime juice (A) and commercially lime juice (B). Acetic acid (2mg/ml) and acid formic (2mg/ml) were used as internal standard

3.3. Citric: isocitric acid ratio in naturally and commercially lime juice by HPLC methods

Organic acids such as acetate, lactate, citrate and isocitrate are caused by biological activity as good indicator of an old juice that may be too spoiled for consumption. The calibration curve range, LOQ, LOD and retention time of citric acid, isocitric acid, formic acid & acetic acid in lime juices are summarized in Table.3. The average retention times were less than 16 and 24 min for isocitric acid and citric acid, respectively. In this regards, levels of citric acid, isocitric acid and their ratio in the lime juice samples were calculated. In this regards, levels

of citric acid, isocitric acid and their ratio in the lime juice samples were was calculated. Although, the mean levels of citric acid in naturally and commercially lime juice had no significant difference ($p>0.05$), the mean level of isocitric acid and citric acid: isocitric acid ratio in naturally and commercially lime juice were significantly different (Table.5; $P<0.001$). The HPLC chromatograms of the naturally and commercially lime juice samples by HPLC methods are shown in Figure.2.

4. Conclusions

Based on the present study, lime juice samples are significantly different in their chemical properties. Lime juice adulteration can't be detected just by measuring one or several chemical properties. However, LC-MS data that includes both chromatographic and mass spectrometric information, 100% lime juice samples were successfully differentiated from adulterated samples containing 30% lime juice. The findings show that this method allows rapid and accurate monitoring of citrus juices and getting more information on quality and possible adulteration of the product. Similar procedures could be used to monitor other fruit juices and quantitative diverse juice blends.

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INVESTIGATION OF PROPERTIES OF POLYSACCHARIDE-BASED EDIBLE FILM INCORPORATED WITH FUNCTIONAL *MELASTOMA MALABATHRICUM* EXTRACT

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ABSTRACT

Edible film can be used to replace the usage of non-biodegradable packaging material to reduce environmental pollution. In this study, three types of polysaccharide-based edible films including chitosan, sodium alginate and carboxymethyl cellulose (CMC) were made and compared for their physical, mechanical and chemical properties. Sodium alginate had the highest value of thickness (0.08 mm), elongation at break (42.70%) and puncture force (13.26 N). Sodium alginate having the most suitable properties, different concentrations of 0- 6% (v/v) of *Melastoma malabathricum* extract was incorporated into sodium alginate edible film and its physical, antioxidant and antimicrobial properties were determined. *Melastoma malabathricum* extract addition decreased the water activity and elongation at break of the film. The incorporation of *Melastoma malabathricum* extract did not significantly affect ($p>0.05$) the solubility, puncture force and the antimicrobial properties of sodium alginate edible film; while the antioxidant properties and the total phenolic content of the edible film improved. Thus, it showed that with the addition of *Melastoma malabathricum* extract into sodium alginate edible film, the functionality of the film as an active packaging was enhanced.

1.Introduction

Food packaging is an essential material used in food industry as it serves as containment and protection for food products to maintain the product quality and extend the shelf life of foods (Klimchuk and Krasovec, 2012). However, some packaging is mostly made up of non-biodegradable materials such as plastic that can cause a huge problem to the environment. Therefore, the development of edible packaging such as edible film has gained a lot of attention from researchers in the recent years to reduce environmental pollution. Edible film is a food grade thin layer of material that forms a physical barrier between food and surrounding, reducing the moisture loss and gas transport (Eca et al., 2014). Edible film can be made from polysaccharides film-forming material such as chitosan, sodium alginate and carboxymethyl cellulose (CMC) that has large variety of structural features with different forming properties, thereby influencing the physical properties of the film formed (Dhanapal et al., 2012).

Chitosan is a non-toxic and biodegradable polymer that is being widely used to form

edible film due to its excellent mechanical and chemical properties (Suput et al., 2015). It has antimicrobial properties and widely used to extend the shelf life of fresh foods (Suput et al., 2015). Sodium alginate is the sodium salt of alginic acid, and it is isolated from brown seaweed (Embuscado and Huber, 2009). The film that made from sodium alginate is strong and soluble in water, acid and alkali. Sodium alginate film protects food against lipid oxidation due to its highly hydrophilic properties (Varela and Fiszman, 2011). Esmaeili and Fazel (2016) highlighted that cellulose derivative edible film is highly resistance to lipid and aromatic compound diffusion.

Edible films can be incorporated with food additives such as herbs and spices that has antimicrobial and antioxidant properties (Han, 2014). Jutaporn et al. (2011) found that the addition of Phayom wood extract into hydroxypropyl methylcellulose inhibits the growth of *L. monocytogenes, S. aureus* and *E. coli*. Chitosan edible film with the incorporation of galangal extract is found to have inhibitory effect against *S. aureus* by damaging the bacteria cell wall and cell membranes (Mayachiew et al., 2010). On the other hand, green tea extract can enhance the free radical scavenging effect of fish skin gelatin film (Wu et al., 2013). Also, in the work of Zaman et al. (2018), the application of edible with incorporated with Garcinia atrovirdis extract have prolonged the shelf life of fish. Choong et al (2019), however, added the musk lime extracts into chitosan edible film for the preservation of squid. Hence, the incorporation of herbs and spices with antimicrobial and antioxidant properties can reduce the growth of bacteria and improve the antioxidant properties of the film.

Melastoma Malabathricum, is known as 'senduduk'. It is a very common plant that can be found in tropical country. It grows as small trees and they can be found easily in forest or old defoliated area throughout the year (Susanti et al., 2007). The leaves, roots and shoots of *Melastoma malabathricum* extract has antimicrobial properties against some Gram-negative bacteria such as *S. aureus* and *B. subtilis* (Anbu et al., 2008). The bioactive compounds of *Melastoma malabathricum* such as quercetin and quercitrin have higher antioxidant effect than Vitamin E (Susanti et al., 2008).

To date, there is lack of studies on comparison of polysaccharide-based edible film. Hence, this study aimed to compare edible films from chitosan, sodium alginate and CMC based on their properties. The favourable polysaccharide-based edible film is determined based on its physical, mechanical and chemical properties. In addition, no report was found on the incorporation of *Melastoma malabathricum* extract into edible film. Thus, this study is followed by the incorporation of *Melastoma malabathricum* extract in different concentration into the chosen polysaccharide-based edible film to study the effect of *Melastoma malabathricum* extract on the physical, mechanical, chemical and antimicrobial properties of the edible film.

2. Materials and methods

2.1. Materials

2.1.1. Samples

Leaves of *Melastoma Malabathricum* were collected from the roadside to Genting Highland, Pahang, Malaysia. All the chitosan, sodium alginate and CMC, glycerol were of food grade, while the chemicals and reagents were of analytical grade.

2.2. *Preparation of Polysaccharide-based Edible Film*

2.2.1. Chitosan

The development of chitosan film was carried out according to Remya et al. (2016) with slight modification. Film forming solution was prepared by dissolving 1.5% (w/v) chitosan in 100 mL of 1% acetic acid solution with constant stirring at room temperature. Glycerol was added into filtered solution at a concentration of 0.75% (v/v) followed by mixing for 15 minutes. Chitosan solution was

casted onto a sterile petri dish and dried in a 40°C oven for 24 hours.

2.2.2. Sodium alginate

Sodium alginate film was prepared in accordance with the procedure described by Benavides et al. (2012) with slight modification. Sodium alginate solution was prepared by dissolving 1.5% (w/v) of sodium alginate powder into 200 mL of distilled water followed by mixing for 30 minutes at temperature of 70°C and allowed to cool. Glycerol (0.75% v/v) was added into the filtered solution as a plasticizer and the solution was stirred for 15 minutes. Sodium alginate solution was casted onto a petri dish and dried at 40°C for 24 hours in an oven.

2.2.3. Carboxymethyl cellulose (CMC)

Preparation of CMC film was conducted according to Savanjali et al. (2011) with slight modification. CMC powder was dissolved in 100 mL distilled water in 1.5% (w/v) basis followed by filtration. Glycerol was added in a concentration of 0.75% (v/v) as plasticizer and the solution was heated on flame for 10 minutes until the temperature of solution reached 85°C. The solution was cooled for 30 minutes followed by casting onto a sterile petri dish. The petri dish was placed into a 40°C oven for 24 hours.

2.3.Development of Melastoma Malabathricum incorporated sodium alginate film

2.3.1. Chitosan

Melastoma malabathricum extract following method by Sarbadhikary et al. (2015) with slight modification. *Melastoma Malabathricum* leaves were washed with tap water followed by rinsing with distilled water. The leaves were grinded into smaller pieces by using a waring blender after drying at ambient temperature for 1 day. Extraction was done by mixing 10 g leaves powder with 250 mL ethanol in a conical flask followed by for 48 hours at 150 rpm. Then, the solution was brought to centrifugation at 7000 rpm for 15 minutes after filtration. Solvent in the supernatant was removed by using rotary evaporator with the temperature of 40°C. Ethanolic extract formed in round bottom flask was weight and dissolved in ethanol to obtain leave extract with the concentration of 50 mg/mL.

2.3.2. Incorporation of Melastoma Malabathricum in sodium alginate film

Melastoma Malabathricum incorporated sodium alginate film was prepared according to Benavides et al. (2012) with slight modification. Film forming solution was prepared by mixing 1.5% (w/v) of sodium alginate powder with 200 mL of distilled water followed by mixing for 30 minutes at temperature of 70°C until dissolved. Glycerol (0.75% v/v) was added into the filtered film solution as a plasticizer. *Melastoma malabathricum* extract was added at concentration of 0%, 2%, 4% and 6% (v/v), followed by homogenization for 3 minutes. Lastly, film forming solution was casted on a petri dish and dried at 40°C for 24 hours in an oven. For antimicrobial analysis, dried films were dipped in 2% CaCl2 solution for 1 minutes to form an insoluble film prior to analysis.

2.4. Antimicrobial Properties

2.4.1. Melastoma malabathricum

Antimicrobial properties of *Melastoma malabathricum* extract was determined by using disc diffusion method as according to Sarbadhikary et al. (2015) with slight modification. Bacteria cultures (*Staphylococcus aureus* and *Escherichia coli*) were prepared and adjusted to the standard of McFarland No. 0.5 to achieve a concentration of approximately 108 cfu/mL. Paper disc in the size of 6 mm diameter was impregnated with 50 µL of *Melastoma malabathricum* extract and placed onto inoculated agar. Antibiotic disc and paper disc impregnated with 50 µL of ethanol was placed on agar as positive control and negative control, respectively. The inoculated plates were then brought to incubation at 37°C for 18-24 hours. The antimicrobial activity of extract

was determined by measuring the zone of inhibition (including the diameter of disc) formed against the bacteria tested.

2.4.2. Melastoma malabathricum incorporated sodium alginate film

Films were cut into 6 mm diameter sphere by using a hole puncture and placed onto the inoculated agar. On the other hand, a 30 µg chloramphenicol disc and film without leaves extract were also placed onto the inoculated agar as positive control and negative control, respectively. The inoculated plates were brought to incubation at 37°C for 18-24 hours. The antimicrobial activity of films was determined by measuring the zone of inhibition (including the diameter of disc) formed against the bacteria tested.

2.5. Antioxidant Properties

2.5.1. Antioxidant activity

Antioxidant effect of *Melastoma malabathricum* extract was determined by using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay, according to Wong et al. (2014) with slight modification. T Melastoma malabathricum extract (0.1 mL) was added into 3.9 mL 0.004% ethanolic DPPH solution in a test tube followed by incubation in dark for 30 minutes. The absorbance of the mixture was measured at 517 nm using UV-Vis Spectrophotometer. Ethanol was used to zero the instrument prior to sample reading. The percentage of DPPH scavenging effect was calculated by using equation below. Leave extract was replaced with film extract that was prepared by dissolving 25 mg of film into 3 mL of distilled water.

$$DPPH\ Scavenging\ effect\ (\%) = \frac{Abs\ of\ DPPH - Abs\ of\ sample}{Abs\ of\ DPPH} \times 100$$

(Eq.1)

where Abs of DPPH is the absorbance value of 0.004% ethanolic DPPH solution and Abs of sample is the absorbance value of extract at 517 nm, respectively.

2.5.2. Total phenolic content

Total phenolic content of *Melastoma malabathricum* extract was determined by using Folin-Ciocalteu method as according to Wong et al. (2014) with slight modification. Ten times diluted *Melastoma malabathricum* was added into a wrapped test tube in the amount of 0.3 mL followed by the addition of 1.5 mL Folin-Ciocalteu's reagent and 1.2 mL sodium carbonate. The mixture was placed in dark for 30 minutes prior to the absorbance reading at 765 nm. Gallic acid solution prepared in specific concentration (0, 0.025, 0.050 and 0.100 mg/g) was used to generate a standard curve. Leave extract was replaced with film extract that was prepared by dissolving 25 mg of film into 3 mL of distilled water.

2.6. Film Analysis

2.6.1. Thickness

The thickness of edible films was measured by using a micrometer with accuracy of 0.01 mm. Measurements were taken at five different locations of each film and the average film thickness was calculated to ensure consistent results (Muppalla et al. 2014).

2.6.2. Water solubility

Water solubility of edible films was determined according to the method by Ma et al. (2016) with slight modification. Edible films were cut into 2 cm x 2 cm dimension followed by drying at 60°C for 24 hours. The weight of dried films (W1) was determined and recorded accordingly. Each dried film was immersed in 25 mL of distilled water for 2 hours at room temperature. The films were then dried in oven at 60°C for 24 hours. Final mass of dried films (W2) was weight and recorded after cooling to room temperature. The water solubility of films was calculated following equation.

$$Water\ solubility = \frac{W_1 - W_2}{W_1} \times 100\%$$

(Eq.2)

2.6.3. Water activity

Water activity of films was determined by using water activity meter (AquaLab Pre, USA). Edible films were cut into 3 cm x 3 cm dimension, placed into a sample cup and sample drawer prior measuring the water activity.

2.6.4. Colour

Colour of the film was determined using colorimeter (ColorFlex EZ, Australia). A 3 cm x 3 cm film was placed on a ColorFlex sample cup for colour determination. The film colour result was generated in terms of L*, a*, and b*, where L* represents darkness when L=0 and brightness when L=100; a* represents green hue in negatives value and red hue in positive value; b* represents yellow-blue with positive value indicates yellow and negative value indicates blue. The colour index (ΔE) that identifies the total colour changes between each sample of *Melastoma Malabathricum* incorporated sodium alginate films was calculated according to the equation [4] (Hernandez-Hernandez et al., 2009).

$$\Delta E = \sqrt{(L* - L)^2 + (a* - a)^2 + (b* - b)^2} \quad \text{(Eq.3)}$$

where L*, a* and b* are the colour parameter values of the pure sodium alginate edible film and L, a, and b are the colour parameter values of the *Melastoma Malabathricum* incorporated sodium alginate films.

2.6.5. Tensile strength and elongation at break

Tensile test was conducted with a universal material tester (LF1096, UK) in accordance with ASTM standard method as described by Remya et al. (2016). The films (60 mm x 10 mm) were clamped between grips with an initial separation of 40 mm, and the cross-head speed was set at 20 mm/min. Tensile strength and elongation at break were measured. Tensile strength and elongation at break were calculated by using the equations below, respectively.

$$Tensile\ strength = \frac{Peak\ load\ (N)}{Cross-sectional\ area\ (mm^2)} \quad \text{(Eq.4)}$$

$$Elongation\ at\ break\ point = \frac{Final\ length\ of\ film\ ruptured\ (mm)}{Initial\ grip\ length\ (mm)} \times 100\% \quad \text{(Eq.5)}$$

2.6.6. Fourier transform infrared spectroscopy (FTIR)

The absorbance spectra of films were determined using Fourier Transform Infrared (FTIR) spectrometry connected to OMNIC Spectra Software in transmission mode. Films were placed on the sample holder and fixed by a tiny probe. Spectra of films were obtained with a resolution of 4 cm^{-1} as the average of 20 scans in the range of 500 cm^{-1} to 4000 cm^{-1} against a background spectrum from an empty cell.

2.7. Statistical Analysis

The experimental data was analyzed using IBM SPSS version 20 software. One-way Analysis of Variance (ANOVA) was used to analyse data obtained from film analysis whereas independent sample T-test was performed for the antimicrobial properties of *Melastoma malabathricum*. The p-value of ≤0.05 was considered as statically significant.

3.Results and discussions

3.1. Comparison between different polysaccharide-based edible films

3.1.1. Physical and Mechanical Properties of Different Polysaccharide-based Edible Films

From the result obtained in Table 1, the thickness of chitosan, sodium alginate and CMC edible film was determined as 0.06 mm, 0.08 mm and 0.04 mm, respectively. Rossi-Marquez et al. (2009) found that film thickness will affect the elasticity (E%) of the film where thicker films had higher elasticity than thinner films due to greater amount of material per unit length, while Ghasemlou et al. (2011) stated that thinner film is difficult to handle. Based on the result, sodium alginate film has the highest thickness value among three edible films

indicating stronger mechanical properties, higher protecting effect for food products.

Water solubility of edible films determines the biodegradability and integrity of the films in an environment with higher relative humidity (Ma et al., 2016). It affects the application of the films on food products (Maizura et al., 2007). Result shows that sodium alginate and CMC are 100% soluble in water whereas chitosan is only 25.60% soluble in water. Alginate and cellulose film have poor water resistance owing to the hydrophilic nature of the materials (Dhanapal et al., 2012). The presence of hydrophobic acetyl group in the structure of chitosan reduces the solubility of chitosan in water (Bangyekan et al., 2006).

Chitosan, sodium alginate and CMC edible film have the water activity value of 0.571, 0.524 and 0.545, respectively (Table 1). The water activity level of all three polysaccharide-based edible films is below the minimum threshold for the growth of microorganism (Barbosa-Cánovas, 2007). Based on the result obtained, there is no significant difference ($p>0.05$) between the water activity of all three edible films.

Colour is one of the important parameters for food packaging as it will affect the appearance of the food product. Chitosan edible film is the lightest among the three edible films, followed by sodium alginate and CMC. The more negative a* value obtained for chitosan film indicate that chitosan has higher intensity of green colour compared to the other two edible films. The physical appearance of chitosan edible film in this study show lighter yellowness and thus it has a positive value (0.74) in b* parameter. Ojagh et al. (2010a) reported that chitosan edible film is slightly yellowish.

Sodium alginate and CMC edible films developed in this study are transparent which indicate that the food product appearance will not be affected by the film. Hence, sodium alginate and CMC are more suitable to be used as food packaging for protecting effect.

Table 1. Physical and mechanical properties of chitosan, sodium alginate and CMC edible films

Films	Chitosan	Sodium alginate	CMC
Thickness (mm)	0.06 ± 0.01^{a}	0.08 ± 0.01^{b}	0.04 ± 0.01^{a}
Water solubility (%)	25.60 ± 0.90^{a}	100.00 ± 0.00^{b}	100.00 ± 0.00^{b}
Water activity	0.571 ± 0.025^{a}	0.524 ± 0.036^{a}	0.545 ± 0.031^{a}
*L**	13.28 ± 0.13^{a}	7.36 ± 0.85^{b}	6.15 ± 0.57^{b}
*a**	-1.67 ± 0.25^{a}	-0.28 ± 0.05^{b}	-0.22 ± 0.04^{b}
*b**	0.74 ± 0.07^{a}	-0.94 ± 0.01^{b}	-0.82 ± 0.14^{b}
Tensile strength (MPa)	15.45 ± 1.25^{a}	17.17 ± 0.16^{a}	12.61 ± 1.46^{b}
Elongation at break (%)	7.83 ± 0.76^{a}	42.70 ± 1.26^{c}	23.84 ± 3.09^{b}
Puncture force (N)	7.17 ± 1.01^{a}	13.26 ± 2.39^{b}	9.24 ± 0.54^{a}

[a-c]Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$ according to Tukey's test.

Tensile strength is the maximum stress that can be withstand by the sample and higher tensile strength is usually preferable for a material (Bourtoom and Chinnan, 2008). Based on the result in Table 1, no significant difference ($p>0.05$) were found between the tensile strength of chitosan and sodium alginate, with the value of 15.45 MPa and 17.17 MPa, respectively. However, the tensile strength of CMC with the value

of 12.61 MPa is lower than chitosan and sodium alginate edible film. Chitosan and sodium alginate edible film have higher tensile strength compared to CMC edible film, indicating that chitosan and sodium alginate edible films are stronger than CMC edible films.

Elongation at break describes the plasticity and the ability of the film to stretch and extend before breaking into half (Maran et al., 2013). Generally, film with higher plasticity or extensibility is favourable, as the integrity of the film can be maintained when applied onto food products (Arham et al., 2016). As shown in Table 1, among the elongation at break of chitosan, sodium alginate and CMC edible film, sodium alginate has the highest elongation at break value. Alginate was reported to produce stronger film (Maizura et al. 2007). Hence, sodium alginate is selected to be a food packaging as it will not break easily.

Puncture force can be defined as the maximum force required to pierce or break a certain type of material (Maran et al., 2013). Based on the result obtained in Table 1, sodium alginate edible film has highest puncture force (13.26 N) among chitosan and CMC edible film (7.17 N and 9.24 N, respectively). It is expected that thicker film has higher puncture force value (Gimenez et al., 2009). Sodium alginate edible film was found to have the highest thickness value among three polysaccharide-based edible films. Higher puncture force value makes sodium alginate more suitable to be used as a food packaging compared to chitosan and CMC edible film as films with higher puncture force may be able to withstand higher external force.

3.1.2. Chemical Properties of Different Polysaccharide-based Edible Films

The analysis of FTIR was carried out to identify the functional groups present in chitosan, sodium alginate and CMC edible films by the bands generated from spectra (Su et al., 2010). Figure 1 (a) represents the absorption spectra of chitosan edible film. The broad spectrum at 3281 cm^{-1} represents the intermolecular hydrogen bonding of chitosan as well as the stretching vibration of N-H and O-H groups (Pranoto et al., 2005a). Absorption peak at 2877 cm^{-1} and 1557 cm^{-1} representing the stretching vibration of C-H bond and N-H band for either amide II or primary amine, respectively (Jawaid et al., 2016). The most intense peak at 1021 cm^{-1} in chitosan edible films represents the C=O and C-N groups stretching.

Figure 1 (b) presents the spectra of sodium alginate edible film. The spectrum has a broad absorption band at 3265 cm^{-1} indicates the stretching vibration of OH groups whereas the absorption peak at 2938 cm^{-1} indicates the C-H and C-O stretching of the carboxyl group (Fang, 2016). Two characteristic absorption band of sodium alginate were identified at 1408 cm^{-1} and 1600 cm^{-1}, indicating the symmetric and asymmetric stretching vibration of COO- group, respectively (Dai et al., 2008). The absorption peak of sodium alginate at 1317 cm^{-1} and 1089 cm^{-1} are a sign of oligosaccharides while the most intense peak at 1025 cm^{-1} referring to the C-O bonds of saccharide structure as well as the presence of guluronic acids (Mishra 2015).

Figure 1 (c) shows the spectra of CMC edible films. The broad absorption band of CMC at 3276 cm^{-1} represents the stretching vibration of hydroxyl group while the absorption peak at 2882 cm^{-1} indicates the stretching vibration of C-H groups (Hebeish et al., 2013). Other absorption peak of CMC edible film was found at 1413 cm^{-1} and 1321 cm-1, which represents the bending vibration -CH_2 and –OH group respectively (Biswal and Singh, 2004).

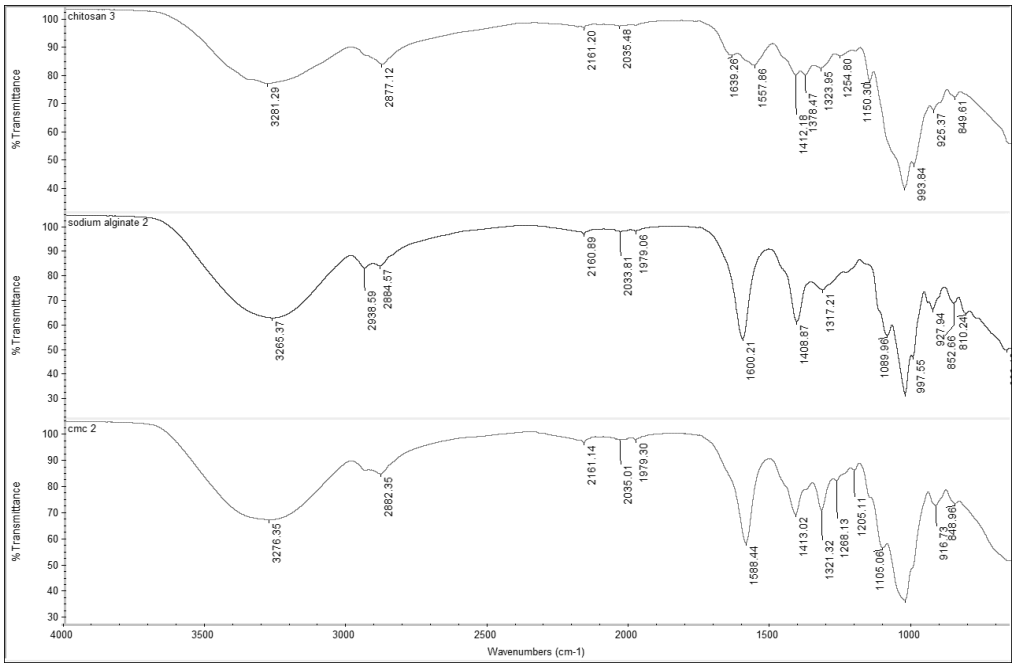

Figure 1. Spectra of Fourier Transform Infrared (FTIR) of (a) chitosan, (b) sodium alginate and (c) CMC edible films.

3.1.3. Selection of polysaccharide-based edible film

Edible film made from sodium alginate has better mechanical properties in the aspect of elongation at break and puncture force as compared to chitosan and CMC edible films. In addition, sodium alginate edible film possesses good physical properties such as colourless and water soluble which make it more suitable to be a packaging material. Hence, sodium alginate was selected for the incorporation of *Melastoma malabathricum*.

3.2. Analysis of *Melastoma malabathricum* extract

3.2.1. Antimicrobial Properties

Melastoma malabathricum extract was found to inhibit the growth of *S. aureus* as it has the inhibition zone of 10.44 mm. However, *Melastoma malabathricum* extract showed no inhibitory effect towards *E. coli*. The structure of the bacterial cell wall. *S. aureus* is Gram-positive bacteria while *E. coli* is Gram-negative bacteria. According to

Nazzaro et al. (2013), the cell wall of Gram-negative bacteria contains lipopolysaccharide, which link the membranes together which create a protecting zone at the cell wall. This unique structure of Gram-negative bacteria causes active compound that has antimicrobial properties are difficult to penetrate through the bacteria cell wall and hence no inhibitory effect was shown.

3.2.2. Antioxidant Properties and Total Phenolic Content

Result shows that *Melastoma malabathricum* extract has the scavenging effect of 82.91%. The antioxidant properties of *Melastoma Malabathricum* is owing to the presence of flavonoid compounds such as quercetin and quercitrin (Susanti et al., 2008). Result shows that *Melastoma malabathricum* contains phenolic content of 909.22 mg GAE/100 g. According to

Suhaimy et al. (2017), total phenolic content value higher than 1000 mg GAE/100 g is considered as high total phenolic content.

3.3. Analysis of *Melastoma malabathricum* sodium alginate films

3.3.1. Physical and Mechanical Properties of Melastoma Malabathricum incorporated sodium alginate films

According to Table 2, the thickness of the films was increased from 0.08 mm to 0.10 mm when the concentrations of *Melastoma malabathricum* extract increased from 0% to 6% (v/v). There is no significant difference ($p>0.05$) in the thickness of sodium alginate with 0% to 4% (v/v) *Melastoma malabathricum* extract. However, the thickness of sodium alginate + 6% *Melastoma malabathricum* extract edible film was 0.10 mm, shown to be higher than the thickness of sodium alginate edible films with 0%, 2% and 4% (v/v) *Melastoma malabathricum* extract.

The addition of *Melastoma malabathricum* extract does not affect the solubility of sodium alginate edible film as there was no significant difference ($p>0.05$) between the solubility of sodium alginate edible films and pure sodium alginate film. The sodium alginate edible films were dissolved completely in water within 2 hours. Water activity of sodium alginate edible films decreased from 0.524 to 0.424 when the concentrations of *Melastoma malabathricum* extract was increased from 0% to 2% (v/v). However, there is no significant difference ($p>0.05$) in the water activity of sodium alginate edible film incorporated with 2%, 4% and 6% (v/v) of *Melastoma malabathricum* extract.

Table 2 shows that the lightness value (L*) of sodium alginate edible films increased when *Melastoma malabathricum* extract concentration increased from 0% to 6% (v/v). A decrement trend of a* value from -0.28 to -2.50 indicating an increasing intensity of greenness. The yellow-blue (b*) value of sodium alginate edible films were increased from -0.94 to 2.58 with the increased concentrations of *Melastoma malabathricum* extract. The yellow intensity of edible films increased when more *Melastoma malabathricum* extract was added into the film. The increment of green and yellow colour may be attributed to the colour nature of *Melastoma malabathricum* extract, which is yellowish-green visually. The addition of *Melastoma malabathricum* extract into sodium alginate edible film has resulted in an increasing trend of total colour change (ΔE) from 5.92 to 13.06. Generally, the incorporation of *Melastoma malabathricum* extract caused the reduction in transparency of sodium alginate edible film where the colour of the films turns yellowish-green after the incorporation of *Melastoma malabathricum*. With increase in concentrations of *Melastoma malabathricum* extract, the green and yellow intensity were increased due to the yellowish-green of *Melastoma malabathricum*.

Table 2. Physical and mechanical properties of sodium alginate edible films with different concentration of *Melastoma malabathricum* extract

Melastoma malabathricum (%)	0	2	4	6
Thickness (mm)	0.08 ± 0.01^{a}	0.08 ± 0.01^{a}	0.09 ± 0.01^{a}	0.08 ± 0.01^{a}
Water solubility (%)	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
Water activity	0.524 ± 0.036^{a}	0.424 ± 0.012^{b}	0.417 ± 0.006^{b}	0.524 ± 0.036^{a}

*L**	7.36 ± 0.85^{a}	13.17 ± 0.38^{b}	17.28 ± 0.32^{c}	7.36 ± 0.85^{a}
*a**	-0.28 ± 0.05^{a}	-1.32 ± 0.12^{b}	-2.06 ± 0.10^{c}	-0.28 ± 0.05^{a}
*b**	-0.94 ± 0.01^{a}	-1.36 ± 0.08^{b}	0.84 ± 0.10^{c}	-0.94 ± 0.01^{a}
Tensile strength (MPa)	-	5.92 ± 0.38^{a}	10.24 ± 0.31^{b}	-
Elongation at break (%)	17.17 ± 0.16^{a}	16.03 ± 0.64^{a}	22.91 ± 1.27^{b}	17.17 ± 0.16^{a}
Puncture force (N)	42.70 ± 1.26^{a}	40.16 ± 0.43^{a}	32.87 ± 1.90^{b}	42.70 ± 1.26^{a}

[a-d]Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$ according to Tukey's test.

The addition of *Melastoma malabathricum* extract from 0% to 6% (v/v) has improved the tensile strength of the edible film, from 17.17 MPa to 24.33 MPa. Tensile strength of sodium alginate edible film incorporated with 6% (v/v) *Melastoma malabathricum* extract was determined as 24.33 MPa, which showed no significant difference ($p>0.05$) to the tensile strength of sodium alginate edible film incorporated with 4% (v/v) *Melastoma malabathricum*; extract whereas the elongation at break of the edible films was in a decreasing trend with the increasing concentrations of *Melastoma malabathricum* extract.

The elongation at break was reduced from 42.70% to 29.05% when the concentrations of *Melastoma malabathricum* extract ranging from 0% to 6% (v/v). According to Hosseini et al. (2009), water is the most abundant plasticizer for most hydrocolloid-based films such as alginate as it can alter the polymer structure. The incorporation of *Melastoma malabathricum* extract into sodium alginate edible film does not significantly affect ($p>0.05$) the puncture force of the films.

3.3.2. Chemical Properties of Melastoma Malabathricum incorporated sodium alginate films

FTIR was used to study the interaction between sodium alginate and *Melastoma malabathricum* extract in this study. Figure 2 provides the spectra of sodium alginate edible films incorporated with different concentrations of *Melastoma malabathricum* extract. All spectra have shown similar absorption peak around 3263 cm^{-1}, 2938 cm^{-1} and 1408 cm^{-1}. According to Fang (2016), the absorption band around 3263 cm^{-1} represents the presence of OH group stretching whereas the peak around 2938 cm^{-1} indicates the C-H and C-O stretching of the carboxyl group. The absorption band around 1408 cm^{-1} specifies to the carboxylic group (COO-) (Pranoto et al., 2005b). Another absorption peak was obtained at around 1600 cm^{-1} and 1025 cm^{-1}, which 1600 cm^{-1} represented the carboxylic group stretching vibration of either phenolic acids or uronic acids whereas 1025 cm^{-1} indicates the C-O stretching for phenolic acids (Khoo et al., 2014).

In the 4% and 6% (v/v) *Melastoma Malabathricum* incorporated sodium alginate films, there is a small peak present at 1231 cm^{-1}, indicating the presence of (C-O) phenolic hydroxyl group in the film (Salbiah et al., 2010). However, there is no peak shown at 1231 cm^{-1} for 2% *Melastoma malabathricum* sodium alginate edible film. This may be caused by insufficient of the phenolic hydroxyl group to cause a peak at 1231 cm^{-1}. The small peak area at 1231 cm^{-1} is due to the presence of phenolic compounds such as kaempferol and naringenin which its structure consists

phenolic hydroxyl group (Susanti et al., 2008).

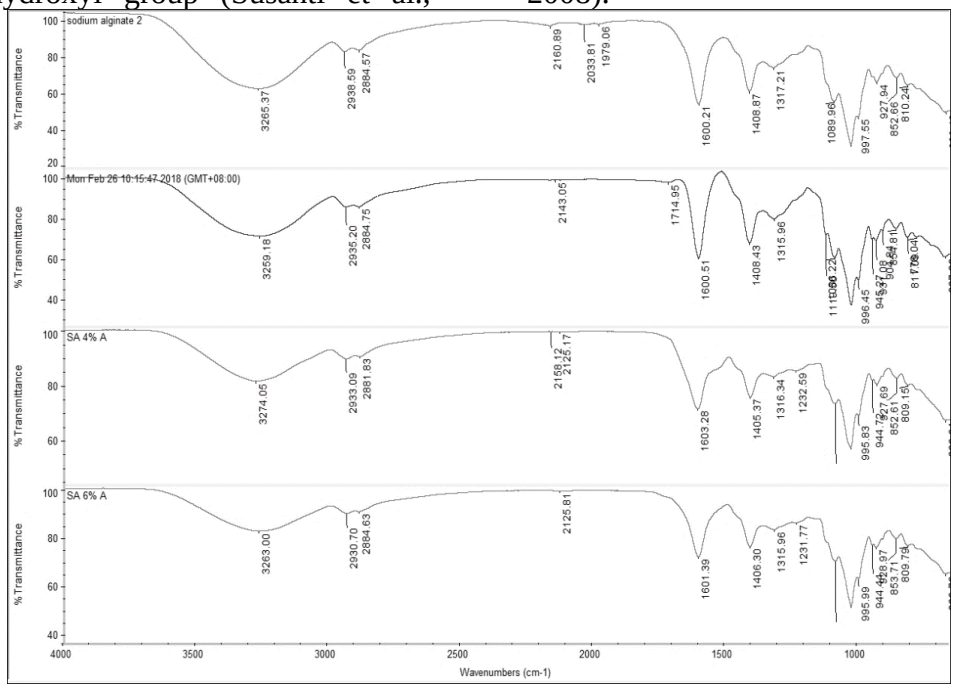

Figure 2. Spectra of Fourier Transform Infrared (FTIR) of *Melastoma malabathricum* sodium alginate edible films (a) 0% extract (b) 2% extract (c) 4% extract (d) 6% extract

Table 3 shows that sodium alginate film has scavenging effect with the value of 5.49%. There is no significant difference ($p>0.05$) found between the scavenging effect of 0% and 2% *Melastoma Malabathricum* incorporated sodium alginate films. With the increase of *Melastoma malabathricum* into sodium alginate edible films from 2% (v/v) to 6 % (v/v), the scavenging effect increase from 7.12% to 26.32%. Based on the result in Table 3, an increment in total phenolic content sodium alginate edible film was observed by the increasing concentrations of *Melastoma malabathricum* extract ranging from 0% to 6% (v/v), where the total phenolic content of films was increased from 28.58 mg GAE/100 g to 621.17 mg GAE/100 g.

Table 3. scavenging effect, total phenolic content and inhibitory effect of sodium alginate edible films with different concentration of *Melastoma malabathricum* extract

Melastoma malabathricum (%)	0	2	4	6
Scavenging effect (%)	5.49 ± 0.73^{a}	7.12 ± 0.74^{a}	19.47 ± 1.42^{b}	26.32 ± 3.69^{c}
Total phenolic content (mg GAE/100 g)	28.58 ± 2.59^{a}	305.42 ± 29.21^{b}	443.10 ± 47.30^{c}	621.17 ± 56.84^{d}
Inhibitory effect against *S. aureus* (mm)	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

[a-d]Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$ according to Tukey's test.

In this study, the incorporation of *Melastoma malabathricum* extract into sodium alginate edible film does not exhibit any inhibitory effect against *S. aureus*. A possible explanation for this might be the concentration of *Melastoma malabathricum* extract is lesser to possess any inhibitory effect against bacteria even at the concentration of 6% (v/v).

4. Conclusions

Edible film can be used to replace non-biodegradable food packaging. Edible film forming materials classified into polysaccharide, protein and lipids.

Thus, this study was conducted to compare three types of polysaccharide-based edible film, which including chitosan, sodium alginate and carboxymethyl cellulose (CMC).

The results showed that sodium alginate edible film has better mechanical and physical properties compared to chitosan and CMC. *Melastoma malabathricum* extract was found to have high free radical scavenging effect and low total phenolic content.

The effect of different concentration of MME to the physical, mechanical and chemical properties of sodium alginate edible film was investigated in this study. With the addition of *Melastoma malabathricum* extract (0% to 6% v/v), the thickness, total color changes, antioxidant and total phenolic content of sodium alginate edible film was improved.

However, there was reduction in moisture content, water activity, elongation at break and no antibacterial activity. In conclusion, the addition of *Melastoma malabathricum* extract into sodium alginate edible film enhanced the functionality of the film as an active packaging.

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OXIDATIVE STABILITY OF MAYONNAISE PREPARED USING VIRGIN COCONUT OIL/FISH OIL BLEND

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ABSTRACT

Chemical changes in mayonnaise prepared using virgin coconut oil (VCO)/ fish oil (FO) blends at different ratios (95:5, 90:10, 85:15, v/v) were monitored throughout 30 days of storage at ambient temperature in comparison with the mayonnaise prepared using soybean oil (SO). Free fatty acid contents in all mayonnaise were increased after the storage of 30 days. Peroxide value, thiobarbituric acid reactive substances, *ρ*-anisidine value and total oxidation values of mayonnaise prepared using VCO were lowest during the storage, indicating the highest oxidative stability among all samples. Lipid oxidation was increased with extended storage time and higher level of FO added in mayonnaise. Highest lipid oxidation took place in mayonnaise containing SO after the storage of 30 days. At day 0, linoleic acid (50.07%) was the dominant fatty acid in SO containing mayonnaise, whereas lauric acid (47.05%) was predominant in VCO containing mayonnaise. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were found in mayonnaise prepared using VCO/FO blends. Lauric acid, myristic acid, EPA and DHA were decreased in all samples after the storage of 30 days. Volatile compounds, mainly hexanal, were increased after storage of 30 days. Mayonnaise prepared from VCO/FO (90:10) blend had no differences in sensorial property with that containing SO. Thus, VCO/FO (90:10) blend could be used to prepare mayonnaise with health benefit and the increased oxidative stability.

1. Introduction

Mayonnaise is one of the most favorite sauces in the world (Huang *et al*., 2016). It is oil-in-water emulsion with acidic pH, comprising three components: vinegar/water as a continuous phase, 70-80% oil as a dispersed phase and egg yolk used as an emulsifier (Li *et al*., 2014). Mayonnaise is oil containing product, in which soybean oil is commonly used. Basically, soybean oil is highly prone to lipid oxidation because of its unsaturated fatty acids. As a result, undesirable components e.g. reactive aldehydes and free radicals are formed (Gorji *et al*., 2016). To conquer the problem or prevent lipid oxidation, synthetic or natural antioxidants have been generally added in mayonnaise (Meyer and Jacobsen, 1996; Jacobsen *et al*., 2001). However, synthetic antioxidants e.g. butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) and ethylene diamine tetraacetic acid (EDTA) have a bad impression due to their toxic effects, particularly when high concentrations are used (Martinez-Tome *et al*., 2001).

Fish oil (FO) has been well known for health-promoting benefits. Therefore, health experts recommend a higher consumption of fish oil rich in polyunsaturated fatty acids (PUFAs), mostly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Hartvigsen *et al*., 2000). Incorporation of PUFAs into foods

can be of consumers' health benefit (Gorji *et al*., 2016). Susceptibility of lipids towards oxidation is determined by the location and number of double bonds. Generally, unsaturated lipids are prone to lipid oxidation than saturated counterpart (McClements and Decker, 2000).

Virgin coconut oil (VCO) comprises medium chain fatty acids (MCFAs), mostly lauric acid (Patil and Benjakul, 2019b). By reason of various health benefits and high stability, VCO has gained the interest for processor and consumer (Patil and Benjakul, 2018). Incorporation of VCO in combination with FO could be a means to prepare a functional mayonnaise. The balance between saturated and unsaturated oil would achieve both targets: health promotion and oxidative stability of resulting mayonnaise. Nevertheless, different types of oil in mayonnaise may give different sensory and physical characteristics. Therefore, this work was undertaken to incorporate VCO and FO at different ratios into mayonnaise. Oxidative stability and physical properties of resulting mayonnaises were examined throughout the storage of 30 days in comparison with that prepared using soybean oil (SO).

2. Materials and methods

2.1. Chemicals

Nile blue A, ammonium thiocyanate, *ρ*-anisidine, sodium hydroxide, and sodium chloride were procured from Sigma (St. Louis. MO, USA). Isooctane, sodium dodecyl sulfate and trichloroacetic acid were obtained from Merck (Darmstadt, Germany). Ethanol, methanol, chloroform, propanol, acetic acid, petroleum ether, n-hexane and hydrochloric acid were procured from Lab-Scan (Bangkok, Thailand). Eggs and soybean oil (SO) were bought from a supermarket in Hat Yai, Songkhla, Thailand.

2.2. Preparation of virgin coconut oil (VCO)

VCO was produced according to the method of Patil and Benjakul (2019b). Coconut milk was firstly hydrolyzed with partially purified seabass protease (PPSP) (10 units/g protein) at 60 °C for 60 mins followed by low speed centrifugation (3585×g) to obtain cream. The cream was frozen at -20 °C for 6 hr and then thawed at room temperature (30±2 °C) for 1 hr. Five freeze-thawing cycles were repeated. Finally, cream was centrifuged at high speed (8000×g) to obtain VCO.

2.3. Preparation of fish oil from seabass viscera

Whole viscera of seabass were purchased from a local market in Hat Yai. Polyethylene bag was used to pack the samples and ice was used to store it using an ice/sample ratio of 2:1 (w/w). Thereafter, samples were carried to the Department of Food Technology, Prince of Songkla University within 30 min. After arrival, depot fat from viscera was separated immediately from other internal organs such as stomach, liver, intestine and pyloric caeca. The obtained depot fat was chopped into small pieces with knife and ground with a blender (National, MX-T2GN, Taipei, Taiwan). The ground sample was used for oil extraction.

2.3.1. Extraction of oil from depot fat

Fish oil was extracted from depot fat following the method of Patil and Benjakul (2019a). Visceral depot fat (100g) was transferred into a round bottom flask equipped with a rotary evaporator (EYELA, N-1000, Tokyo Rikakikai, Co., Ltd., Tokyo, Japan). The extraction was performed at 70 °C for 20 mins under vacuum. After extraction, oil was placed in an Erlenmeyer flask containing anhydrous sodium sulfate (approximately 3-4 g), shaken well and decanted into a centrifuge tube through a Whatman No. 4 filter paper. The mixture was centrifuged at 10,000×g for 20 mins at 4 °C using a refrigerated centrifuge (CR22N, Hitachi, Hitachi Koki Co., Ltd., Tokyo, Japan). Pasteur pipette was used to collect oil. The oil sample was transferred to the amber bottles and purged with N_2 gas. The amber bottles were capped tightly and kept at −40 °C until further use.

2.4. Preparation of mayonnaise

Mayonnaise was prepared following the method of Patil and Benjakul (2019a). Formulation (% on weight basis) included 8% fresh egg yolk, 4% vinegar, 1% salt, 14% sugar, 3% distilled water and 70% oil. For oil samples,

VCO (100%) or VCO/FO blends with different VCO: FO ratios (95:5, 90:10, 85:15, v/v) were used. Soybean oil (100%) was used as the reference. Mayonnaise samples were designated as S: soybean oil (100%), V: VCO (100%), V-F5: VCO (95%) + FO (5%), V-F10: VCO (90%) + FO (10%), V-F15: VCO (85%) + FO (15%). The resulting mayonnaise samples were used for further analysis.

2.5. Sensory evaluation

For sensory evaluation of all samples, 9 – point hedonic scale was used; 9 is the most likeness and 1 is the most dislikeness (Meilgaard *et al*., 2006). Fifty panelists were recruited for sensory evaluation. They were the staffs and students from the Department of Food Technology, who were familiar with mayonnaise. The samples were served at room temperature with freshly made bread. Appearance, color, odor, flavor, texture, and overall likeness were evaluated. Panelists were asked to rinse their mouth between the samples using mineral water.

2.6. Chemical changes of mayonnaise during the storage of 30 days

Mayonnaise samples containing SO, VCO and VCO/FO blends at different ratios were analyzed during storage of 30 days. All mayonnaise samples were kept in zip lock bag and stored at room temperature (30-32 °C) in the incubator (Memmert, Schwabach, Germany). Oil from mayonnaise samples was extracted every 5 days during the storage for analysis.

2.6.1. Extraction of oil from mayonnaise

The oil was extracted from mayonnaise sample using the method of Bligh and Dyer method (Bligh and Dyer, 1959). Obtained oil was transferred into amber bottle, flushed with N_2 gas and used for further analysis.

2.6.2. Determination of free fatty acid content (FFA)

Free fatty acid content (FFA) was determined following the method as described by Patil *et al*. (2016).

2.6.3. Measurement of lipid oxidation products

Lipid oxidation products in mayonnaise samples were determined. Peroxide values (PV), *ρ*-anisidine values (AnV) and thiobarbituric acid reactive substances (TBARS) values were measured following the method tailored by Takeungwongtrakul *et al*. (2012) and Patil *et al*. (2016). Totox value was calculated by the following formula: Totox value = 2 PV + AnV (Huimin *et al*., 2014).

2.6.4. Analysis of fatty acid profiles

Oil samples extracted from mayonnaise at day 0 and day 30 were determined for fatty acid profile following the method described by Muhammed *et al*. (2015). Fatty acid content was calculated, based on the peak area ratio and expressed as g fatty acid/100g oil.

2.7. Determination of volatile compounds

Mayonnaise sample rich in both medium chain fatty acid and PUFA, with the likeness score equivalent to that of reference sample (S) was collected at day 0 and 30 for analysis of volatiles. Volatile compounds were determined using a solid-phase micro-extraction gas chromatography mass spectrometry (SPME-GC-MS) as described by Sae-leaw and Benjakul (2017). The volatile compounds were expressed as abundance of each identified compound.

2.8. Statistical analysis

Experiments were carried out in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range test. T-test was used for pair comparison (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussions

3.1. Sensory characteristics

Likeness score of mayonnaise prepared using SO, VCO and VCO/FO blends with different ratios is presented in Table 1. Higher likeness scores for appearance and color were obtained for S sample ($p<0.05$). However, appearance, color, and texture likeness scores were decreased by incorporation of VCO and VCO/FO blends.

Table 1. Likeness score of mayonnaise containing different oils.

Samples	Appearance	Color	Odor	Flavor	Texture	Overall
S	8.47±0.51a	8.50±0.63a	6.90±1.30a	7.00±0.79a	7.87±0.63a	7.57±0.97a
V	7.00±0.79b	7.07±0.98b	7.37±0.67a	7.73±0.94a	6.83±0.99b	7.43±0.89a
V-F5	7.57±0.73b	7.33±1.03b	6.87±0.94a	7.67±1.06a	7.00±0.91b	7.10±0.61a
V-F10	7.47±0.82b	7.60±1.07b	6.87±0.90a	7.07±0.94a	7.13±0.90b	7.07±0.53a
V-F15	7.73±0.98b	7.50±1.22b	5.57±0.57b	6.00±0.95b	7.30±0.95b	5.77±0.97b

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil (VCO), V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

Values are mean ± standard deviation (n=50).

Different lowercase letters in the same column indicate significant difference ($p<0.05$)

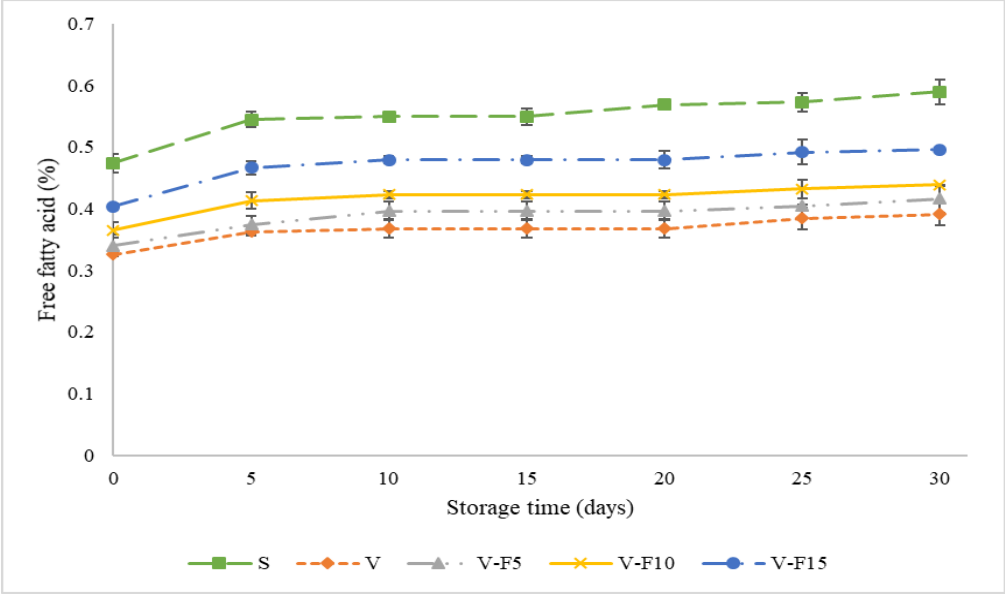

Figure 1. Free fatty acid (FFA) content of mayonnaise containing different oils during the storage of 30 days at room temperature. Bars represent standard deviations (n=3).

S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil (VCO), V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

For odor likeness score, no difference was observed between S, V, V-F5 and V-F10 samples ($p>0.05$). Nonetheless, V-F15 showed lower score for odor likeness ($p<0.05$). The results demonstrated that VCO might be able to mask fishy odor when FO up to 10% was used. At high concentration of FO (15%), panelists could detect the fishy odor in the mayonnaise sample. Therefore, low likeness score was obtained. Lowest flavor likeness score was also gained for V-F15 sample ($p<0.05$). It was noticed that there was no difference between S and V-F10 ($p>0.05$). VCO has natural distinctive coconut odor and flavor (Patil *et al.*, 2016). Results suggested that incorporation of VCO was able to improve the flavor of mayonnaise containing FO at low content but it was unable to mask the flavor of fish oil when highest level of FO (15%) was used. For texture likeness, the highest score was found for S sample ($p<0.05$). No difference between mayonnaise containing VCO and VCO/FO

blends was attained ($p>0.05$). The difference in texture between S sample and other samples might be governed by different fatty acid compositions. VCO rich in saturated fatty acids (Patil *et al*., 2016) became solidified. This might increase the viscosity or consistency of samples. The overall likeness was decreased when FO added was at levels more than 10% ($p<0.05$). Nevertheless, no difference was observed among the rest of samples ($p>0.05$). The results suggested that VCO could be used instead of SO for the production of mayonnaise. Additionally, FO could be added up to 10% in VCO/FO blend (V-F10) for mayonnaise preparation. To increase the level of FO, the masking agent or potential removal of off-odor, particularly fishy odor, should be implemented prior to making the blend for mayonnaise preparation.

3.2. Free fatty acid (FFA)

FFA contents of mayonnaise prepared using SO, VCO and different VCO/FO blends throughout the storage of 30 days at room temperature are depicted in Figure 1. At day 0, all mayonnaise samples showed different FFA content. The lowest FFA content was found in V sample ($p<0.05$). Generally, VCO has low FFA content (Patil *et al*., 2016). FFA content was increased with increasing FO level in the blends. Some FFA were presented in FO, used for the preparation of blends for mayonnaise. Therefore, increasing concentration of FO eventually increased FFA content of the resulting mayonnaise. FFA content was gradually increased after storage of 5 days in all the samples ($p<0.05$). During the storage, all samples showed the similar pattern, in which no drastic change was observed. FFA content of V sample was lowest amongst all samples throughout the storage of 30 days ($p<0.05$). The result suggested that the ester bonds of triglyceride were hydrolyzed at lower rate. However, significant difference was noticeable between day 0 and day 30 for all the samples ($p<0.05$), probably due to the microbial activity. Lactic acid bacteria (acid tolerant) might exist in mayonnaise (continuous phase) (Kishk and Elsheshetawy, 2013). Moreover, these increases were more likely attributed to the hydrolytic enzyme, which exists in eggs (Abu-Salem and Abou-Arab, 2008). The results suggested that type of oil used for preparation of mayonnaise could affect the FFA content of resulting mayonnaise at the beginning and after the storage.

3.3. Oxidative stability of mayonnaise during storage

3.3.1. Peroxide value (PV)

Changes in PV of mayonnaise prepared using SO, VCO and different VCO: FO blends throughout the storage of 30 days are shown in Figure 2 (A). At day 0, all mayonnaise samples showed different PV. It was lowest in V sample ($p<0.05$). The highest PV was observed for S sample, compared to other samples ($p<0.05$). PV is generally employed to measure lipid oxidation at the initial stage, where hydroperoxides are formed. For all the samples, PVs were continuously increased with increasing storage time ($p<0.05$). The increase in PV of all samples suggested that the samples were in propagation stage of lipid oxidation, while decomposition of hydroperoxides took place at a lower rate. Among all the samples, S sample showed the highest PV throughout the storage than those containing VCO or VCO/FO blends ($p<0.05$). In general, mayonnaise is susceptible to lipid oxidation because of their large surface area of oil droplets which facilitates the interactions between the oil, water and air (Gorji *et al*., 2016). McClements and Decker (2000) documented that oxidation of lipid is usually started at oil-in-water interface, where pro-oxidants such as transition metals in the continuous phase are able to interact with the hydroperoxides situated at the surface of droplet. Moreover, the pH is the main factor affecting oxidation of lipid in mayonnaise. In mayonnaise, as the emulsifier, egg yolk is used. Yolk contained a high amount of iron (734 μM) (Jacobsen, 1999). The iron is able to make cation bridges between the protein phosvitin and other components at pH 6. At the low pH (3.8-4) found in the mayonnaise, the bridges of iron between low-density lipoprotein and phosvitin are destroyed and the iron is released, leading to enhanced oxidation of lipid (Jacobsen, 1999).

The lowest increase in PV was observed in the V sample, compared to PV of all samples ($p<0.05$), indicating that VCO was less prone to lipid oxidation. This was plausibly owing to the low unsaturated fatty acid content (Patil *et al*., 2016). However, PV was increased with increasing concentration of FO in VCO/FO blend. This might be caused by the increase in unsaturated fatty acids, which were susceptible to oxidation. The results suggested that differences in PV between mayonnaises with different oils more likely resulted from differences in their chemical structure and fatty acid compositions.

3.3.2. TBARS

TBARS values of mayonnaise prepared using SO, VCO and VCO/FO blends at different ratios during 30 days of storage are depicted in Figure 2 (B).

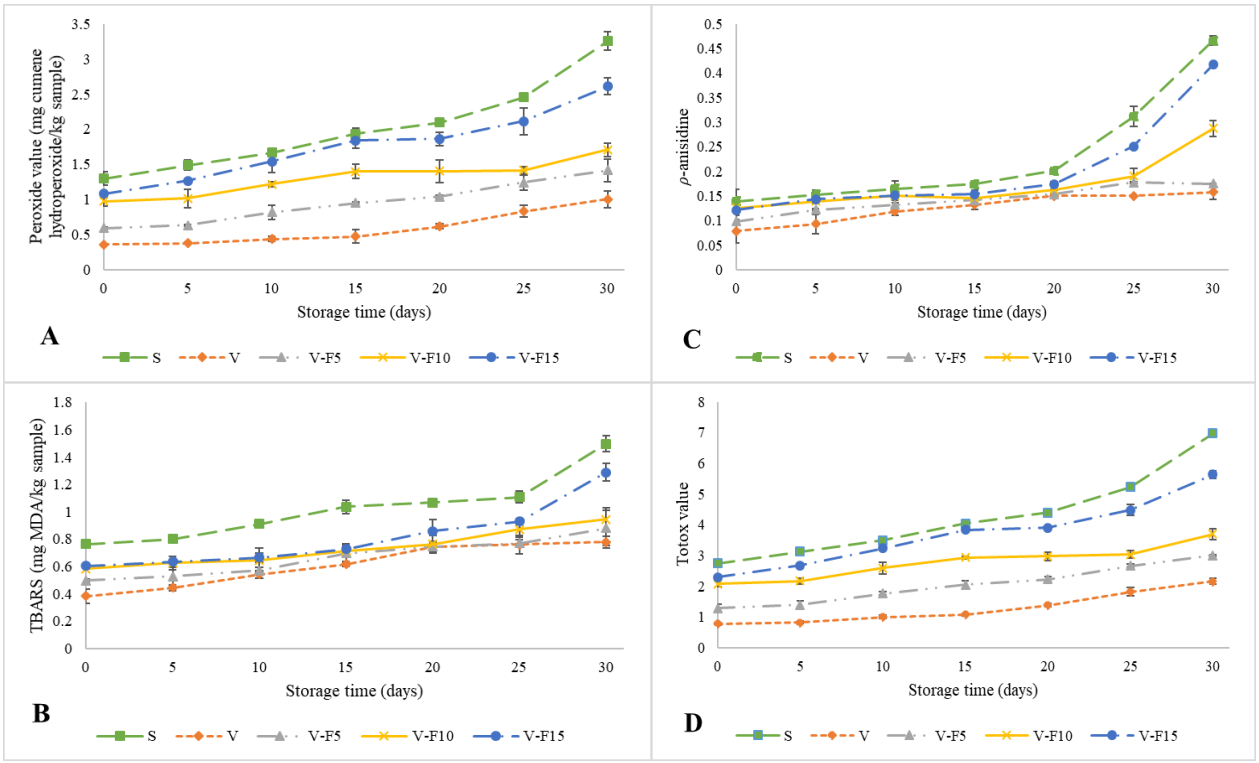

Figure 2: Peroxide value (PV) (A), thiobarbituric acid reactive substances (TBARS) (B), *ρ*-anisidine value (AnV) (C) and totox value (D) of mayonnaise containing different oils during the storage of 30 days at room temperature. Bars represent standard deviations (n=3).
S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil (VCO), V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%).

TBARS values of all samples were continuously increased with extended storage time ($p<0.05$). Compared to all other samples, the S sample showed the higher TBARS values throughout 30 days of storage ($p<0.05$). Generally, lipids with increasing TBARS value suggested that secondary lipid oxidation products were formed in samples. TBARS value is an index of decomposition of hydroperoxides into the secondary oxidation products in the later stages of lipid oxidation (Sae-leaw and Benjakul, 2017). Hydroperoxides are decomposed to malonaldehyde, which contributes to off-flavor of oxidized lipids (Zhang *et al*., 2013). As storage time increased, some prooxidants could accelerate lipid oxidation at higher extent. Conversely, the lowest TBARS values were observed for V

sample throughout 30 days. The results reconfirmed that VCO in mayonnaise was less prone to lipid oxidation. The slight difference was observed between TBARS values of samples added with FO until day 25. Nonetheless, the sharp increase in TBARS was found in V-F15 at day 30 ($p<0.05$). Overall, PVs were much lower than that reported by Chotphruethipong and Benjakul (2017) for mayonnaise enriched with fish oil. The result suggested that lipid oxidation rate was dependent on the type of oil used for the preparation of mayonnaise.

3.3.3. ρ-anisidine values (AnV)

AnV of mayonnaise prepared with SO, VCO and VCO/FO blends throughout the storage of 30 days is shown in Figure 2 (C). All samples had slight increase in AnV until day 20 ($p<0.05$). Thereafter, S, V-F15 and V-F10 samples had an apparent increase in AnV till the end of storage ($p<0.05$). S sample showed the highest AnV, as compared to other samples throughout 30 days of storage ($p<0.05$). The increase in AnV suggested the development of the secondary lipid oxidation products, mainly non-volatile compounds (mostly 2,4-alkadienals and 2-alkenals) in lipids (Choe and Min, 2006). When comparing AnV of all the samples at day 0, the lowest AnV was observed for the V sample, whereas the highest AnV was found in the S sample ($p<0.05$). The results showed that SO might contain some oxidative products at the beginning or during mayonnaise preparation. Overall, AnVs were much lower than that reported by Chotphruethipong and Benjakul (2017) for mayonnaise enriched with fish oil. The difference was most likely caused by the difference in oils used. Chotphruethipong and Benjakul (2017) prepared mayonnaise using SO blended with FO at ratio of SO:FO (90:10 v/v). The secondary oxidation products are important in food products for human consumption, since usually they have strong odor, while primary lipid oxidation products are flavorless and colorless (Osborn and Akoh, 2004). McClements and Decker (2000) documented that lipid oxidation of oil is affected by its chemical structure, mainly, location and number of double bonds. The results suggested that VCO used for mayonnaise was able to retard lipid oxidation of mayonnaise.

3.3.4. Total oxidation value (Totox value)

Totox value measures hydroperoxides as well as their breakdown products and gives a well estimation of the progressive oxidative deterioration of oil (Shahidi and Zhong, 2005). For all the samples, the Totox value was increased with extended storage time ($p<0.05$). S sample showed the highest increase in Totox value ($p<0.05$). Conversely, the sample containing VCO showed the lowest totox value throughout 30 days of storage, compared to others ($p<0.05$). Overall, a similar trend was noticed in comparison with PVs. Totox value was calculated from PV and AnV. Primary oxidation products and secondary oxidation products, together with free radicals, constitute the basis for measuring the oxidative deterioration of mayonnaise (Shahidi and Zhong, 2005). Toxic reaction products are generated due to the oxidation of lipids in mayonnaise (Coupland and McClements, 1996). Unaccepted off-flavors decreased shelf-life of mayonnaise (Alemán *et al*., 2015). In general, mayonnaise showed high oxidative stability when VCO was used, while FO at higher ratio increased oxidation.

3.4. Fatty acid composition

Fatty acid compositions of mayonnaise prepared with SO, VCO and VCO/FO blends at day 0 and day 30 are given in Table 2. At day 0, fatty acid composition of S sample was observed to be different from V sample and those containing VCO/FO blend. In S sample, linoleic acid (50.07%) was the predominant fatty acid. Oleic acid (24.18%) and palmitic acid (11.16%) were also found. The results were in agreement with previous report for fatty acid composition of mayonnaise prepared using SO, in which linoleic acid (50.4%), oleic acid (26.1%) and palmitic acid (11.6%) were documented (Enig *et al*., 1983). For V sample, lauric acid (47.05%) was predominant fatty acid, and myristic acid (19.57%) and palmitic acid (9.55%) were also present. Patil *et al*. (2016) documented that VCO predominantly comprised medium chain fatty acids (MCFA), mainly lauric acid (49.74-

51.18%), followed by myristic acid (18.70-19.84%). VCO with MCFA, mainly lauric acid, is responsible for health benefits (Patil and Benjakul, 2018). FO extracted from seabass visceral depot blended with VCO showed the marked difference in fatty acid composition in the resulting mayonnaise. Sae-leaw and Benjakul (2017) documented that oil extracted from depot fat of seabass viscera contained oleic acid (25.49%), palmitic acid (21.8%), linoleic acid (13.84 %), docosahexaenoic (DHA) (6.91%) and eicosapentaenoic (EPA) (2.09%). Saturated fatty acid, mainly lauric acid and myristic acid, were decreased in V-F5, V-F10 and V-F15 samples with increasing FO concentration ($p<0.05$). Conversely, palmitic acid was increased with increasing FO concentration. On the other hand, unsaturated fatty acids, mainly EPA and DHA were significantly increased in mayonnaise prepared with VCO/FO blends as the FO ratio increased.

After storage of 30 days, the different fatty acid profiles were observed in all samples, compared to those found at day 0. Fatty acids, particularly lauric acid, myristic acid, DHA and EPA were decreased after 30 days of storage ($p<0.05$). Conversely, linoleic acid, oleic acid, palmitic acid were increased after 30 days of storage ($p<0.05$). The changes in fatty acid profile might be owing to the microorganisms or oxidative and hydrolytic enzymes in eggs (Karas *et al*., 2002). It was postulated that hydrolytic enzyme was able to cleave ester bond from glycerol backbone and release free fatty acids. This result was in concomitant with FFA content, which significantly increased after the storage of 30 days for all the samples (Figure 1). Moreover, lipid oxidation also contributes to the changes in fatty acid composition. After the 30 days of storage, changes in fatty acid profile of unsaturated fatty acids were observed. The results were coincidental with the increased oxidation of lipids after 30 days of storage as observed by the increases in PV, TBARS, AnV and totox value of all samples (Figure 2 A, B, C, D). The results suggested that changes in fatty acid compositions were mainly governed by hydrolysis and oxidation process during the extended storage.

3.5. Volatile compounds

Selected volatile compounds in V-F10 sample at day 0 and day 30 of storage at ambient temperature are shown in Table 3. Generally, volatile compounds noticed in mayonnaise at day 0 were lower in abundance than those found at 30 days. At day 0 of storage, hexanal was found as the major compound in the sample. Hexanal has been reported as a good indicator of lipid oxidation (Fuller *et al*., 1992). The result indicated that oxidation occurred before or during mayonnaise preparation. Other aldehydes including 3-methyl-butanal, pentanal, benzaldehyde, heptenal, propanal, (E, E)-2,4-heptadienal and octanal were also found at the low levels. Hexanal and heptenal were major compounds, which contributed to rancid odor and fishy odor (Yarnpakdee *et al*., 2012). Moreover, volatile alcohols (1-pentanol and 1-cyclobutylcyclopropanol) and furans (2-ethyl-furan and 2-pentyl-furan) were found at low abundance at day 0. However, ketones were not detected at day 0 and 30.

After storage of 30 days, higher formation of volatile compounds was noticeable. Aldehydes were the most prominent volatiles detected in the samples. Aldehydes, ketones and alcohols have been known to be related with oxidation of lipids (Sae-leaw and Benjakul, 2014), which might occur during storage. Furans were increased after 30 days of storage. Furans and their derivatives such as 2-ethyl-furan and 2-pentyl-furan are generated by the decomposition of hydroperoxide of EPA and DHA (Maqsood and Benjakul, 2011). The results were in agreement with the increases in PV and decreases in PUFA in V-F10 sample. DHA and EPA are prone to oxidation and may cause off-flavor in mayonnaise samples owing to low sensory threshold values of oxidation products (Depree and Savage, 2001).

Table 2. Fatty acid profile of mayonnaise containing different oils at different storage time.

Fatty acids (g/100 g oil)	S		V		V-F5		V-F10		V-F15	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
C6:0 (Caproic)	ND	ND	0.45±0.00a	0.38±0.00b	0.41±0.00a	0.37±0.00b	0.38±0.00a	0.35±0.00b	0.36±0.00a	0.33±0.00b
C8:0 (Caprylic)	ND	ND	6.34±0.00a	5.91±0.00b	5.91±0.01a	5.69±0.00b	5.53±0.00a	5.35±0.01b	5.18±0.00a	5.05±0.00b
C10:0 (Capric)	ND	ND	5.49±0.01a	5.32±0.00b	5.15±0.00a	5.09±0.00b	4.83±0.00a	4.78±0.00b	4.52±0.00a	4.48±0.00b
C12:0 (Lauric acid)	0.30±0.00a	0.07±0.00b	47.05±0.10a	43.09±0.03b	44.14±0.02a	41.62±0.20b	41.62±0.02a	39.15±0.01b	38.81±0.02a	36.44±0.01b
C14:0 (Myristic acid)	0.23±0.00a	0.1±0.00b	19.57±0.05a	16.66±0.00b	18.61±0.01a	16.65±0.01b	17.80±0.01a	15.90±0.02b	16.81±0.00a	14.99±0.01b
C15:0 (Pentadecanoic)	ND	ND	ND	ND	ND	0.04±0.00	0.06±0.00b	0.07±0.00a	0.09±0.00a	0.09±0.00a
C16:0 (Palmitic)	11.16±0.00a	10.63±0.00b	9.55±0.01b	10.00±0.00a	10.49±0.01b	10.93±0.01a	11.15±0.00b	11.66±0.01a	11.88±0.00b	12.37±0.02a
C16:1 (Palmitoleic)	0.24±0.00a	0.08±0.00b	ND	0.20±0.00	0.37±0.00b	0.51±0.00a	0.64±0.00b	0.82±0.00a	0.95±0.00b	1.09±0.00a
C17:0 (Heptadecanoic)	0.10±0.00a	0.09±0.00b	ND	ND	ND	ND	0.062±0.00b	0.07±0.00a	0.09±0.00b	0.10±0.00
C18:0 (Stearic)	4.17±0.00a	4.12±0.00b	3.55±0.00a	3.45±0.00b	3.70±0.00a	3.58±0.00b	3.80±0.00a	3.69±0.00a	3.91±0.00a	3.85±0.00b
C18:1 cis 9 (Oleic)	23.47±0.00b	24.18±0.00a	5.78±0.00b	9.25±0.00a	7.26±0.2b	10.01±0.10a	8.54±0.00b	11.02±0.02a	9.68±0.00b	11.98±0.00a
C18:2 cis 9,12 (Linoleic)	50.07±0.00b	52.90±0.00a	1.13±0.00b	4.07±0.01a	1.85±0.00b	3.08±0.00a	2.38±0.00b	3.42±0.00a	2.99±0.00b	3.99±0.00a
C20:0 (Arachidic)	0.32±0.00b	0.34±0.00a	0.10±0.00a	0.09±0.00b	0.11±0.00a	0.10±0.00b	0.13±0.00a	0.10±0.00b	0.13±0.00a	0.12±0.00b
C18:3 cis 6,9,12 gamma (gramma-Linolenic)	0.49±0.00b	0.53±0.00a	ND	ND	ND	ND	0.06±0.00b	0.07±0.00a	0.10±0.00a	0.10±0.00a
C20:1 cis 11 (cis-11-Eicosenoic)	0.47±0.00b	0.50±0.00a	ND	ND	0.092±0.00a	0.09±0.00b	0.06±0.00a	0.05±0.00b	0.08±0.00a	0.08±0.00a
C18:3 cis 9,12,15 alpha (alpha-Linolenic)	5.28±0.00b	5.58±0.00a	ND	0.24±0.00	0.09±0.00b	0.13±0.00a	0.16±0.00b	0.19±0.00a	0.23±0.00b	0.26±0.00a
C20:0 (Docosanoic)	0.36±0.00b	0.38±0.00a	ND	ND	ND	ND	ND	ND	ND	ND
C20:2 cis 11,14 (cis-11,14-Eicosadienoic)	ND	ND	ND	ND	ND	ND	0.11±0.00b	0.12±0.00a	0.16±0.00b	0.17±0.00a
C20:3 cis 8,11,14 (cis-8,11,14-Eicosatrienoic)	ND	ND	ND	ND	ND	ND	ND	ND	0.06±0.00a	0.06±0.00a
C22:1 cis 13 (Erucanoic)	ND	ND	ND	ND	ND	ND	0.06±0.00a	0.06±0.00a	0.09±0.00a	0.09±0.00a
C23:0 (Tricosanoic)	ND	ND	ND	ND	0.06±0.00b	0.10±0.00a	0.12±0.00b	0.16±0.00a	0.18±0.00b	0.23±0.00a
C24:0 (Lignoceric)	0.11±0.00b	0.12±0.00a	ND	ND	ND	ND	ND	ND	ND	ND

C20:5 cis 5,8,11,14,17 EPA (cis-5,8,11,14,17-Eicosapentaenoic)	ND	ND	ND	ND	0.21±0.00a	0.19±0.00b	0.39±0.00a	0.38±0.00b	0.59±0.00a	0.57±0.00b
C22:6 cis 4,710,13,16,19 DHA (cis-4,710,13,16,19-Docosahexaenoic)	ND	ND	ND	ND	0.42±0.00a	0.38±0.00b	0.75±0.00a	0.73±0.00	1.13±0.00a	1.10±0.00b

ND: not detected. S: mayonnaise containing soybean oil, V: mayonnaise containing virgin coconut oil (VCO), V-F5: mayonnaise containing VCO (95%) + fish oil (5%), V-F10: mayonnaise containing VCO (90%) + fish oil (10%), V-F15: mayonnaise containing VCO (85%) + fish oil (15%). Values are mean ± standard deviation (n=3). Different lowercase letters in the same row under the same sample indicate significant difference ($p<0.05$).

Table 3. Volatile compounds in mayonnaise containing VCO/FO (90:10) blend at day 0 and day 30 of storage at room temperature

Volatile compounds	Peak area (Abundance) x10^5	
	day 0	day 30
Furans		
2-Ethyl-furan	8.15	183.69
2-Pentyl-furan	5.70	79.53
Aldehydes		
Propanal	4.39	7.52
2-Methyl-butanal	ND	ND
3-Methyl-butanal	11.15	63.31
Pentanal	7.81	283.20
Hexanal	113.16	682.89
(E)-2-Hexenal	ND	ND
Heptenal	4.78	68.49
(E, E)-2,4-heptadienal	2.37	11.06
Octanal	1.51	11.15
(E)-2-decenal	ND	ND
Benzaldehyde	6.38	35.49
Alcohols		
1-Cyclobutylcyclopropanol	3.40	ND
1-Pentanol	10.59	3.68
(Z)-2-pentenol	ND	ND
(E)-2-hexenol	ND	ND
1-Methyl-4-(1-methylethenyl)-cyclohexanol	ND	ND
1-Octen-3-ol	1.70	ND
(Z)-1,5-octadien-3-ol	ND	ND

ND: not detectable

4. Conclusions

VCO could be used instead of SO for the production of mayonnaise. Addition of FO up to 10% in VCO/FO blend could yield the mayonnaise with sensorial acceptability. However, masking agent or potential removal of off-odor was still required when FO higher than 10% was added. Type of oil used for preparation of mayonnaise affected FFA content of resulting mayonnaise. Oxidative stability varied with mayonnaises containing different oils. Mayonnaise sample with VCO was less prone to lipid oxidation throughout storage of 30 days. Overall, VCO in combination with FO at an appropriate ratio could be used instead of SO to prepare a functional mayonnaise with increased oxidative stability.

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EFFECT OF THERMAL PROCESSING AND FERMENTATION ON THE CHEMICAL COMPOSITION, PROTEIN DIGESTIBILITY AND FUNCTIONAL PROPERTIES OF BAMBARA PROTEIN ISOLATE

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Abstract

Protein isolate offers huge opportunity in the expansion of a new class of formulated foods. In this study, the effect of thermal processing and fermentation on the chemical composition, protein digestibility and functional properties of Bambara nut protein isolate were investigated. Thermal processing reduced the yield of the protein (53.4% and 51.6%) while fermentation increased the yield of the protein (54%) when compared to the raw sample (53.8%). Protein digestibility of the isolate significantly increased due to thermal processing and fermentation. The proximate composition of the isolate revealed that fermentation increased the crude protein content of protein isolate (83.2%) compared to the raw sample (82.3%) while all the processing method used increased the ash content significantly. Processing had no effect on the water absorption capacity, foaming capacity and stability of the protein isolate. However, the emulsion stability of all the samples improved with processing. Protein digestibility, yield, protein content, and emulsion stability were improved by processing especially fermentation, these attributes may improve the possible use of Bambara nut protein isolates as an excellent protein ingredient in the food system.

1. Introduction

The emphasis of our research recently has been on the use of vegetable proteins for the formulation of new food products and functional foods. This is because animal protein is still luxurious in developing countries than plant protein (Arise et al., 2015). However, in order to advance in the use of vegetable proteins for food ingredients, their physicochemical and functional properties must be assessed. Many legumes consumed in Africa are important sources of nutrients, especially proteins and a good source of complex carbohydrates as reported by many studies which makes them a perfect food to combat malnutrition (Ogodo et al., 2017).

Bambara groundnut *(Vigna subterranean* (L.) Verdc.) is a native of African legume that has been cultivated in Africa for periods. It originated from the Sahelian region which is the present-day West Africa with its name comes from the Bambara tribe who lives in Mali (Boateng et al., 2013). In Africa, Bambara has been classified as the third most significant legume after groundnut (*Arachis hypogea*) and Cowpea (*Vigna ugniculata*) (Adebowale et al., 2007, Arise et al., 2015). The protein content of Bambara groundnut varies between 15-27% (Arise et al., 2015, Murevanhema and Jideani, 2013).It is interesting to note that Bambara seed grain contains a good balance of essential amino acid with a relatively high proportion of lysine (6.5-6.8%) and a considerable amount of methionine which is unusual in other legumes (Arise et al., 2017). A major attribute of Bambara groundnut is its forbearance to drought, poor soils and ability to yield in conditions where other legumes fail entirely.

It can moderately resist pest and disease, this gives it a greater advantage over soybean and groundnut (Mazahib et al., 2013). Processing through heat application and fermentation has been stated to reduce the antinutritional influences and toxic contents in nuts and legumes thereby exposing the seed to utilization (Arinola and Adesina, 2014, Iyenegbe et al., 2017). Roasting, apart from lowering the antinutrients, has also been found to improve the palatability and nutrients of food products thereby increasing shelf life. The chemical composition, as well as functional properties of cashew nut, were stated to have been enhanced by processing methods (Fagbemi et al., 2006). Protein extracts have superior functions and are more successfully used in the formulation of foods compared to cereal flour (Boye et al., 2010). Protein isolate offers enormous opportunity in the development of a new class of formulated foods. The high concentration of protein results in colour, flavour and functional properties of greater value which makes protein isolates an ideal raw material for use in beverages, infants & children milk food, textured protein products and certain types of specialty foods (Boye et al., 2010).Therefore, the aim of this study is to investigate the effect of thermal processing and fermentation on the protein digestibility, chemical and functional properties of the protein isolates.

2. Materials and methods

2.1.Materials

Bambara groundnut was obtained from *Oja-Oba* market in Ilorin. The seeds were screened to remove defective seeds. Other materials include; bowls, weighing scale, polythene bags, electric oven, autoclave, and milling machine. All apparatus, chemical, and equipment used were obtained from the food processing laboratory in the department of Home Economics and Food Science, University of Ilorin. The nuts were washed to remove dirt, dehulled and divided into four parts. A part was boiled at 100^{o}C for 30 minutes, another portion was roasted at 120^{o}C for 45 minutes and the third part was fermented in water for 4 days at room temperature while the fourth part was used in the raw form. The de-hulled seed was milled into flour using hammer mill and then sieved through a 0.4 mm mesh size, packaged in high-density polyethylene bag and stored in a refrigerator at 4^{o}C till further used.

2.2. Preparation of defatted Bambara nut flour and protein isolates

The various samples of Bambara nut were defatted using the method of Arise et al. (2015). Briefly, Bambara seed flours were each defatted with n-hexane in the ratio 1:5 (flour: solvent) for 3 hours. Defatted flours were placed in a fume hood overnight to remove the remaining hexane. The fat content was less than 0.01% after defatting using the soxhlet extractor. Protein content (N x 6.25) of the defatted flours was determined using the Kjedahl method (AOAC, 2012). Protein isolates were prepared from the defatted flour samples using isoelectric precipitation method as described by Arise et al. (2015) with slight modification. Precisely, the defatted flour was mixed with distilled water (1:10 w/v) and the pH of the mixture was adjusted to 9.0 with 1 M NaOH so as to facilitate protein solubilization. The suspension was stirred for 4 h at 32 °C in a shaking water bath. Centrifugation was thereafter carried out. The centrifuge was set at 4000 *g* for 30 min at 4 °C. After centrifugation and recovery of the supernatant, the precipitate was resuspended in half the volume of initial water and extraction was carried out as described above. The supernatants were pooled together and pH adjusted to 4.0 with 0.5 M HCL to precipitate the protein concentrates, which were recovered by centrifugation at 5000 *g* for 30 min at 4 °C. The precipitated protein was washed in three volumes of distilled water and neutralized to pH 7 with 1M NaOH. The neutralized protein isolate was freeze-dried, the resulting powder was held in a tight container and stored at 4 ^{o}C for further analysis.

2.3. Proximate composition of the isolates

Moisture, fat, ash and protein contents were determined using AOAC methods (AOAC, 2012). The micro kjeldahl nitrogen method was used in the determination of the crude protein

content. A conversion factor of 6.25 was used to convert the nitrogen content to protein. The carbohydrate content was determined by difference (100-[moisture + total ash + crude fat + crude fibre + protein]).

2.4. Protein yield

The yield of protein concentrate was determined as the dry weight of protein concentrate after precipitation and solubilization respectively per weight of the defatted flour as shown below (Arise et al., 2015)

$$Yield = \frac{protein\ concentrate\ recovery\ X\ protein\ content\ of\ concentrate\ (\%) \times 100}{Protein\ content\ of\ defatted\ flour\ (\%)} \quad \text{(Eq.1)}$$

2.5. Determination of the *in-vitro* Protein Digestibility

In-vitro protein digestibility was carried out by the method of Chavan et al. (2001). A known weight sample containing 16 mg nitrogen was taken in triplicate and hydrolysed with 1 mg pepsin in 15 ml of 10% (w/v) trichloroacetic acid (TCA). The mixture was then filtered quantitatively through Whatman No. 1 filter paper. The TCA soluble fraction was assayed for nitrogen micro-Kjedahl method. Digestibility was calculated using the following formula:

$$Protein\ digestibility\ \% = \frac{(N\ in\ supernatant - N\ in\ blank)}{N\ in\ sample} \times 100 \quad \text{(Eq.2)}$$

2.6. Colour parameters

Colour of Bambara protein isolates were measured at room temperature using a Chroma Meter CR-400 (Minolta Co. LTD. Japan). Colour analysis was done by putting the device on the freeze-drying Bambara protein isolate at least in triplicates. Colour of isolate was measured as the average of 3 readings for each sample after calibrating the instrument. Colour was measured as lightness (L*), redness (a*), and yellowness (b*). Whiteness was calculated by the equations: L* -3b as referred by Codex Alimentarius

2.7. Functional properties

2.7.1. Water and Oil absorption capacity

The water absorption capacity (WAC) and oil absorption capacity (OAC) were determined as described by Arise et al. (2015). Briefly, sample (1.0 g) was weighed into a 15 ml pre-weighed centrifuge tube to which 10 ml of distilled water (for WAC) or 10 ml of soybean oil (for OAC) was added stepwise with continuous stirring at room temperature for 10 min. The suspension in the tube was centrifuged at 2,500 × g for 20 min and the volume of supernatant measured. The WAC or OAC was calculated as the difference between the initial volume of water or oil used and the final volume of the decanted supernatant and calculated in percentages, taking into consideration the density of the oil.

2.7.2. Least gelation concentration

The least gelation concentration (LGC) was determined according to the method described by Abbey and Ibeh (1988). The sample was weighed and mixed with 5 ml of distilled water in a test tube to obtain 2%–20% (w/v) concentrations. The test tube was heated for 1 hr. in a boiling water bath followed by rapid cooling under running tap water and further cooled for 2 hr. in a refrigerator at 4 °C. The LGC was regarded as the least concentration at which the sample from the inverted tube did not fall or slip.

2.7.3. Foaming Capacity and Stability

Foaming capacity (FC) and foaming stability (FS) were carried out as described by Arise et al., (2015). The sample (0.5 g) was dispersed in 50 ml of distilled water in a 100 ml graduated cylinder and the solutions homogenized at a speed of 1,600 × g for 5 min. The volume was recorded before and after whipping. FC was expressed as the volume (%) increase due to whipping. This was then stored for 1 hr and the foam- volume changes in the graduated cylinder were recorded as FS.

$$\text{Foaming capacity} = \frac{Volume\ after\ homogenization - Volume\ before\ homogenization}{Volume\ before\ homogenization}\ X\ 100 \quad \text{(Eq.3)}$$

2.7.4.Emulsion capacity (EC) and Stability

This was determined according to the method of Chavan et al. (2001). One gram of the sample in 25 ml distilled water was homogenized at a speed of 5,000 × *g* for 1 min at 27 °C. The protein solution was then mixed with 25 ml of soybean oil followed by homogenization at 10,000 × *g* for 1 min. The emulsion volume was then used in calculating the EC as shown below:

$$\text{Emulsifying capacity (\%)} = \frac{Height\ of\ emulsified\ layer\ in\ the\ tube}{Height\ of\ the\ total\ content\ in\ the\ tube} \text{ X } 100 \quad \text{(Eq.4)}$$

The emulsion stability (ES) was measured by re-centrifugation as above and heating for 30 min at 80°C. The ES was calculated as shown below

$$\text{Emulsifying stability (\%)} = \frac{Height\ of\ remaining\ emulsion\ layer}{Height\ of\ initial\ emulsion\ layer} \text{ X } 100 \quad \text{(Eq.5)}$$

2.8. Statistical analysis

Statistical analysis Experiments were conducted in triplicate. Mean scores of some of the results and their standard deviation were reported. Data were subjected to analysis of variances, and Duncan multiple range test was used to separate the means (Duncan, 1955)

3. Results and discussion

3.1. Protein yield of the isolates

Protein yield of Bambara protein isolate ranges from 51.60 to 54.00% (table 1). The protein yield of the isolates obtained in this research is in agreement with the result obtained by Arise et al. (2015) which ranges from 49.50 to 54.50%. However, the result obtained is higher than 27.05-29.41% reported for two Bambara nut landraces by Boateng et al. (2013). High protein yield and content gotten in this study may be attributed to adjustment in pH using NaOH (pH 9.5), which may enhance extraction. Protein has been stated to display higher solubility at pH above their isoelectric point (Adebowale et al., 2007). This study shows that the processing method increased the yield of the Bambara nut protein isolate. The yield of the fermented and roasted sample increased compared to the raw isolate while the yield of the boiled sample reduced. The low yield recorded in the boiled sample may be due to leaching of some soluble components in water coupled with denaturation by heat.

Table 1. Protein yield of the isolates

Sample	Protein Yield (%)
Raw	$53.8^{b} \pm 0.1$
Roasted	$53.4^{b} \pm 0.4$
Fermented	$54.0^{c} \pm 0.1$
Boiled	$51.6^{a} \pm 0.3$

Values with similar superscripts in a column do not differ significantly (P <0.05).

Table 2. Proximate Composition of Raw and Processed Bambara Groundnut Protein isolate

Samples	Crude protein (%)	Moisture content (%)	Ash Content (%)	Fat (%)	Crude fibre (%)	Carbohydrate (%)
Raw	$82.3^{c}\pm2.0$	$3.2^{b}\pm0.0$	$3.0^{a}\pm0.1$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$11.5^{b}\pm0.0$
Fermented	$83.2^{d}\pm0.8$	$3.2^{b}\pm0.0$	$4.7^{d}\pm0.1$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$8.9^{a}\pm0.0$
Roasted	$80.4^{a}\pm1.2$	$3.0^{a}\pm0.0$	$4.5^{c}\pm0.2$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$12.1^{b}\pm0.0$
Boiled	$81.3^{b}\pm0.5$	$3.5^{c}\pm0.0$	$3.8^{b}\pm0.2$	$0.0^{a}\pm0.0$	$0.0^{a}\pm0.0$	$11.4^{b}\pm0.0$

Values with similar superscripts in a column do not differ significantly (P <0.05).

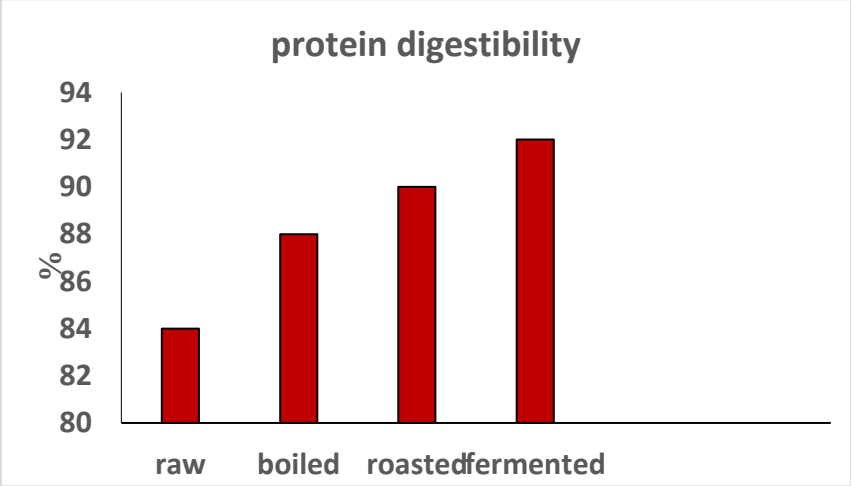

Figure 1. *In-vitro* protein digestibility of Bambara groundnut protein isolate

3.2. Proximate composition of the protein isolates

The effect of processing (boiling, roasting, and fermentation) on the proximate composition of Bambara groundnut protein isolate on a dry weight basis are presented in table 2. The result revealed that boiling, roasting, and fermentation significantly affect the crude proteins, ash and moisture content. The samples had high protein content ranging from 80 to 83%. These values were lower compared to the 86% protein reported by Eltayeb et al. (2011) and higher than the 80% reported by Gbadamosi et al. (2012). The result for the protein content of all protein isolates was consistent with criteria for legume protein isolates, in which protein must be higher than 70% (Butt and Batool, 2010). The high protein content recorded can be considered for protein supplements in human. There was a significant difference among the samples in ash content. The protein isolates contained ash content that ranges from 3 to 5%. It can be inferred from this result that ash content reduces on soaking/steeping in water as in the case of fermented isolate and increases due to the application of heat, which come to an agreement with the findings of Abdulsalami and Sheriff (2010), for Bambara groundnut seeds. Processing had no significant effect on the crude fiber, fat and carbohydrate content of the isolate. This result is in line with what was reported by Iyenagbe et al. (2017) for boiled and roasted conophor nuts.

3.3. In-vitro protein digestibility of the isolates

The effect of fermentation and thermal processing on the percentage *in-vitro* protein digestibility of the Bambara groundnut protein isolate samples is shown in figure 1. The values range from 84 to 92%, this shows that processing of Bambara nut protein isolate enhances its digestibility. All processing methods used, led to an increase in the digestibility of protein. However, among the three samples, the fermented sample gave the uppermost value of 92% while the boiled sample gave the lowest. The changes in the *in-vitro* protein digestibility differ significantly ($p<0.05$) in the processed sample compared to the raw sample. The increase in protein digestibility compares favorably with the finding of Ogodo et al. (2017), especially for the fermented sample. This author reported that fermentation increased protein digestibility from 68.70% to 85.24%. Improvements in the protein digestibility of the protein isolates may be due to the modifications that occur in protein during n atural fermentation.

Table 3. Colour parameter of Bambara groundnut Protein isolate

Samples	L*	a*	b *
Raw	65.8^{ab}±2.3	8.4^{b}±0.3	6.3^{ab}±0.3
Fermented	71.5^{a}±4.4	7.9^{b}±0.5	7.4^{b}±0.7
Roasted	57.3^{c} ±6.2	10.1^{a}±0.5	6.3^{ab}±1.6
Boiled	62.2^{bc}±2.3	8.1^{b}±0.2	5.0^{a}±0.7

Mean±SD. Mean with different superscript along the column are significantly different (p<0.05)

3.4. Colour parameters

The effect of thermal processing and fermentation on the colour parameters of Bambara protein isolate are presented in table 3. All colour data were expressed by hunter l, a and b values corresponding to lightness, redness, and yellowness respectively. The colour of the Bambara protein isolate was significantly affected by the processing method employed. The roasted sample seemed slightly brown in colour with low lightness (l*), high redness (a*) and yellowness (b*) values, the other boiled and fermented sample have a brighter colour with increased lightness (l*), low redness (a*) and yellowness (b*). This result agrees with the findings of Nikmaram et al. (2011) who worked on the effect of cooking methods on veal muscle. The study revealed that roasting reduced brightness and increased redness on the surface of the meat due to the formation of dark pigments while boiling improved the brightness.

3.5. The water absorption capacity of Bambara protein isolate

The values gotten for the WAC of the raw, roasted, fermented, and boiled Bambara nuts protein isolates are 218.50%, 159.00%, 135.50%, and 185.50% respectively as shown in table 4. The raw sample gave the highest WAC which implies that pre-treating the samples had no effect on the WAC of the protein isolates. The value obtained in this study for WAC for the raw Bambara nuts protein isolate are higher than 205.22% for peanuts protein isolate by Sibt-e-Abbas et al. (2015), 174.65% for Bambara protein isolate by Adeleke et al. (2018) but similar to 221.83% reported for Bambara protein isolate by Eltayeb et al. (2011). The higher WAC of raw Bambara protein isolate may be due to the higher polar amino acid residues of Bambara proteins having an affinity for water molecules. A high WAC was observed in the fermented sample of protein isolates which may be attributed to the water binding sites on the side chain groups of protein units (Adebowale et al., 2007). The increased WAC in fermented sample suggests their use in aqueous food formulations especially those involving dough formation like bread and cookies (Adebowale et al., 2011).

3.6. The oil absorption capacity of Bambara protein isolate

The OAC of the raw, roasted, fermented and boiled is 56.00%, 72.00%, 72.00% and 95.50% respectively (table 4). The protein-protein interaction could have been aided by the high protein content in the concentrate which could be responsible for the increase in OAC of the isolates. All the processing methods used in this study increased the OAC with the boiled sample having the highest OAC while roasted and fermented samples have the same oil absorption capacity. Oil absorption capacity is an essential parameter since oil increase the mouth feel of food and act as flavour retainer in certain food products. Although the values for OAC obtained in this study are lower compared to 100.67% obtained from peanuts (Sibt-e-Abbas et al., 2015), the Bambara protein isolate still has the possibility of been used as an ingredient in pie-filling, meat extenders and meat substitutes for sausages.

Table 4. Functional properties of processed Bambara nuts protein isolates

Samples	Raw	Roasted	Fermented	Boiled
WAC %	218.50±2.1[a]	159.00±1.4[a]	135.50±0.9[a]	185.50±0.5[a]
OAC %	56.00±0.9[c]	72.00±2.83[b]	72.00±0.0[c]	95.50±0.7[b]
FC %	15.00±0.0[d]	5.00±0.0[f]	10.00±0.0[e]	10.00±0.0[e]
EC %	15.00±0.0[d]	15.00±0.0[d]	16.50±0.7[d]	14.00±0.0[d]
ES %	5.50±0.7[b]	7.50.00±0.7[e]	8.00±0.00[f]	6.50±0.7[f]
LGC %	62.00±0.0[b]	42.00±0.0[c]	83.00±0.0[b]	42.00±0.0[c]

In each of the rows, any means not followed by the same superscripts are significantly different (p<0.05>) Sample Keys**:** WAC: Water absorption capacity, OAC: Oil absorption capacity ,FC: Foaming capacity EC: Emulsion capacity**,** ES: emulsion stability**,** LGC: Least gelation concentration

3.7. Foaming capacity and foaming stability

Foam is a colloid of many gas foams trapped in a liquid or solid. Small air bubbles are surrounded by thin liquid films. Foam can be produced by whipping air into a liquid as much and as fast as possible (Sikorski, 2006). The values obtained for FC of the isolates ranged from 5.00% to 15.00%. The values obtained are low and are not improved with processing, this may be due to the pre-treatment (roasted, boiled, fermented) which may have destroyed the structure of the protein preventing it from forming a cohesive visco-elastic film, or it may have been from the preparations of the samples. There was no foam stability observed after 10 mins. This indicates that the pre-treated Bambara protein isolate has very poor foaming capacity and stability.

3.8. Emulsion capacity and stability

Emulsion capacities of the raw, roasted, fermented and boiled are 15.00%, 15.00%, 16.50% and 14.00% respectively, Whereas the values for emulsion stability of the protein isolates are 5.50%, 7.50%, 8.00%, and 6.50% respectively. The protein isolates emulsion capacity and stability were significantly increased by roasting and fermentation. A similar observation was observed for conophor nut protein isolates (Iyenagbe, et al., 2017). The emulsion capacity and stability observed in this study points out that it can be used as an ingredient in mayonnaise and sausage in the food industry.

3.9. Least gelation concentration

The values obtained for LGC in this study are in the ranges of 42.00% - 83.00% as shown in table 4 below. The roasted and boiled samples have the lowest LGC for the protein isolate. With these results, it shows that Bambara nut protein isolate may not be a good gelling agent when compared to soybean protein isolates (Padilla et al., 1996) and hemp protein isolate (Malomo et al., 2015). However, the protein gel texture can be improved by the addition of non – protein component. For instance, an improvement in protein gel texture was also reported when carbohydrates were added (Adebowale et al., 2007).

4. Conclusions

The results obtained from this research shows that fermentation is the most suitable processing method. The fermented sample gave the highest yield for protein isolate, also in terms of the nutritional composition of the isolate, the fermented sample had the highest protein and ash content. Furthermore, in terms of functional properties, the fermented sample had a better emulsion capacity and stability. Generally, thermal processing and fermentation resulted in an increase in the digestibility of the protein isolate. Therefore, we can conclude that raw Bambara nut should undergo fermentation before protein isolation for use in the food industry.

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USE OF CHITOSAN AND XANTHAN GUMS TO EXTEND THE SHELF LIFE OF MINIMALLY PROCESSED BROCCOLI (*Brassica oleracea L. Italica)*

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Abstract

The objective of this work was to apply edible coatings based on xanthan and chitosan gums in minimally processed broccoli and evaluate their physical, chemical, microbiological and sensory characteristics, during 12 days of refrigerated storage. The coatings were applied to the florets of broccoli. After the application of coatings, the broccoli heads were stored under refrigeration. 3 treatments were generated, being T1 the control treatment, T2 treatment with xanthan (1.5%) and T3 treatment with chitosan (1.5%). Of the evaluated treatments, the one which contained chitosan was the best in relation to the attributes of color, texture, loss of vitamin C and weight and also in relation to the sensory attributes when compared with the other treatments. The treatment containing xanthan was also efficient when compared with the control sample for all the performed analyzes. The presence of total coliforms and thermotolerant coliforms and *Escherichia coli*, as well as *Salmonella* for all evaluated treatments was not detected. Of the researched coatings, the treatment with chitosan, was the one that presented the best results, showing that they were able to reduce the microbial growth and extend the life-span of minimally processed broccoli for up to 12 days in refrigerated storage.

1. Introduction

Broccoli (*Brassica oleracea L. Italica*), are plant species with low calories, which contains a high content of components (Caleb *et al.*, 2016), but their metabolism is relatively high (Esturk *et al.*, 2014), causing them to be highly perishable after harvesting, and this can be noticed due to loss of green coloring and yellowing. Various types of storage and techniques of treatments have been tested with the aim of improving the quality of post-harvest and extend the broccoli's lifespan (Li *et al.*, 2014).

The production and consumption of minimally processed foods have become popular. Thus, fruits and vegetables have pleased consumers due to their interest in natural products and a change of life style (Yousuf and Srivastava, 2015).

Due to the increase in the preference of consumers in relation to food ready for consumption, the economic importance in the industries of fresh fruits has become increasingly significant. The main target of the fresh products technology is to provide consumers with ease and products with nutritional and sensory desirable characteristics (Yousuf *et al.*, 2018).

An alternative technology to increase the life-span of minimally processed vegetables is the employment of edible coatings. These coatings are not intended to replace the use of conventional materials of packaging or even permanently eliminate the use of low temperatures, but present a functional performance and supporting role, contributing to the preservation of the texture and nutritional value, reducing the gaseous exchange surface and the excessive loss or

gain of water (Assis and Britto, 2014). Various materials have been used in the production of edible films, including gelatin, starch, gum, pectin and chitosan (Palou *et al.*, 2015).

Chitosan is a linear polysaccharide derived from the chitin deacetylation. This component can be used for the development of edible coatings at industrial level due to their biofuncional, biodegradable, biocompatible, non-toxic, film formator and antimicrobial characteristics (Sun *et al.*, 2016).

The xanthan gum is a high molecular weight polysaccharide obtained from the fermentation of bacteria *Xanthomonas campestris*. When added in aqueous medium gives stability to heat and the acidic and basic conditions, due to its rigid structure. The xanthan gum is an important component when mixed with other biopolymers due to the synergism of its viscosity as a result of intermolecular interaction with other materials (Bak and Yoo, 2018; Jo *et al.*, 2018).

Edible coatings could assist in the conservation of minimally processed vegetables. Thus, the objective of this work was to apply edible coatings based on xanthan and chitosan gums in minimally processed broccoli and evaluate their physical, chemical, microbiological and sensory characteristics, during 12 days of refrigerated storage at a temperature of 4 ±1 °C.

2. Material and methods

2.1. Material

The broccoli (*Brassica oleracea L*. var. *Italica*) used was purchased at local shops of the city of Dourados, MS, and was selected according to the weight (approximately 0.4 kg), color and appearance. The broccoli was transported in Styrofoam boxes, where they were stored at a temperature of 4±1 °C for 12 hours until the achievement of minimal processing.

2.2 Methods

2.2.1. Preparation of samples and coatings

The processing was carried out at a temperature of approximately 10 °C, with the utensils previously sanitized in organic chlorine solution (sodium dichloroisocyanurate), at a concentration of 2 g L^{-1}. The broccoli was sanitized in organic chlorine solution at a concentration of 0.2 g L^{-1}, for 15 minutes and then the florets were cut using a stainless-steel knife. Then, the pieces were rinsed with chlorinated water (0.2 g L^{-1}) in order to eliminate the cellular overflowed juice. The water was drained for 2 to 3 minutes on sieves and the florets were reserved until the preparation of the coatings.

The coatings used were xanthan (Shandong Fufeng) and chitosan (Polymar®) gums.

For the xanthan-based coating, the same was dissolved slowly in the proportion of 1.5% in water at a temperature of 25 °C, under agitation, after its complete dissolution, it was heated at 60 °C for 20 minutes.

For the 1.5% chitosan-based coating, a dissolution in acetic acid solution 1% (v/v) was performed, and the same was agitated for 30 minutes at ambient temperature.

After the preparation of the coatings, the florets of broccoli were completely submerged in the solutions prepared for 5 minutes and then drained using sieves. They were waited to dry in an oven with air circulation at ambient temperature for 10 minutes. Then they were stored in PET packaging - Polyethylene terephthalate, with cover (SANPACK).

Three treatments were generated: T1 - control treatment (only the florets of broccoli without cover; T2 - Treatment using 1.5% xanthan gum and T3 - Treatment using 1.5% chitosan gum.

The packages were stored under refrigeration at 4±1 °C for a period of 12 days (day zero to day 12). Being that the physical, chemical, microbiological and sensory analyzes were performed at the time of storage 0, 1, 3, 5, 7, 9 and 12 days. Each day of analysis generated data independent of each other, following a normal distribution.

2.2.2. Physical, chemical and microbiological analysis

2.2.2.1. Texture analysis

The measures of texture of minimally processed florets of broccoli were determined using a texturometer (Stable Micro Systems model TA.XTplus), in accordance with the methodology of Chevalier *et al.* (2018).

2.2.2.2. pH analysis

For the pH analysis, approximately 20 g of samples were crushed in 100 mL of distilled water, and then the measurement was performed using a pHmeter Marconi (PA 200). The analysis was performed according to the official Method (AOAC, 2000).

2.2.2.3. Content of vitamin C

For the determination of the content of vitamin C the standard method of AOAC (2000) was used.

2.2.2.4. Loss of weight

For the loss of weight analysis, the methodology described by Cortez-Vega *et al.* (2014) was used.

2.2.2.5. Color analysis

The color analysis was determined according to the methodology of Chevalier *et al.* (2018), using a Minolta colorimeter (CR400) where random measures were taken on the external surface of the florets of broccoli. The parameters analyzed were luminosity (L*), Chromas a* and b*.

2.2.2.6. Microbiological analysis

Microbiological analyzes were performed for total coliforms and thermotolerant coliforms, *Escherichia coli*, *Salmonella*, aerobic mesophilic and molds and yeasts, during 12 days of storage following analytical methodology described by APHA (2001).

2.2.2.7. Sensory analysis

For the sensory analysis, the methodology described by Chevalier *et al.* (2018) was followed. 12 trained appraisers were used. For each treatment the following attributes were evaluated: texture, color, aroma and overall evaluation. It was established a scale that ranged from 5 to 3, where 5 meant sample of excellent quality (fresh, aromatic and without darkening); 4 meant regular (little fresh, less intense odor and moderate darkening); 3 the sample of poor quality (without freshness and intense odor and with a high degree of darkening and the presence of mold).

2.3. Statistical analysis

The research was carried out in a completely randomized design, with three treatments and twelve storage times. Data was subjected to an analysis of variance (ANOVA), with comparison of means by the Tukey test ($P < 0.05$), using the Statistica® 7.0 software (StatSoft, Inc., Tulsa, USA). All data was presented as mean ± standard error. All the assumptions of the analysis of variance were verified to ensure the validity of the statistical analysis.

3. Results and discussions

3.1. Physical and chemical analyzes

3.1.1. pH analysis

Table 1 presents the values of pH of minimally processed broccoli during storage for 12 days.

Table 1. pH of minimally processed broccoli for the different treatments in 12 days at 4 °C

Parameter analyzed	Days	Treatments		
		T1	T2	T3
pH	0	5.83 ± 0.15^{bA}	5.83 ± 0.15^{cA}	5.83 ± 0.15^{bA}
	1	5.87 ± 0.41^{bA}	5.89 ± 0.09^{bcA}	5.87 ± 0.07^{abA}
	3	6.02 ± 0.23^{bA}	5.93 ± 0.22^{abcA}	5.89 ± 0.21^{abA}
	5	6.38 ± 0.11^{abA}	6.08 ± 0.05^{abcB}	6.07 ± 0.09^{abB}
	7	6.75 ± 0.17^{aA}	6.31 ± 0.17^{aA}	6.24 ± 0.26^{abA}
	9	6.28 ± 0.32^{abA}	6.24 ± 0.14^{abA}	6.24 ± 0.03^{abA}
	12	$6.32\pm.016^{abA}$	6.31 ± 0.08^{aA}	6.30 ± 0.15^{aA}

Equal lowercase letters in the column and equal uppercase letters in line does not present a significant difference at the level of 5% ($p<0.05$) by the Tukey test. (T1) control; (T2) 1.5% xanthan; (T3) 1.5% chitosan.

In Table 1 it is possible to observe that at the beginning of the experiment, the broccoli pH value was 5.83. Value close to that (6.08) was reported by Padula *et al.* (2006) in minimally processed organic broccoli.

Between the fifth and seventh day there were more accentuated increases in pH values for both treatments, being that the control treatment showed the largest increase (6.75). These high values of pH may be associated to the use of organic acids in excess as a respiratory substrate (Araújo and Shirai, 2016). Thus, it is possible to deduce that the xanthan and chitosan coatings were effective as a barrier to oxygen diffusion, therefore reducing the rate of breathing and maintaining a pH more stable until the seventh day of storage.

It was observed that after the seventh day, there was a decrease of pH to the control treatment, this can be related with the microbial growth and production of acids that may occur during the deteriorating period.

3.1.2. Loss of weight

Table 2 presents the loss of weight of minimally processed broccoli during storage, for the different treatments.

There was an increase in the loss of weight of minimally processed broccoli. The control treatment (T1) showed the greatest loss (17.41%), followed by the treatment T2 (14.63%) and finally with smaller loss of weight in 12 days of treatment T3 (11.57%). With this, it is realized that the treatments T2 and T3 were more efficient to reduce the permeability to water vapor of the product to the environment. In their work on the impact of edible coating and shocks of moderate heat on the quality of minimally processed broccoli, Ansorena *et al.* (2011) observed that the loss of weight of broccoli without the coating was significantly higher than the coated broccoli. This observation agrees with the present study, because the use of coatings significantly decreased the loss of weight of the coated samples.

Araújo and Shirai (2016) in their work on the application of chitosan coating in minimally processed broccoli also observed that during storage there was a gradual increase in the percentage of loss of weight of the treatments.

The broccoli coated with chitosan of those authors, had a smaller loss of weight in relation to other coatings used, agreeing with the values of loss of weight of this work, which was also lower than the control treatment or when xanthan was used.

Table 2. Loss of weight (%) of minimally processed broccoli with different treatments during storage

Parameter analyzed	Days	Treatments		
		T1	**T2**	**T3**
Loss weight (%)	0	0 ± 0.0^{fA}	0 ± 0.0^{fA}	0 ± 0.0^{gA}
	1	2.78 ± 0.19^{eA}	1.31 ± 0.07^{fB}	1.42 ± 0.24^{fB}
	3	4.16 ± 0.42^{eA}	3.27 ± 0.16^{eA}	3.04 ± 0.31^{eA}
	5	5.93 ± 0.78^{dA}	5.78 ± 0.71^{dA}	5.16 ± 0.07^{dA}
	7	9.79 ± 0.21^{cA}	8.65 ± 0.15^{cB}	7.58 ± 0.13^{cC}
	9	13.16 ± 0.46^{bA}	12.03 ± 0.31^{bAB}	9.68 ± 0.09^{bB}
	12	17.41 ± 0.33^{aA}	14.63 ± 0.18^{aB}	11.57 ± 0.27^{aC}

Equal lowercase letters in the column and equal uppercase letters in line does not present a significant difference at the level of 5% ($p<0.05$) by the Tukey test. (T1) control; (T2) 1.5% xanthan; (T3) 1.5% chitosan.

Table 3. Content of ascorbic acid in minimally processed broccoli with different treatments during storage

Parameter analyzed	Days	Treatments		
		T1	T2	T3
Ascorbic acid content (mg/100g of broccoli)	0	117.14 ± 0.57^{aA}	117.14 ± 0.57^{aA}	117.14 ± 0.57^{aA}
	1	101.25 ± 0.71^{bB}	112.16 ± 0.47^{bA}	113.48 ± 0.37^{bA}
	3	70.18 ± 0.83^{cC}	83.15 ± 0.88^{cA}	80.98 ± 0.22^{cB}
	5	68.51 ± 0.24^{dB}	80.11 ± 0.29^{dA}	80.08 ± 0.17^{cA}
	7	65.48 ± 0.32^{eB}	77.42 ± 0.57^{eA}	76.92 ± 0.41^{dA}
	9	60.17 ± 0.42^{fC}	72.63 ± 0.41^{fB}	76.03 ± 0.18^{deA}
	12	58.65 ± 0.16^{gC}	71.16 ± 0.63^{fB}	75.59 ± 0.47^{eA}

Equal lowercase letters in the column and equal uppercase letters in line does not present a significant difference at the level of 5% ($p<0.05$) by the Tukey test. (T1) control; (T2) 1.5% xanthan; (T3) 1.5% chitosan.

3.1.3. Analysis of content of vitamin C

Table 3 presents the variation of ascorbic acid (vitamin C) of broccoli, during refrigerated storage for 12 days.

In Table 3 it is possible to observe that in all treatments, there was a decrease in the content of vitamin C. This can be explained by the presence of oxygen, which, in contact with the broccoli, reduces the content of ascorbic acid (Qiu *et al.*, 2013).

The control treatment presented a greater loss of vitamin C with as the days passed by of storage 49.93%), followed by treatment with xanthan (39.25%).

The chitosan showed better retention of vitamin C than the other treatments. This indicates that the incorporation of this coating reduced the oxygen diffusion, preserving the contents of ascorbic acid (Qiu *et al.*, 2013). This behavior was similar to that found by Ansorena *et al.* (2011).

3.1.4. Texture analysis

Table 4 shows the results found for texture (N) in all the evaluated treatments.

Texture is an important factor at the time of purchase of certain products, because it is associated with the sensory factor. When there is loss of weight, contact with oxygen and temperature variation, the values of texture increase in relation to the fresh product, making the taste unpleasant. Checking the Table 4 it was realized that the values of texture increased at the days of storage went by, being that the control treatment presented a higher texture in 12 days of storage. This increase of the texture is related with the loss of weight of broccoli, and there was a greater loss of water and consequently leaving the tissue more rigid and thus increasing the florets texture. The T3 treatment showed the lowest variance (17.18%) of texture when compared to the other treatments.

Moreira *et al.* (2011) in their study on the effect of chitosan coating in broccoli observed that the application of chitosan coating did not affect the texture and inhibited the florets opening, this being an important advance in the quality of broccoli. The preservation of floret texture of broccoli was also observed in the present study when chitosan was used as coating.

Table 4. Values of texture (%) of minimally processed broccoli with different treatments during storage

Parameter analyzed	Days	Treatments		
		T1	T2	T3
Texture (N)	0	6.75 ± 0.52^{dA}	6.75 ± 0.52^{dA}	6.75 ± 0.52^{cA}
	1	6.81 ± 0.24^{dA}	6.8 ± 0.17^{cdA}	6.81 ± 0.18^{cA}

	3	7.19±0.15^{dA}	7.05±0.09^{cdA}	6.97±0.08^{bcA}
	5	8.72±0.54^{cA}	7.58±0.17^{bcB}	7.08±0.21^{bcB}
	7	9.55±0.11^{bcA}	8.04±0.25^{cB}	7.75±0.42^{abB}
	9	10.12±0.33^{bA}	9.08±0.41^{aB}	8.16±0.15^{aC}
	12	11.21±0.13^{aA}	9.85±0.22^{aB}	8.21±0.46^{aC}

Equal lowercase letters in the column and equal uppercase letters in line does not present a significant difference at the level of 5% ($p<0.05$) by the Tukey test. (T1) control; (T2) 1.5% xanthan; (T3) 1.5% chitosan.

3.1.5. Color analysis

Table 5 presents the parameters of color evaluated for minimally processed broccoli during storage for 12 days.

There was an increase in the values of brightness as the storage days passed by. The higher the value of L* the clearer the sample, and thus it is possible to notice that the control sample showed a higher brightness (48.91) with 12 days of storage (44.11), presenting a coloring that was previously dark green to a color tending to be yellowish. Whereas the treatment with chitosan showed the smallest increase of this parameter, showing that it was capable of retaining to retain the broccoli original color for more time.

Regarding the parameter Chroma a*, all samples showed negative values, showing the green color of broccoli, but as time went by, there was a tendency for loss of green, being that in the control treatment there was a tendency in 12 days for positive values. The treatment T2 and T3 had final values closer to the beginning of the study and showed no significant difference between them in 12 days of storage.

The Chroma b* values also declined as time of storage went by and the treatments T2 and T3 presented smaller yellowing of the florets. The T3 treatment showed stability of values in 12 days of storage, which did not differ among themselves during this evaluated period, which demonstrates a better efficiency of treatment with chitosan as coating.

Araújo and Shirai (2016) found that the color parameters to evaluate the effect of acetic acid and chitosan in relation to the control group, showed no significant variation ($p<0.05$) in 10 days of storage. These results disagree with the present study, because the use of chitosan as coating was effective to preserve the florets of broccoli color for a longer time. The results found in this study agree with Ansorena *et al.* (2011), who once worked with broccoli coated with carboxymethyl cellulose and chitosan and they kept the green color and the degradation of chlorophyll occurred in lower proportion when compared with the control sample.

Table 5. Parameters of color of minimally processed broccoli with different treatments during refrigerated storage

Parameter analyzed	**Days**	**Treatments**		
		T1	**T2**	**T3**
L*	0	30.19±0.88^{dA}	30.19±0.82^{eA}	30.19±0.82^{cA}
	1	30.27±1.09^{dA}	30.31±0.44^{eA}	30.45±0.53^{cA}
	3	35.57±2.13^{cA}	33.11±0.65^{dA}	32.71±1.79^{bcA}
	5	39.21±1.45^{cA}	37.92±1.53^{cAB}	35.38±0.77^{bB}
	7	42.87±0.35^{bA}	40.33±0.55^{bcB}	39.38±1.56^{aB}
	9	43.23±1.17^{bA}	41.71±1.17^{bAB}	40.22±1.07^{aB}
	12	48.91±1.41^{aA}	45.07±0.96^{aAB}	44.11±0.49^{aB}
	0	-8.86±0.52^{aA}	-8.86±0.52^{aA}	-8.86±0.52^{aA}

Chroma a*	1	-8.88±0.29^{aA}	-8.79±0.19^{aA}	-8.89±0.41^{aA}
	3	-8.11±0.44^{aA}	-8.65±0.22^{aA}	-8.80±0.73^{aA}
	5	-7.65±1.02^{aA}	-8.03±0.58^{aA}	-8.06±0.34^{abA}
	7	-6.16±0.23^{bB}	-7.11±0.33^{bA}	-7.57±0.12^{bA}
	9	-5.31±0.17^{bB}	-6.91±0.72^{bA}	-7.31±0.40^{bA}
	12	-5.09±0.24^{bB}	-6.54±0.17^{bA}	-6.97±0.21^{bA}
Chroma b*	0	32.56±1.28^{aA}	32.56±1.28^{aA}	32.56±1.28^{aA}
	1	32.49±0.76^{abA}	29.65±3.46^{abcA}	28.89±4.58^{aA}
	3	33.38±1.22^{aA}	30.21±1.54^{abA}	31.82±1.48^{aA}
	5	31.05±0.55^{abA}	29.89±0.67^{abcA}	30.11±0.48^{aA}
	7	27.57±2.73^{bcA}	28.02±1.67^{abcA}	29.41±1.37^{aA}
	9	23.88±3.13^{cdA}	26.56±1.44^{bcA}	27.09±2.55^{aA}
	12	19.57±0.96^{dB}	25.07±0.77^{cA}	26.89±3.54^{aA}

Equal lowercase letters in the column and equal uppercase letters in line does not present a significant difference at the level of 5% ($p<0.05$) by the Tukey test. (T1) control; (T2) 1.5% xanthan; (T3) 1.5% chitosan.

3.1.6. Microbiological analysis

The obtained results showed that the presence was not detected of total coliforms and thermotolerant coliforms and *Escherichia coli* ($<10^2$ CFU.g^{-1}), as well as of *Salmonella* (absence in 25g) in samples of minimally processed broccoli, confirming the effectiveness of hygienic care and action of the organic chlorine in the samples disinfection.

In Brazil, there is no specific legislation for minimally processed vegetables with tolerated limits of counts. However, there is legislation for fresh products, in natura (peeled or selected or fractionated), sanitized, refrigerated or frozen, establishing maximum values of thermotolerant coliforms of 5×10^2 CFU/g and the absence of *Salmonella* spp. in 25 g of sample (Brasil, 2001). The present study is, therefore, within these specifications imposed by the legislation.

Figure 1 shows the growth curve for mesophilic aerobic microorganisms.

There was an increase in the growth of mesophilic aerobic microorganisms as the days went by being that the control treatment presented a higher growth, significantly differing from the other treatments. The T3 treatment was the one that showed the lowest growth over 12 days of storage, showing that chitosan is a good barrier to microbial growth. More homogeneous edible coatings are desirable because they provide a more effective barrier between the plant and the external environment (Tezotto-Uliana *et al.*, 2014), thus hindering the transfer and microorganisms respiratory activity (Aquino *et al.*, 2015).

Moreira *et al.* (2011), researching the impact of edible coatings together with different thermal shocks on quality of minimally processed broccoli during refrigerated storage, observed that the chitosan did not act effectively against the mesophilic aerobic microorganisms. The present study demonstrated the contrary, because chitosan used in the proportion of 1.5% was effective to retard the growth of this microorganism.

Figure 2 presents the microbial growth for molds and yeasts, for florets of broccoli minimally processed during 12 days in refrigerated storage.

At the end of 12 days it was observed that there was significant difference among all the evaluated treatments. There was a greater growth of yeasts and molds in the treatment without coating (T1), and lower growth in the treatment with chitosan. This may be related to the fact that chitosan due to having a high content of deacetylation presents a greater antimicrobial power (Dutta *et al.*, 2009). Because the same acts against bacteria through their positive charges in the molecule allows

the interaction and formation of polyelectrolyte complexes and surface polymers of bacterial cell (Durango *et al.*, 2006).

Ansorena *et al.* (2011), in their work with edible coating of chitosan and different levels of thermal shock, also found a reduction in the growth of yeasts and molds.

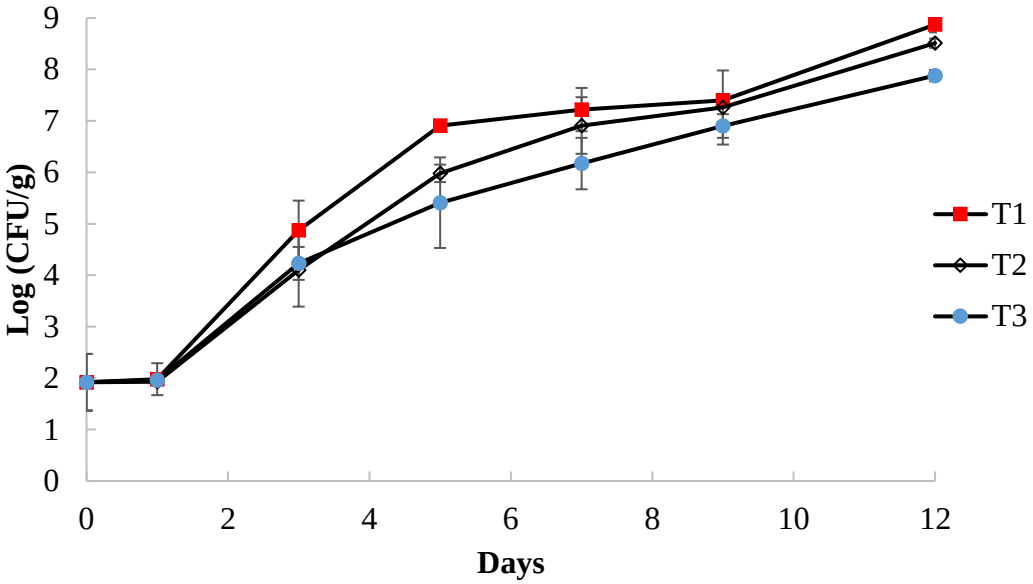

Figure 1. Growth of mesophilic aerobic microorganisms in minimally processed broccoli for 12 days
Where: (T1) control; (T2) 1.5% xanthan; (T3) 1.5% chitosan.

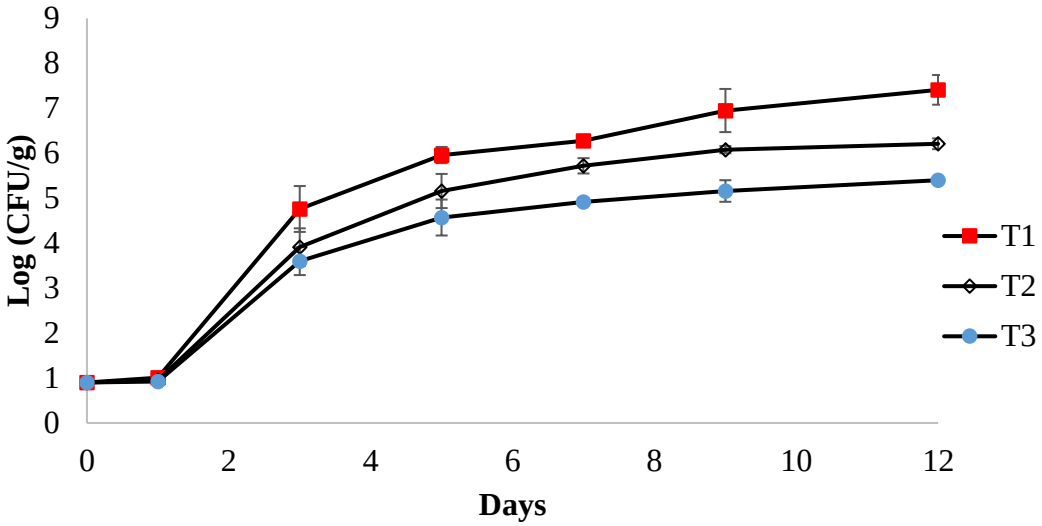

Figure 2. Growth of molds and yeasts in florets of broccoli minimally processed over 12 days
Where: (T1) control; (T2) 1.5% xanthan; (T3) 1.5% chitosan.

3.1.7. Sensory analysis

Table 6 shows the values of the sensory analysis found for minimally processed broccoli and stored at 4±1 °C for a period of 12 days.

It can be observed in Table 6, that in all the evaluated attributes, there was a decrease of acceptability by the appraisers, and the control

treatment was the one that presented a greater reduction of values as the evaluation days

passed by, showing a significant difference in relation to the treatments T2 and T3.

During the storage some reactions occurs which affect the structure of the plants cell wall. For the texture attribute, the treatments T2 and T3 were above the value 3, which was established as a minimum limit of acceptability, demonstrating that the pectin and chitosan were capable of maintaining the texture for a longer time. This reduction of the texture during storage, can be explained due to the hydrolysis of pectic acids of the cell wall caused by the action of the enzymes pectinase, cellulase, β-galactosidase and glycosidases (Chitarra and Chitarra, 2005).

In relation to the parameter of color, odor and overall evaluation, a reduction was observed in these attributes in all the samples during storage. A greater variation was observed in the treatment T1, being that it differed significantly from the other treatments for the evaluated attributes, demonstrating that the xanthan gum (T2) and chitosan (T3) succeeded in delaying the changes that degrade and diminish the quality of minimally processed broccoli.

Pizarro *et al.* (2006) working with different plastic packages found values of odor lower to the other treatments in comparison with the control sample. These results are not in agreement with the present study, because higher values were found on the last day of storage for the remaining treatments (T2 and T3) in relation to the control sample. Chevalier *et al.* (2018) when worked with the application of Whitemouth croaker protein isolate in minimally processed melon, also managed to maintain the sensory characteristics of this fruit for more time.

Table 6. Evaluation of the sensory attributes of texture, color, aroma and overall evaluation of minimally processed broccoli, stored at 4 ºC for 12 days

Sensory attributes	Days	Treatments		
		T1	T2	T3
Texture	0	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}
	1	4.9 ± 0.0^{bB}	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}
	3	4.6 ± 0.1^{cB}	4.9 ± 0.1^{aA}	4.9 ± 0.1^{aA}
	5	3.8 ± 0.1^{dB}	4.5 ± 0.1^{bA}	4.6 ± 0.1^{bA}
	7	3.4 ± 0.1^{eB}	4.2 ± 0.1^{cA}	4.2 ± 0.1^{cA}
	9	2.2 ± 0.1^{fB}	3.6 ± 0.1^{dA}	3.8 ± 0.1^{dA}
	12	1.5 ± 0.1^{gB}	3.1 ± 0.1^{eA}	3.2 ± 0.1^{eA}
Color	0	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}	5.0 ± 0.1^{aA}
	1	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}
	3	4.5 ± 0.1^{bB}	4.8 ± 0.1^{bA}	4.9 ± 0.1^{aA}
	5	3.9 ± 0.1^{cB}	4.6 ± 0.1^{bA}	4.6 ± 0.1^{bA}
	7	2.8 ± 0.1^{dB}	3.6 ± 0.1^{cA}	3.8 ± 0.1^{cA}
	9	2.1 ± 0.1^{eB}	3.1 ± 0.1^{dA}	3.3 ± 0.1^{dA}
	12	1.4 ± 0.1^{fB}	2.5 ± 0.1^{eA}	2.7 ± 0.1^{eA}
Aroma	0	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}
	1	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}
	3	4.3 ± 0.1^{bB}	4.9 ± 0.1^{aA}	4.9 ± 0.1^{aA}
	5	3.8 ± 0.1^{cB}	4.5 ± 0.1^{bA}	4.6 ± 0.1^{bA}

	7	3.2 ± 0.1^{dB}	4.1 ± 0.1^{cA}	4.2 ± 0.1^{cA}
	9	2.3 ± 0.1^{eC}	3.0 ± 0.1^{dB}	3.3 ± 0.1^{dA}
	12	1.3 ± 0.1^{fC}	2.7 ± 0.1^{eB}	3.0 ± 0.1^{dA}
Overall Evaluation	0	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}
	1	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}	5.0 ± 0.0^{aA}
	3	4.4 ± 0.1^{bB}	4.9 ± 0.1^{aA}	4.9 ± 0.1^{aA}
	5	3.9 ± 0.1^{cB}	4.6 ± 0.1^{bA}	4.6 ± 0.1^{bA}
	7	3.0 ± 0.1^{dB}	4.0 ± 0.1^{bA}	4.1 ± 0.1^{cA}
	9	2.2 ± 0.1^{eC}	3.0 ± 0.1^{cB}	3.4 ± 0.1^{dA}
	12	1.3 ± 0.1^{fB}	2.8 ± 0.1^{dA}	3.0 ± 0.1^{eA}

Equal lowercase letters in the column and equal uppercase letters in line does not present a significant difference at the level of 5% ($p<0.05$) by the Tukey test. (T1) control; (T2) 1.5% xanthan; (T3) 1.5% chitosan.

4. Conclusions

The different coatings used in this study were efficient in the conservation of minimally processed broccoli, when compared to the control sample.

The use of xanthan and chitosan showed great potential to be applied as edible coatings for delaying the changes in coloring, maintain physical and chemical characteristics and reduce the microbiological changes.

However, the use of chitosan was more efficient in increasing the shelf life, leaving the minimally processed broccoli safe for consumption for more time.

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MICROWAVE-ASSISTED EXTRACTION OF PHENOLIC COMPOUNDS FROM GINGER (*ZINGIBER OFFICINALE* ROSC.)

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ABSTRACT

The main purpose of this study is to determine the best microwave-assisted extraction conditions such as type of solvent, solvent concentration, material/solvent ratio, microwave of power and extraction time. These factors affect strongly total polyphenol content (TPC) and antioxidant activity (AC). The achieved best parameters for the extraction process were aqueous ethanol concentration of 50%, material/solvent ratio of 1/40 (w/v), extraction time of 3 minutes and microwave power of 127 W. TPC and AC peaked at 22.79±0.29 mg GAE/g DW and 9.85±0.03 mmol Fe/g DW, respectively. Besides, the treatment by microwave can affect the cell structure of material which was observed by scanning electron microscope (SEM).

1. Introduction

Ginger is the herbal plant and precious spice for humans. Ginger *(Zingiber officinale* Rosc.) belongs to the Zingiberaceae family and distributes everywhere in Vietnam. Local citizens usually use fresh or dried ginger root as a spice (Do, 2014). Ginger is also an antioxidant food and good for human health. Ginger root contains many bioactive compounds such as flavonoids, tannins, β carotene and vitamin C which have a strong antioxidant capacity (Shirin *and* Prakash, 2010). Besides, ginger also has many uses to treat many diseases such as nausea, cough, digestive aid, inflammation, swelling (Truong, 2001), digestion, treatment of bronchitis (Pham, 2014). It can slow down or prevent the development of cancerous tumors, etc. In addition, ginger roots extract contains an amount of polyphenols compounds as high antioxidant agents. The antioxidant properties of ginger root are an extremely important activity, they can be used as a preventative agents against certain diseases. In addition, ginger also has antibacterial effects and inhibits the growth of *Escherichia coli*, *Proteus* sp., *Staphylococci* and *Salmonella* (Gupta *and* Sharma, 2014).

Nowadays, there are some methods to extract phenolic compounds from the plant such as maceration, soxhlet, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), etc. However, the demands of a good extraction method are environmentally friendly (reduced use of organic solvents), saved time and increased extraction yield (Hue, 2002). In there, MAE is the method that can adapt these demands and it was used in many previous studies to extract phenolic compounds from the plants, for instance, tea (Pan et al., 2003), rosemary (Švarc-Gajic *et al.*, 2013), *Polygonum multiflorum* Thunb. roots (Quoc *and* Muoi, 2016), etc. In addition, according to Wakte *et al.* (2011), MAE used a low amount of solvent, reduced production costs and had high-quality products compared to other methods. Therefore, using the MAE method has many

benefits in food technology and chemical technology.

Until now, no studies have presented the MAE method for the extraction of phenolic compounds from ginger in Vietnam and evaluate the effects of extraction factors on TPC and AC. Hence, based on the above judgment, this study investigates the extraction conditions of phenolic compounds from the ginger extract supported by microwaves such as type of solvent, solvent concentration, the ratio of material/solvent, extraction time and microwave power.

2. Materials and methods

2.1. Plant material and sample preparation

Ginger *(Zingiber officinale)* were harvested from Nghe An province (Vietnam), cleaned and then sliced (2 mm) and dried at 60℃ for nearly 5 hours until the moisture is lower than 12%. The slices were ground into a fine powder (<0.5 mm), packed in vacuum and then stored in the dark at room condition (25℃).

2.2. Chemicals and reagents

Gallic acid and Folin-Ciocalteu (FC) reagent were purchased from Sigma-Aldrich (USA). All organic solvents and other chemicals were of analytical reagent grade.

2.3. Polyphenols extraction process

Dried ginger powder (2 g) is extracted in four solvents such as 50% ethanol, 50% acetone, 50% methanol and deionized water. Appropriate solvents will be carried out at different solvent concentrations of 30, 40, 50, 60 and 70% (v/v); the ratios of material/solvent are 1/20, 1/30, 1/40, 1/50 and 1/60 (w/v); microwave power are 74, 127, 195, 327, 610 W; extraction times are 1, 3, 5, 7 and 9 minutes, respectively. The extract is filtered for removal of the residue by the vacuum filtration system, then TPC and AC of extract were determined.

2.4. Determination of total polyphenol content (TPC)

The TPC in the extract was slightly modified and determined by the Folin-Ciocalteu method. The results were based on a standard curve obtained with gallic acid measured at 738 nm (Siddiqua *et al.*, 2010). TPC was calculated as milligram of gallic acid equivalent per gram of dry weight (mg GAE/g DW).

2.5. Determination of antioxidant capacity (AC)

The AC in the extract is determined by the slightly modified method of the 1.10-phenanthroline solution in methanol. The reaction between Fe (II) and 1,10-phenanthroline forms complex orange-red complexes. AC was measured by a standard curve obtained at a wavelength of 510 nm. Antioxidant activity was expressed as mmol Fe equivalents per gram of dry weight (mmol Fe/g DW) (Szydowska-Czerniak *et al.*, 2008; Songsungkan *and* Chanthai, 2014).

2.6. Scanning electron micrographs (SEM)

Ginger powder was observed by SEM (Jeol/JSM-6480LV, Japan) to indicate changes of the material in particle morphology before and after extraction.

2.7. Data analysis

All experiments were performed in triplicates, the results were expressed in the form of mean±standard deviation (SD) and analyzed by the Statgraphics Centurion XV software, version 15.1.02 (USA). The one-way analysis of variance (ANOVA) at $\rho<0.05$ was used to determine significant differences between the means by LSD (Fisher's least significant difference) method.

3. Results and discussions

3.1. Effect of solvent type on the extraction of polyphenols

The dried ginger powder was extracted with four solvents (deionized water, 50% acetone, 50% ethanol and 50% methanol) under the same extraction condition as follows: sample/solvent ratio of 1/30 (w/v), microwave power of 195 W and extraction time of 5 minutes. The results are shown in Table 1 below.

Table 1 shows that there are significant differences between different types of solvents regarding TPC as well as AC ($\rho<0.05$) and the extraction yield of solvents reduces in order of 50% ethanol > 50% methanol > 50% acetone > deionized water. For using ethanol as solvent, TPC and AC obtained the highest values (18.87±0.23 mg GAE/g DW and 9.05±0.14 mmol Fe/g DW, respectively), while the lowest values of TPC and AC were 9.72±0.22 mg GAE/g DW and 2.84±0.06 mmol Fe/g DW, respectively, for using water as solvent. The differences between TPC and AC may occur because the polarities of solvents are completely different. In this case, the best extractable polarity is the ethanol concentration of 50%, which is similar to the study results concerning the extraction of TPC on the pomegranate peel (Kaderides *et al.*, 2019).

In fact, there are many solvents that can extract phenolic compounds, they depend on the purpose of use. Typically, this research result shows that the TPC is higher than that of Kubra *et al.* (2013), they also extracted phenolic compounds (TPC=16.4±0.2 mg GAE/g DW) from ginger with 50% ethanol as solvent by MAE method. Besides, the TPC of this case is lower than that of other material with the same solvent, for instance, pomegranate peels (Kaderides *et al.*, 2019), *Limnophila aromatica* (Do *et al.*, 2014). These differences can be due to different materials, extraction methods, genes, etc. At present, ethanol was used in many previous studies because of its advantages such as low cost, clean and safe solvent in food. Based on the results obtained, 50% ethanol is selected as the solvent for the next test.

Table 1. TPC and AC of extract at different solvents

	Solvents			
	Deionized water	**50% Acetone**	**50% Ethanol**	**50% Methanol**
TPC (mg GAE/g DW)	9.72^{a}±0.22	13.06^{b}±0.36	18.87^{d}±0.23	18.02^{c}±0.42
AC (mmol Fe/g DW)	2.84^{a}±0.06	6.02^{b}±0.21	9.05^{d}±0.14	7.68^{c}±0.27

Different lowercase letters in the same row indicate a statistically significant difference between various solvents ($\rho<0.05$)

3.2. Effect of ethanol concentration on the extraction of polyphenols

The effects of ethanol concentration on TPC and AC were illustrated in Table 2. The results show that there is a statistically significant difference between various ethanol concentrations with $\rho<0.05$. The TPC reaches the highest value at ethanol concentration of 70% (24.28±0.55 mg GAE/g DW), while the AC obtains the best value at ethanol concentration of 50% (9.08±0.07 mmol Fe/g DW). Hence, the level of changes between TPC and AC is not identical in this case.

The ethanol concentration plays a major role in polyphenols extraction and the high ethanol concentrations can cause the protein denaturation, prevent the dissolution of polyphenols and thus it affects the rate of the extraction process (Yang *et al.*, 2009). It can be seen that the changes in any parameter do not mean that TPC and AC will increase or decrease accordingly. Both of TPC and AC depend on many factors, especially the polarity of the solvent. The addition of water into the solvent often creates the changes of polarity and phenolic compounds were easily dissolved into the solvent. Solvent properties in this study were changed by a combination of ethanol and water. Choosing the appropriate solvent concentration can improve TPC and AC; minimizes the extraction expenditure and limits unwanted impurities (Tomaz *et al.*, 2019).

This result is similar to studies of Kaderides *et al.* (2019) and Kubra *et al.* (2013), which also shows that ethanol concentration of 50% is the suitable solvent concentration to extract phenolic compounds from pomegranate peels and ginger. In contrast, Quan *et al.* (2006) and Dahmoune *et al.* (2015) noticed that the optimal ethanol concentration to extract

phenolic compounds from fresh tea shoot and *Myrtus communis* L. leaves are 40% and 60%, respectively. This can be explained because of the different polarities of phenolic compounds in materials. Through the received results, the ethanol concentration of 50% was selected to carry out the subsequent studies.

Table 2. TPC and AC of extract at different ethanol concentrations

	Ethanol concentration (%)				
	30	**40**	**50**	**60**	**70**
TP (mg GAE/g DW)	11.74^{a}±0.03	14.34^{b}±0.11	18.67^{c}±0.22	22.78^{d}±0.39	24.28^{e}±0.55
AC (mmol Fe/g DW)	1.59^{a}±0.35	1.99^{b}±0.12	9.08^{d}±0.07	6.27^{c}±0.22	6.17^{c}±0.14

Different lowercase letters in the same row indicate a statistically significant difference between various ethanol concentrations ($\rho<0.05$)

3.3. Effect of material/solvent ratio on the extraction of polyphenols

The effect of material/solvent ratio on TPC and AC was presented in Table 3. TPC and AC have a statistically significant difference ($\rho<0.05$) for the changes in the material/solvent ratios. TPC and AC have the highest values (21.82±0.38 mg GAE/g DW and 9.76±0.53 mmol Fe/g DW, respectively) at the material/solvent ratio of 1/40.

The ratio of raw material/solvent is an important parameter of the extraction method and affects TPC and AC strongly. The volume of solvent must be sufficient to ensure the entire sample is soaked in solvent. For the MAE method, an increase in excessive solvent volume will reduce microwave adsorption of material mainly because the solvent absorbed almost all energy of the microwave. Therefore, the breakdown of the cell wall and mass transmission might be negatively affected (Li *et al.*, 2010). In addition, material size has a significant effect on extraction efficiency. Smaller materials reduce cell diffusion distance in solids, increase the concentration of the gradient, thereby leads to higher extraction rates (Tomaz *et al.*, 2019).

The material/solvent ratio of this study is lower than that of study of Calinescu *et al.* (2017), who extracted polyphenols from sea buckthorn leaves at the material/solvent ratio of 1/20 with microwave support combined with a coaxial antenna and a cooling system; this ratio also is similar to that of study of Quoc and Muoi (2016), they extracted polyphenols from *Polygonum multiflorum* Thunb. roots and it is higher than that of the study of Kaderides *et al.* (2019), who extracted polyphenols from pomegranate peels at the material/solvent ratio of 1/60 with MAE method. It can be seen that different materials can produce different proportions of material/solvent. Through the above research results, the material/solvent ratio of 1/40 was selected for the next steps.

Table 3. TPC and AC of extract at different material/solvent ratios

	Material/solvent ratios (w/v)				
	1/20	**1/30**	**1/40**	**1/50**	**1/60**
TPC (mg GAE/ g DW)	15.49^{a} ±0.27	18.53^{b}±0.31	21.82^{d}±0.38	21.97^{d}±0.25	21.12^{c}±0.17
AC (mmol Fe/g DW)	6.68^{c}±0.02	8.80^{d}±0.24	9.76^{e}±0.53	5.38^{b}±0.05	4.67^{a}±0.21

Different lowercase letters in the same row indicate a statistically significant difference between various material/solvent ratios ($\rho<0.05$)

3.4. Effect of microwave power on the extraction of polyphenols

Table 4 shows that the maximum TPC and AC were 22.37±0.19 mg GAE/g DW and 9.20±0.2 mmol Fe/g DW at 127 W, respectively. Microwave power significantly affects TPC and AC ($\rho<0.05$). Extraction temperature depends on microwave power in

this case; as the microwave power increases, the solution temperature also increases. The yield of the process increases correlatively from 74 W to 127 W and then drops slowly during the rest scale because the sensitive phenolic compounds are degraded at high temperature.

At the high temperature, surface tension and solvent viscosity decrease, the solvent has high dissolve ability, which improves extraction yield (Li *et al.,* 2010). Thereout, the microwave irradiation redoubles the rate of cell destruction by the internal pressure increase the plant cell, which promotes the destruction of the sample surface and diffuses free compounds in the plant cells into the surrounding solvent (Maran *et al.*, 2014).

The microwave power of 127 W is not high and quite suitable for this study because using low microwave power with extending extraction time can improve the extraction yield. In contrast, high microwave power could reduce purity and create very high temperatures leading to the degradation of phenolic compounds (Liew *et al.,* 2016). This may explain that the high irradiation power provides residual energy for solvent and it disturbs molecular interactions (Maran *et al.,* 2015).

The microwave power of this study was similar to that of the study of Quoc *and* Muoi (2016), who extracted the phenolic compounds from *Polygonum multiflorum* Thunb. roots. Besides, it is higher than that of study of Bouras *et al.* (2015), they extracted the phenolic compounds from *Quercus* bank at the optimal microwave power of 45 W, while Kaderides *et al.* (2019) extracted the phenolic compounds from pomegranate peels at the optimal microwave power of 600 W. This may also explain that the structure of the initial raw materials and type of polyphenols in materials are different leading to the difference of microwave power. Thus, the microwave power of 127 W was chosen for subsequent studies.

Table 4. TPC and AC of extracts at various microwave powers

	Microwave powers (W)				
	74	**127**	**195**	**327**	**610**
TPC (mg GAE/g DW)	17.52^{a}±0.38	22.37^{d}±0.19	21.76^{c}±0.43	20.28^{b}±0.18	17.69^{a}±0.13
AC (mmol Fe/g DW)	5.33^{a}±0.19	9.20^{d}±0.2	8.91^{cd}±0.22	8.78^{c}±0.14	8.23^{b}±0.13

Different lowercase letters in the same row indicate a statistically significant difference between various microwave powers ($\rho<0.05$)

3.5. Effect of extraction time on the extraction of polyphenols

The result of the study shows the changes of TPC and AC when the extraction time changes from 1 to 9 minutes (Table 5). At 3 minutes, the TPC and AC have the best values (22.79±0.29 mg GAE/g DW and 9.85±0.03 mmol Fe/g DW, respectively). Extraction yield depends on the extraction time; as the extraction time increases, the extraction yield also increases. However, the extraction yield in this study decreases after 3 minutes because the sensitive phenolic compounds are destroyed at high temperatures and long extraction time. Thus, choosing appropriate extraction time can improve extraction efficiency as well as shorten the time, saved cost.

This result is similar to that of an extraction study by the MAE method from pomegranate peel of Kaderides *et al.* (2019). However, the extraction time in this case is shorter than that of many previous studies from various materials, for instance, extracting polyphenols from *Polygonum multiflorum* Thunb. roots for 5 minutes (Quoc *and* Muoi, 2016), *Quercus* bark for 60 minutes (Bouras *et al.*, 2015), sour cherry Marasca for 9 minutes (Garofulic *et al.*, 2013); while it is longer than that of study of Wu *et al.* (2012), who extracted polyphenols from potato downstream wastes by MAE method for 2 minutes.

Basically, the MAE method is a good extraction process with effective microwave energy transmission to material by molecular interaction under electromagnetic fields in a

short time compared to conventional methods. Energy is quickly transferred to solvent and material. However, extending extraction time causes degradation of compounds and extraction yield (Huang *et al.*, 2017). Nevertheless, the short extraction time can not completely extract the desired compounds. Therefore, the extraction time of 3 minutes is the best choice in this study.

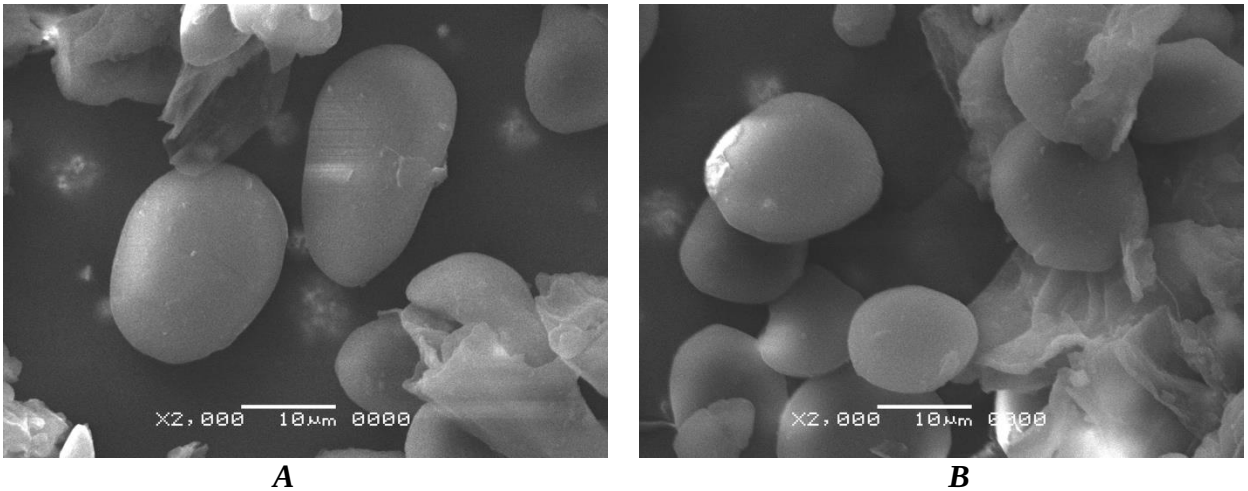
Table 5. TPC and AC extracts at various extraction times

	Extraction time (min)				
	1	**3**	**5**	**7**	**9**
TPC (mg GAE/g DW)	21.21^{b}±0.24	22.79^{d}±0.29	21.88^{c}±0.33	21.62^{bc}±0.33	19.69^{a}±0.21
AC (mmol Fe/g DW)	9.62^{d}±0.04	9.85^{d}±0.03	8.69^{c}±0.36	7.84^{b}±0.06	4.92^{a}±0.06

Different lowercase letters in the same row indicate a statistically significant difference between various extraction times ($\rho<0.05$)

3.6. Effect of microwave assisted extraction on structure of material

Based on the obtained results, it was found that the structure of ginger powder before and after the extraction was different at x2000 magnification. In the beginning, the ginger powder had an intact structure (Figure 1A). After the microwave-assisted extraction, few cells are ruptured and the cell wall is damaged and sticky, specifically, the surface was peeled and cracked (Figure 1B). However, these changes are not significant compared to the initial material. These changes are similar to the study of Quoc *and* Muoi (2016), who used the MAE methods to extract polyphenols from *Polygonum multiflorum* Thunb. roots. In addition, this phenomenon in the MEA method is also similar to the UEA method, for instance, extracting polyphenol from *Myrtus communis* by the UEA method allows solvents to penetrate the cell walls and the bubbles produced by acoustic cavitation aid in the disruption of the cell wall (Xia *et al.*, 2011). The changes in the shape of material occur due to the following causes: the temperature and the internal pressure increases dramatically because of microwave energy in the extraction process leading to break the cell wall and releases bioactive compounds.



A ***B***

Figure 1. Structure of material before (A) and after (B) treatment by MAE

4. Conclusions

Ginger is rich in phenolic compounds with high antioxidant activity. The results proved that the extraction factors directly affect total polyphenol content and antioxidant capacity. The best extraction conditions are ethanol

concentration of 50%, material/solvent ratio of 1/40, microwave power of 127 W and extraction time of 3 minutes. Total polyphenol content and antioxidant capacity of the extract obtain the highest value. After microwave treatment, the cell wall of the material changes insignificantly.

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