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CONTENT

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EFFECTS OF MULTI-STRAIN PROBIOTICS ON THE GROWTH AND HEMATOLOGICAL PROFILE IN JUVENILE CARP (*CYPRINUS CARPIO*, LINNAEUS 1758)

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ABSTRACT

The potential individual probiotic microorganisms to act synergistically or as an additive when mixed present a great promise for future use in the treatment of various diseases in aquaculture. Besides inhibiting pathogens and improving the immune response, multi-strain probiotics also assist in promoting the growth of the host. The experiment was performed in independent breeding units such as recirculating systems that allowed the comparative evaluation with control of the action of the three commercial probiotics with applicability in human and zootechnical consumption being tested for use as feed bio additive to grow carp juveniles. This study involved assessing the mode of action of these multiple strains of probiotics on the evolution of the nonspecific immune response as a tool to highlight their effect being used in equal concentrations of 3.2 $\times 10^9$ CFU/kg feed, the following commercial products: *BioPlus® 2B* (mixture of *Bacillus licheniformis and Bacillus subtilis in a ratio of 1: 1*), *BetaPlus®* (mixture of *Bacillus licheniformis and Bacillus subtilis* with betaine) and *Lactobact Premium* (mixture of *Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium lactis, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus, Streptococcus thermophilus* - lactic acid bacteria - LAB). The study aims were to evaluate the effect of multi-strains probiotics with *Bacillus* and *LAB* on the growth performance and hematological profile of juvenile carp. In conclusion, the use of multi-strain probiotics, could use a positive effect on growth performance and improved some hematological of juvenile carp.

1. Introduction

In the last two decades, there has been a massive expansion of research and the use of probiotics in aquaculture. Recent results have shown that groups of about 20 bacterial genera have been recognized as potential probiotic candidates, and most species with probiotic potential belong to the genus *Bacillus spp.* and the group of lactic acid bacteria (LAB) (Knipe et al., 2020).

Recently, the probiotic microorganisms most commonly used in aquaculture belong to *Bacillus spp.*, *Lactobacillus spp.*, *Bifidobacterium spp.*, *Enterococcus spp.*, *Streptomyces spp.*, *Carnobacterium spp*. and yeast (Van Doan et al., 2019).

Multi-strain probiotics had been recommended as a necessity for the shared use of these microorganisms to allow development in favorable conditions, such as those in the gastrointestinal tract (GIT), and dominate the specific resident microbiota. Therefore, it has also been suggested that a multispecies probiotic would be more successful than a monospecies supplement Kumar et al. 2016; Zorriehzahra et al. 2016;

Feckaninova et al. 2017; Ringø et al. 2018; Dawood et al. 2019; Ringø et al. 2019; Soltani et al. 2019; Wang et al. 2019; Kuebutornye et al. 2019b; Melo-Bolivar et al. 2020). According to previous research, multi-strain probiotics (MSP) are confirmed to be more beneficial to the host organism than the use of a single probiotic in certain aspects. Multi-strain probiotics may also be called mixed probiotic mixtures or combinations of probiotics that consist of a mixture of two or more strains or species of bacteria that have been previously demonstrated to provide various benefits for the host. Also, a combination of different species different gen, or strains of the same genus could be called probiotics with several species. The effectiveness of one probiotic varies depending on the type of host to which it is applied, and multi-strain probiotics can be used to increase their influence (Wang et al., 2019).

Probiotics, living microorganisms, confer health benefits to the host by improving the intestinal microbial homeostasis and nutrient digestion, regulating immunity, and suppressing the pathogens infection it can reduce the use of antibiotics and has received more and more attention because of its high abundance, low cost and convenient application in the aquaculture industry (Sharifuzzaman and Austin, 2017; Wang et al., 2019).

A single administration of *Bacillus velezensis*, *Bacillus cereus,* and *Lactobacillus casei* has been found to confer immunomodulatory effects and improve host health (Safari et al., 2017; Wang et al., 2019a; Yang et al., 2019). However, to the knowledge of the authors, they were not used by incorporated into the growth of any animal species still. In addition, multi-strain probiotic (MP) is much more effective in enhancing the growth and immunity of aquatic animals (Salinas et al., 2008; Wang and Xu, 2006; Wang et al., 2019b).

Probiotics have been used as an integral part of aquaculture for a long time to grow crop species. Probiotics are also considered beneficial in disease control and improving water quality in aquaculture (Aslam Hosain and Liangyi, 2020). Probiotics administered to fish can be divided into the large dominant group of $Gram^+$ bacteria such as *Lactobacillus species* (LAB), *Bacillus*, and *Bifidobacterium* and the group of $Gram^-$ bacteria (several strains of *Aeromonas, Vibrio, Pseudomonas* and *Enterobacteriaceae*). Among the strains of probiotic bacteria applied in aquaculture *Bacillus spp., Lactobacillus spp.,* and *Streptococcus spp.* are used more widely, while biomedicine contains colonies of strains of *Lactobacillus spp., Bifidobacterium spp.,* and *Sterptococcus spp*. as a mixture of feed probiotics.

Feeding aquatic organisms with acceptable amounts of probiotics incorporated in the administered feed modified the intestinal microflora by replacing pathogens with microorganisms beneficial. In addition, they could promote enzyme digestion, improve the immune system response, and growth promotion (Wang et al. 2002; Hoseinifar et al. 2017; Sookchaiyaporn et al. 2020; Doan et al. 2020).

The direct incorporation of the probiotic in granulated feed is one of the most important and applicable methods of their administration in feeding. Probiotics are applied directly in this form of spores in feed pellets (Assefa and Abunna, 2018).

According to Melo-Bolivar et al. (2021), to obtain optimal efficiency of probiotics in aquaculture fish it is necessary to determine the correct dose, the time of administration, and the stage of development of the fish during administration, and the method of administration.

Most probiotic mixtures have been tested only once, which could make it difficult to

determine the beneficial effects that could be replicated in other studies (Melo-Bolvar et al., 2021). Only three mixtures of probiotics were replicated in other articles, namely*: B. subtilis* and *B. licheniformis (Bio-Plus 2B*; Chr. Hansen A/S; Merrifield et al. 2010a; Merrifield et al.2010b); *B. subtilis, B. licheniformis* (*BioPlus 2B*; Chr. Hansen A/S) and *E. faecium* (*Lactosan* GmbH & Co. KG; Merrifield et al.2010a; Merrifield et al. 2010b); *and Bacillus sp., Enterococcus sp., Pediococcus sp*. and *Lactobacillus sp*. (*AquaStar, Biomin* GmbH; Ramos et al. 2013; Ramos et al. 2015).

2. Materials and methods

2.1. Materials

2.2.1. Experimental design the fish rearing.

To determine the effect of multi-strains probiotics, the units of the growth were populated with an equal number of 10 specimens, being comparable biomass, so that there is the possibility of comparison between the four experimental variants. The breeding system was populated with 4.65 kg of juvenile carp with an average individual weight of 38.82 g/specimen (*Cyprinus carpio*) aged three months, from the Brateş farm, Institute for Research and Development in Aquatic Ecology, Fishing, and Aquaculture from Galați. The experimental variants and multi-strains of commercial probiotics that were used in this study to test their effect on the physiological state and growth performance of carp juveniles are the following:

I. Control variant (V1) - batch fed with granulated feed treated only with the probiotic fixing binder (but without probiotic);
II. *BioPlus® 2B* probiotic variant (V2) - added in a concentration of 3.2×10^9 UFC/kg feed; *BioPlus® 2B* (veterinary use), is represented by a mixture of *Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750) in a ratio of 1: 1;
III. *BetaPlus®* probiotic variant (V3) - added in a concentration of 3.2×10^9 CFU/kg feed; *BetaPlus®* (veterinary use), consists of *BioPlus® 2B* (*Bacillus licheniformis* (DSM 5749) and *Bacillus subtilis* (DSM 5750) in a ratio of 1: 1) and betaine (nitrogenous substance);
IV. *Lactobact premium* probiotic variant (V4) - added in a concentration of 3.2×10^9 CFU/kg feed; *Lactobact Premium* (human use) is a mixture of equal proportions of *Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium lactis, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus, Streptococcus thermophilus* (the product does not contain lactose, gluten, and yeast).

The *BioPlus® 2B* and *BetaPlus®* probiotics used in this experiment came from the Biochem company in Lohne, Germany, through the Romanian subsidiary Biochem Animal Health and Nutrition affiliated with the one in Lohne and located in Cluj-Napoca.

Preparation of experimental diets. In this experiment, the ratio used was 3.5% of body weight (BW). The total amount of feed calculated for one day was administered in five meals that were distributed manually every two hours. The fish were fed *CLASSIC EXTRA 1P* feed - 2.5 mm pellets with a protein content of 41%. The composition of *CLASSIC EXTRA 1P* feed includes the following ingredients: fishmeal, fish oil, hemoglobin, soybean oil, wheat gluten, sunflower flour, wheat and wheat products, BTH.

The working protocol for the incorporation/addition of feed with probiotic products was updated according to the ratio administered during the period of the experiment.

This protocol goes through the following steps:

a. Dissolve the probiotic in 4 (6.8) ml distilled water;

b. Stirring the solution for 10 minutes;

c. Preparation of 2% gelatin solution on a water bath;

d. Cooling the gelatin solution to 30 ^{0}C;

e. Mixing probiotics and gelatin solutions in a ratio of 2: 1 (4 (6.8) ml probiotic solution: 2 (3.4) ml gelatin solution/variant);

f. Spraying the final solution on the surface of the feed granules by continuous stirring;

g. Drying in oven T^0 = 20 ^{0}C, for 12 hours.

The study falls into the category of experimental investigations, conducted in Pilot Research Stations of the Department of Food Science, Food Engineering, Biotechnology and Aquaculture, Faculty of Food Science and Engineering, University "Dunărea de jos" of Galati. From a constructive point of view, the growth system in which the experiment took place consists of the following parts: growth units - represented by four aquariums made of glass with a thickness of 10 mm (figure 1). Each aquarium has the dimensions of 40 × 50 × 100 cm and was divided into three enclosures equal to the dimensions of 40 × 50 × 32 cm and a volume of 45 l/subvariant (figure 2).

Figure 1. Growth system units intensive for testing multi-strain probiotics.

Figure 2. Compartmentation of growth units in three enclosures/rehearsals.

Water quality recirculation and conditioning equipment at the level of each subvariant was represented by *Tetratec EX 400* filters (figure 3). Physico-chemical parameters represented by dissolved oxygen, temperature, and pH were monitored with the help of the portable instrument *HANNA* instruments type HI 7100042. Also, nitrogen compounds ($N\text{-}NO^-_2$, $N\text{-}NO^-_3$, $N\text{-}NH^+_4$) were determined with the Spectroquant Nova 400 spectrophotometer using Merk compatible kits.

Figure 3. Water quality recirculation and conditioning equipment.

2.2. Methods

2.2.1. Growth performance and conversion ratio.

At the end of the experiment, after the fish were weighed and measured, the following parameters were calculated: weight gain, feed conversion factor, specific growth rate, and efficiency of protein utilization using the following equations:

a) Weight gain (WG) = Final weight (Wt) – Initial weight (W0) (g);
b) Food conversion ratio (FCR) = Total feed (F)/Total weight gain (W) (g/g);
c) Specific growth rate (SGR) = 100 x (ln Wt – ln W0) / t (% BW/day);
d) Protein efficiency ratio (PER) = Total weight gain (W)/amount of protein fed.

2.2.2. Microbiological analyzes.

Sampling for microbiological analysis was performed from the water of the breeding units and from the feed to verify the viability of multi-strains probiotics. The protocol used for the cultivation and determination number of germs is described in the paper "Bacteria from Fish and Other Aquatic Animals - A Practical Identification Manual" By Nicky B. Buller 2009.

2.2.3. Blood sample and hematological analysis.

The collection of blood samples for hematological determinations was performed at the end of the experimental period to identify the changes between the control variant (control) and the variants with different probiotics. For an accurate assessment of the hematological indicators, blood samples were taken from 6 fish/growth units (representing 85% of the biomass) totaling a total of 72 blood samples.

Before blood sampling, fish were anesthetized with 2-phenoxyethanol in order to reduce handling stress. Research has shown that 2-phenoxyethanol anesthetic had no effect on the hematological profile (Velíšek et al., 2007). Blood analysis was performed by a method used in fish hematology described by Bocioc et al., 2015. This analysis consisted of the determination of red blood cells count - RBCc ($\times 10^6/\mu l$), hemoglobin - Hb (g/dl), and hematocrit – PVC (%). For the determination of erythrocyte, the number was used the Neubauer hemocytometer. The hematocrit was performed by duplicate using capillary tubes centrifugated for 5 minutes at 12000 rpm in a microhematocrit centrifuge. The hemoglobin concentrations were measured spectrophotometrically with SPECORD 210 Analytikjena at λ-540 nm, using Drabkin reagent. Then, using standard formulas described by Svobodova, (2001) were calculated the erythrocyte constants: mean corpuscular volume - MCV (μm^3), mean corpuscular hemoglobin - MCH (pg), mean corpuscular hemoglobin concentration - MCHC (g/dl).

The relative and an absolute number of leukocytes were obtained by microscopic examination of 200 leukocytes on blood smears (two per fish), using Zeiss Axio Imager microscope and immersion objective (10 oc. X 100 ob.). The absolute number of circulating blood leukocytes and thrombocytes was determined in comparison with 1000 erythrocytes counted on a hemocytometer, per blood volume unit. The blood smears were colored with May-Grünwald Giemsa panoptic method (MGG) and the type of leukocytes was determined based on identification characters listed by Svobodova et al. (1991).

2.2.4. Statistical analysis.

Data were analyzed by the one-way analysis of variance (ANOVA). All statistical analysis of data was performed using SPSS for Windows version 20.0 (SPSS Inc., Chicago, IL, USA) and the program PRIMER 7.

3. Results and discussions

3.1. Growth performance

One of the objectives of this experiment was to evaluate the effect of multi-strains of the three commercial probiotic products on the technological performance of juvenile carp. For this purpose, the rearing system was populated with 4.65 kg of juvenile carp and an average individual weight of 38.82 g/fish. The four rearing units corresponding to the experimental variants, each with three repetitions, were randomly populated with many ten fish/repetition. The initial and final values of the culture biomass from each experimental variant are presented in table 1. The statistical analysis of the weight data at the end of the experimental period showed a uniformity at the point of the average weights of the fish at the level of each variant (the averages of the three replicas did not differ statistically).

Regarding the results obtained at the end of the growth period in Table 1, a significant variation was observed between the experimental variants ($p<0.05$), where the data analysis showed a difference between the control variant (V1) and the *BioPlus®2B* probiotic variant (V2) also confirmed by Duncan test which revealed the existence of 2

homogeneous subsets. In figure 4 highlights the distribution of the averages of the individual weights from the four experimental variants.

Table 2 summarizes the technological performance indicators in the four experimental variants. The data obtained are closely related to the quality of the culture medium and the living space provided by the growing units. The physicochemical parameters of the water were monitored and maintained within the optimal limits for growth by changing the water volume of 50%.

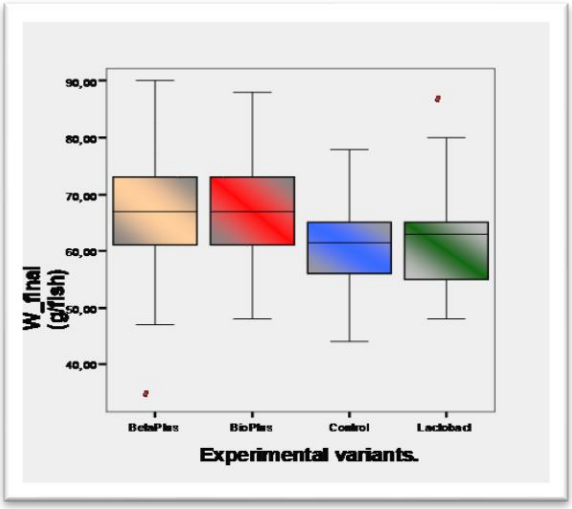

Figure 4. Individual average weight distribution/variant at the end of the experimental period.

Table 1. Variation of the average individual weights at the level of replicas/variants in the experimental period.

Experimental variant	The biometric parameter	Replication	Mean	Stdev.
V1 (Control)	W_initial (g/fish)	1	37.80	5.92
		2	38.30	4.16
		3	40.30	7.32
	W_final (g/fish)	1	62.00	6.23
		2	59.90	9.74
		3	61.70	5.80
V2 (*BioPlus® 2B*)	W_initial (g/fish)	1	40.00	6.93
		2	40.10	4.86
		3	37.50	7.85
	W_final (g/fish)	1	67.90	8.75
		2	69.80	6.68
		3	64.00	11.90
V3 (*BetaPlus®*)	W_initial (g/fish)	1	38.90	8.29
		2	38.00	6.99
		3	39.10	6.74
	W_final (g/fish)	1	67.10	11.48
		2	66.30	9.66
		3	65.20	12.54
V4 (*Lactobact premium*)	W_initial (g/fish)	1	38.70	6.46
		2	38.00	4.92
		3	39.10	6.23
	W_final (g/fish)	1	63.20	9.13
		2	62.20	9.99
		3	62.40	8.64

Table 2. The technological performance indicators of carp treated with multi-strains of probiotics.

Experimental variants	**Control (V1)**				**BioPlus® 2B (V2)**				**BetaPlus® (V3)**				**Lactobact premium (V4)**			
Indicator/ growth unit	**R_1**	**R_2**	**R_3**	**Unit 1**	**R_1**	**R_2**	**R_3**	**Unit 2**	**R_1**	**R_2**	**R_3**	**Unit 3**	**R_1**	**R_2**	**R_3**	**Unit 4**
Probiotic concentration (CFU/kg feed)	0.00	0.00	0.00	0.00	3.2×10^9	3.2×10^9	3.2×10^9	0.00	3.2×10^9	3.2×10^9	3.2×10^9	0.00	3.2×10^9	3.2×10^9	3.2×10^9	0.00
Survival rate (%)	100	100	100	100.00	100	100	100	100.00	100	100	100	100.00	100	100	100	100.00
Initial Biomass(g)	266	275	276	817.00	288.00	288.00	266.00	842.00	282.00	278.00	274.00	834.00	271.00	260.00	272.00	804.00
Final Biomass (g)	448	418	433	1299.00	481.00	492.00	438.00	1411.00	478.00	461.00	451.00	1390.00	443.00	434.00	435.00	1312.0
Biomass gain (g)	182	143	157	482.00	193.00	204.00	172.00	569.00	196.00	183.00	177.00	556.00	171.00	174.00	163.00	508.00
Mean initial weight (g/fish)	38.00	39.00	39.00	38.66	41.00	41.00	38.00	40.00	40.00	40.00	39.00	39.66	39.00	37.00	39.00	38.33
Mean final weight (g/fish)	64.00	60.00	62.00	62.00	69.00	70.00	63.00	67.33	68.00	66.00	63.00	65.66	63.00	62.00	62.00	62.33
Individual weight gain (g)	26.00	20.00	22.00	22.66	28.00	29.00	25.00	27.33	28.00	26.00	25.00	26.33	24.00	25.00	23.00	24.00
Daily growth rate (g/day)	5.20	4.09	4.49	4.59	5.51	5.83	4.91	5.41	5.60	5.23	5.06	5.29	4.89	4.97	4.66	4.84
Specific growth rate (%/day)	1.49	1.20	1.29	1.32	1.47	1.53	1.42	1.47	1.51	1.45	1.42	1.46	1.39	1.46	1.34	1.39
Feed conversion ratio (g feed/g biomass gain)	1.74	2.22	2.02	1.99	1.64	1.55	1.84	1.67	1.62	1.73	1.79	1.71	1.85	1.82	1.94	1.87
Protein efficiency ratio (g/g)	1.40	1.10	1.21	1.23	1.48	1.57	1.32	1.45	1.51	1.41	1.36	1.42	1.32	1.34	1.25	1.30

The most significant technological indicators are the specific growth rate (SGR) and the feed conversion ratio (FCR). These indicators recorded the best values in the experimental variant *BioPlus® 2B* (V2). In the control variant (V1), an average value of SGR of 1.32 g%/day and an FCR of 1.99 g feed/g growth increase was obtained, while variant *BioPlus® 2B* (V2) recorded an SGR value of 1.47 g%/day and an FCR of 1.67 g feed/g growth increase. In figure 5 we can see the inverse correlation established between the evolution of SGR and FCR. Also, the feed conversion factor (FCR) varied inversely with the protein efficiency coefficient (PER) in all experimental variants. From the analysis of the variants treated with probiotics, it was found that the best PER coefficient was registered for the basins of the *BioPlus® 2B* variant where it varied between 1.32-1.57 g/g, while the treatment with *Lactobact premium* led to lower values 1.25-1.34 g/g (average 1.27).

Figure 5. Variation in specific growth rate (SGR) and feed conversion ratio (FCR) between experimental variants.

The authors, Yanbo and Zirong (2006) found that *Bacillus spp.* used alone or in combination with an unidentified photosynthetic bacterium has improved the growth performance of juvenile common carp. Mukherjee et al. (2019) demonstrated that a mixture of *Bacillus methylotrophics* and *Bacillus licheniformis* compared to single strains contributed to disease resistance against *Aeromonas hydrophila*, immune response, and rohu carp growth performance. In the present study, juvenile carp, *Cyprinus carpio* fed diets containing supplementation with multi-strains probiotics of *Bacillus* and *LAB* had better average weight gain compared to the control diet with fed fish. Supplementing the fodder diet with multi-strains of probiotics consisting of *Bacillus* and *LAB* can improve the growth performance of juvenile carp. Compared with our study, previous studies on fish such as canal catfish (Thurlow et al., 2019), Pengze crucian carp (Cao et al., 2019; Yang et al., 2019), grass carp (Tang et al., 2019), carp fingerlings (Amit et al., 2021) also demonstrated the positive effect of *B. cereus*, *B. subtilis* and *L. plantarum* on growth performance.

3.2. Evaluation of microbiological parameters.

During this experiment, microbiological testing was performed by determining the total number of germs in the water, and analyzing the evolution of its quality. The test was performed at an interval of 8 hours after receiving the water and even before replacing the volume of water (2 samples), at an interval of one week to have a picture of the diurnal evolution of the total number of germs in the water culture. The dynamics of the microbial load of water are shown in figure 6 highlighting, as expected, the depreciation quality of water from a microbiological point of view with the accumulation of catabolism products in pool water.

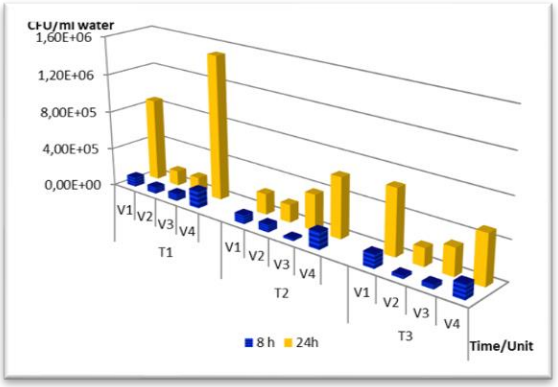

Figure 6. Dynamics of the total number of germs in the water of the growth units.

The maximum value determined was 1.5 x10^6 CFU/ml which indicates very impure water in all experimental variants but a significantly lower number was observed in variants V2 and V3 in which the feed was administered with *Bacillus* (10^4, 10^5 CFU/ml). The highest values were recorded in the growth unit of the variant treated with lactic acid bacteria (V4).

The verification of the existence and viability of the multiple strains of probiotics incorporated in the fodder administered to the carp juvenile during the experimental period was performed by performing the sowing on a nutritious and selective culture medium. Following the cultivation of these species of probiotic bacteria, their existence and viability in embedded feed were observed (figure 7).

Figure 7. Multi-strains probiotics in feed (a - *Bacillus subtilis, Bacillus licheniformis;* b – lactic acid bacteria).

3.3. Evaluation of hematological indicators

Blood indicators are significant tools that show us the response to physiological stress and the health of fish to nutritional and environmental changes (Kader et al., 2012). Knowledge and research of hematological parameters can facilitate the development of indicators of the health status of fish in response to changes related to nutrition, water quality, and disease.

The hematological indicators studied were hemoglobin, hematocrit, and red blood cell count. Red blood cell indices including mean corpuscular hemoglobin (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated and compared among the groups.

3.1.1. Hemoglobin (Hb)

The mean hemoglobin values recorded in the four experimental variants are presented in figure 8 and it can be seen that it falls within the normal values (6.5-10.6 g/dL by Bocioc et al., 2015) of the species *Cyprinus carpio*, without significant differences between the variants with multi-strains probiotics and control. As you can see, at the end experimental period, hemoglobin (Hb) ranged from 9.59 g/dL in the control variant (V1) case to 12.85 g/dL for Lactobact variant (V4).

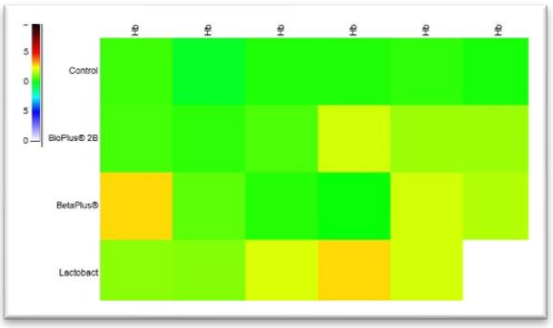

Figure 8. Matrix of mean hemoglobin values.

3.1.2. Hematocrit (PVC)

Hematocrit is the ratio of the volume of red blood cells to the total volume of blood that can be affected by the number of cells. In experimental variants with multi-strains probiotics, there was an increase in hematocrit compared to the control variant (from 29.00 % in a control group to 37.00 % in variants *BioPlus® 2B* and *Lactobact*) in general, all batches with values within the normal range (32-43.8% by Bocioc et al., 2015) for carp (figure 9).

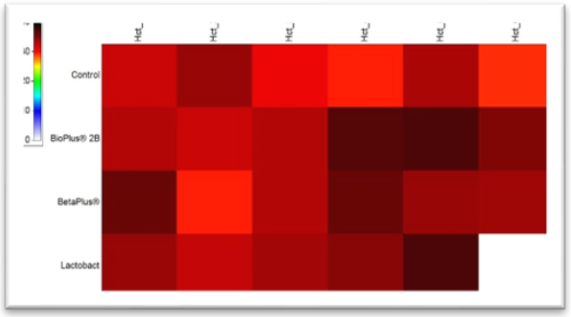

Figure 9. Matrix of mean hematocrit values.

3.1.3. Red blood cells count (RBCc)

The red blood cells count in the experimental period registered an increasing evolution in the carp specimens treated with multi-strain probiotics, the average values indicating an increase from 1.37×10^6/µL in the control variant to 1.65×10^6/µL in the *BetaPlus* variant and 1.77×10^6/µL in the variant treated with LAB (Lactobact). Statistical analysis revealed a significant difference between the control groups and *Lactobact* variant (figure 10), and the values recorded fall within the normal range of *Cyprinus carpio* ($1.10\text{-}2.20\times10^6$/µL by Bocioc et al., 2015). This evolution indicates stimulation of erythropoiesis in the variants treated with multi-strain probiotics implying positive consequences on the transport of oxygen to the tissues.

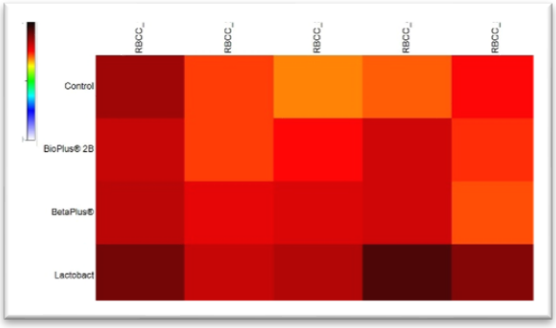

Figure 10. Matrix of mean red blood cells count values.

After determining the hematological indices, erythrocyte constants of juvenile carp were calculated which helps to detect physiological lesions in the process of hemoglobin formation, and provides information about the size, shape, and hemoglobin charge of the erythrocyte.

3.1.4. Mean corpuscular volume (MCV)

The mean corpuscular volume was maintained in the range of normal values for *Cyprinus carpio* species (152-364 µm^3 by Bocioc et al., 2015) observing a statistically insignificant difference between the groups treated with multi-strains probiotics and the control group. In the variant with *BioPlus® 2B*, there was a slight increase at 255.70 µm^3 compared to the control (190.12 µm^3). This indicates that the volume occupied by a single erythrocyte is higher in the group treated with the probiotic based on *Bacillus* compared to the control group (figure 11).

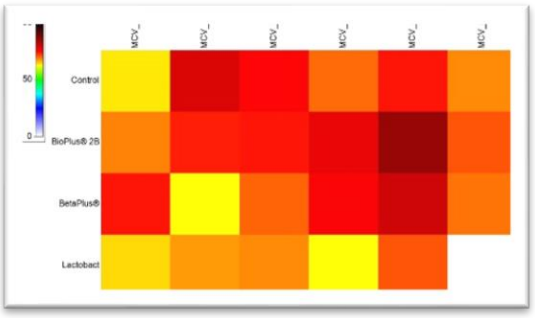

Figure 11. Matrix of mean corpuscular volume values.

3.1.5. Mean corpuscular hemoglobin (MCH)

The mean corpuscular hemoglobin registers average values that do not fall within the normal range of variation (50-63 pg by Bocioc et al., 2015) which indicates an increase in the variants fed with probiotics containing *Bacillus* species of 83.97 pg compared to 62.64 pg in the control variant. Given that hemoglobin is the major component of erythrocytes (95% of erythrocyte cytoplasmic proteins) and that MCV is higher in *Bacillus* variants (figure 12), this suggests that the adaptive response consists in stimulating the function of hemoglobin synthesis in these carp specimens.

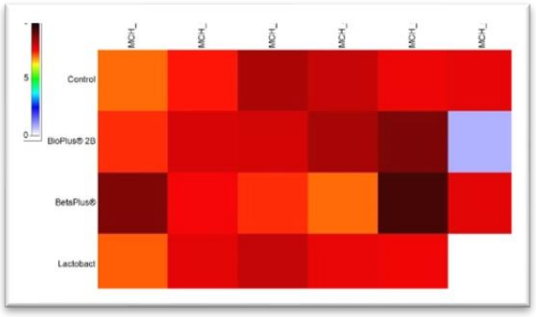

Figure 12. Matrix of mean corpuscular hemoglobin values.

3.1.6. Mean corpuscular hemoglobin concentration (MCHC)

The mean corpuscular hemoglobin concentration that measures the average Hb concentration in a given volume of erythrocytes indicates an increased response in all experimental variants compared to the normal range (15-25 g/dL by Bocioc et al., 2015), the control group recording values less than 28.19 g/dL compared to the *Lactobact* variant where 37.24 g/dL was recorded (figure 13).

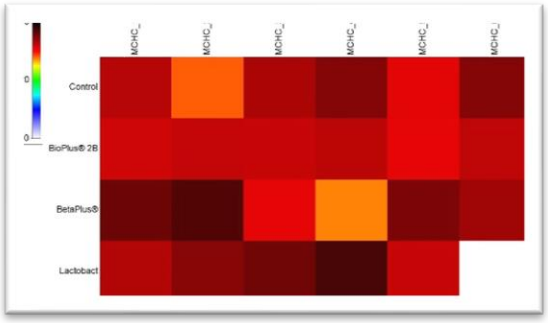

Figure 13. Matrix of mean corpuscular hemoglobin concentration values.

According to our results, the use of multiple probiotic strains determined a positive effect on hematopoiesis because red blood cell count and hematocrit were higher in the experimental groups with *Bacillus* and *LAB* compared to the control group.

The ecophysiological response of carp chicks to adaptation to new supplements of multiple strains of probiotics has consisted of some quantitative or qualitative changes in blood characteristics that suggest positive stimulation of metabolic health and immune response, especially nonspecific cellular. Similarly, feeding common carp (*Cyprinus carpio*) with a mix of *Bacillus subtilis* and *Bacillus licheniformis* could increase serum immunoglobulin levels and some immune parameters (Wang et al., 2017). Normal reference ranges of hematologic indicators are considered significant for assessing and monitoring the health status of fishes. In recent years more normal reference ranges of hematologic parameters of cultured and wild fish have been established (Witeska et al., 2016; Bocioc et al., 2015, Fazio et al., 2012b; Fazio et al., 2013a, 2013b)

3.1.7. Evaluation of leukocyte reactions in carp juveniles

A study of erythrocyte and leukocyte series on panoptical stained blood smears by the May-Grunwald Giemsa (MGG) method did not show any qualitative changes or morphological aspects in terms of their shape, size, or color. A comparative leukogram of the averages of the carp from the variants experimental is shown in table 3 and the variation in the absolute number of leukocytes in Table 4. Total WBC Count indicates a significant increase in leukocytes compared to other white blood cell types in the experimental variants with multi-strains probiotics. Lymphocytes contribute significantly to the total white blood cells. Usually, lymphocytes are responsible for the immune response. An increase in lymphocytes triggers the production of antibodies (Volbana et al., 2018). Granulocytes in fish blood are usually neutrophils. Reducing the number of granulocytes in the blood may be associated with increased disease resistance (Venkatalakshmi et al., 2015).

The interaction between probiotics and the host's immune system depends on several aspects, namely: source, type, strain, and species of probiotics. Therefore, there is the probability that when a probiotic strain was singularly supplemented in feeding to a particular host, may not have a positive effect on the host's

immune system. On the contrary, the combination of different genera and species of probiotics in a multi-strain probiotic can act synergistically and improve the host's immune response (Nayak, 2021).

Abdulrahman and Ahmed (2015) reported that white blood cells and lymphocyte levels were improved by supplementing diets with synbiotics for common carp. Similarly, the effects of different IMBO symbiotic concentrations, such as *Enterococcus faecium* (as a probiotic) and FOS (as a prebiotic) on the survival, growth performance, and digestive enzyme activities of the common carp juvenile (Dehaghani et al ., 2015).

Table 3. Variation of carp leukocyte formula at the end experimental period.

Leukogram (%)		**V1**	**V2**	**V3**	**V4**
Lymphocytes	Min	91.00	94.50	91.50	89.50
	Max	98.50	99.50	99.00	99.00
	X±SD	95.14±1.94	94.59±1.45	96.38±2.05	97.13±2.27
Monocytes	Min	0.00	0.00	0.00	0.00
	Max	3.00	0.50	0.50	1.00
	X±SD	0.47±0.75	0.013±0.12	0.15±0.23	0.13±0.29
Neutrophils	Min	1.50	0.50	1.00	1.00
	Max	6.50	4.50	8.50	10.00
	X±SD	4.23±1.64	2.16±1.13	3.44±1.99	2.63±2.17
Eosinophils	Min	0.00	0.00	0.00	0.00
	Max	1.00	2.50	0.50	1.50
	X±SD	0.15±0.34	0.22±0.65	0.02±0.12	0.10±0.38

Table 4. Variation in the absolute number of leukocytes at the end experimental period.

		V1	**V2**	**V3**	**V4**
Leukocyte ($x10^3$cell/µL)	Min	46.02	50.65	59.78	77.87
	Max	96.50	117.82	147.83	147.55
	X±SD	63.55±14.05	85.90±17.23	102.93±24.47	99.41±19.93
Lymphocytes ($x10^3$cell/µL)	Min	43.26	50.14	57.69	70.73
	Max	92.64	113.69	139.70	144.60
	X±SD	60.54±13.77	83.78±16.68	99.12±23.27	96.68±20.08
Monocytes ($x10^3$cell/µL)	Min	0.00	0.00	0.00	0.00
	Max	1.47	0.48	0.74	0.91
	X±SD	0.26±0.38	0.03±0.12	0.15±0.27	0.11±0.26
Neutrophils ($x10^3$cell/µL)	Min	0.84	0.50	1.05	0.90
	Max	4.60	4.12	10.50	7.90
	X±SD	2.64±1.04	1.87±1.11	3.62±2.63	2.50±1.69
Eosinophils ($x10^3$cell/µL)	Min	0.00	0.00	0.00	0.00
	Max	0.83	2.62	0.44	1.66
	X±SD	0.10±0.23	0.21±0.67	0.02±0.10	0.11±0.42
Thrombocytes ($x10^3$cell/µL)	Min	0.02	0.01	0.01	0.01
	Max	0.07	0.05	0.09	0.11
	X±SD	0.05±0.01	0.02±0.01	0.03±0.02	0.03±0.02

4. Conclusions

In the present study, we investigated the effects of multi-strain probiotics from three different commercial products with probiotics on the growth and hematological indicators of juvenile carp for 35 days. According to the results of this study, the use of *Bacillus subtilis, Bacillus licheniformis*, and *LAB* caused a positive increment in growth performance and hematological factors of juvenile carp. The highest growth and feed utilization were recorded in a fish group with *BioPlus® 2B* compared to control fish. The analysis of the results regarding the hematological profile shows stimulation of erythropoiesis (increase in the number of erythrocytes in the circulating blood); hemoglobin synthesis; leukopoiesis (increase in the absolute number of leukocytes) by a significant increase in the absolute number of lymphocytes (lymphocytosis) and a slight decrease in the number of neutrophils (neutropenia) in a fish group with multi-strain probiotics compared to control experimental variant.

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VARIATION OF ELECTROLYTES, AMINO ACIDS AND REDUCING SUGARS IN COCONUT WATER OF DIFFERENT AGES FROM AN INLAND REGION OF BANGLADESH

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ABSTRACT

Coconut water, extracted from the fruit of *Cocos nucifera* L., is a popular drinks throughout the tropics. The variable nature of the composition of the drinks had been established multiple times before, with regards to age, location and genetic variation. However, in Bangladesh, such studies were not carried out before. Thus, this study aims to compare electrolytes, amino acids and carbohydrates concentration in coconut water of different age collected from an inland region of the country. To determine electrolyte concentration atomic absorption spectrophotometry and Mohr titrimetric method were applied. Determination of carbohydrate and amino acid concentration required two separate high performance liquid chromatography (HPLC) methods. Regarding electrolytes concentration, rise of potassium, calcium and chloride ion concentration and fall of sodium and magnesium ion concentration were apparent. Potassium ion was the most abundant cation (50.88-67.56 mEq/L) while steep rise of magnesium ion concentration from 4 month to 6 months (4.14±0.17 to 12.72±2.52 mEq/L) was observed. Fructose and dextrose concentrations also escalated with coconut age. Amino acid concentrations varied as well. Histidine (0.43 g/100ml), arginine (0.053 g/100ml) and arginine (0.142 g/100ml) were the most abundant amino acids found in water 4, 6 and 8 months old coconuts. Overall, the trends of variation of components show intake of coconut water can bring different physical outcomes to different consumers and therefore, must be chosen carefully for patients with electrolytic imbalance and other medical complications.

1. Introduction

As a tropical tree, the global distribution and familiarity of the coconut tree (*Cocos nucifera* L.) is overwhelming. The tree conquered from East Indies to West Indies, from the remotest pacific islands to neo-tropical realm (Nayar, 2006). The consumable part of the tree is the coconut fruit. Numerous products can be extracted from the fruit such as coconut water, coconut milk, coconut meat, coconut apple, etc. Among them, coconut water had been more popular and subjected to extensive research due to its unique composition: a fine admixture of solution with electrolytes, carbohydrates, amino acids, vitamins, lipids, organic acids, enzymes, phytochromes (Yong et al., 2009).

The coconut water is drinkable and a popular source of hydration in tropical countries (Santoso et al., 1996). The electrolytic composition of coconut water is different from blood regarding the concentration of potassium (K+), sodium (Na+) and chloride (Cl-) ions. Yet, it provides essential ions to the body in cases of dehydration when taken orally (Saat et al., 2002). This replenishing drink had been popular to tropics and it is also commercialized in various countries, sometimes as sports drinks (Petroianu et al., 2004; Kalman, et al., 2012). Apart from the electrolytes, the coconut water was found to contain different carbohydrates: sucrose, glucose, fructose, galactose, mannose, arabinose, mannitol and xylose and different essential amino acids (del Rosario et al., 1984; Santoso et al., 1996). Moreover, coconut water also contains higher ratio of arginine, alanine, cysteine and serine, greater than those found in cow's milk (Santoso et al., 1996, Prades et al., 2012).

The proportion of nutrients in coconut water varies as the fruit grows old. The immature coconut, in which the endosperm is not well developed, is the most popular refreshing drinks in the tropics. The best time to harvest a coconut for drinking is at age 6-7 months, just as the jelly-like endosperm begins to form. At this stage the water has maximum sweetness and low acidity (Rolle, 2007; Chuku et al., 2014). This is due to the fact that as coconuts mature, their composition and physicochemical properties alter with the white kernel lining the inner shell becoming opaque and hard (Tan et al., 2014). For example, Child et al. (1950) reported that the sugar content of it become concentrated as the water volume decreases with age. Additionally, they reported that after a certain age, the taste of coconut water changes due the rise of pH value, turbidity and minerals concentration. The quantity of electrolytes in coconut water will be varied in Bangladesh due to the reasons such as geographical variation, temperature and humidity (Santoso et al., 1996). Genetic variation and variation among cultivars are another two prominent examples of content differences in coconut water (Santoso et al., 1996; Solangi et al., 2011).

Many research works had been done to determine the exact amount of minerals in coconut water (Saat et al., 2002; Solangi et al., 2011; Waziri et al., 2013). Few researches showed a variation of mineral components in ripe and unripe coconut (Vigliar et al., 2006; Adegoke et al., 2012). Many works describe composition of carbohydrates, but only few describes the variation in the maturation stage (Vigliar et al., 2006; Prades et al., 2012). On the other hand, there had been occasional variation in determination of protein and amino acid compositions from various authors (Santoso et al., 1996; Campbell-Falck et al., 2000). There has been very little research on coconut water quality from Bangladesh. Some of them focused on arsenic contents of coconut water (Safiullah et al., 2013). However, no article had been published to describe the electrolytes, carbohydrates and amino acid composition of coconut water of any varieties in the geographical area of Bangladesh.

In the present study, approaches have been made to evaluate the concentration of those components of the coconut water of Bangladesh and variation of the composition regarding different ages of maturity of the fruit has been observed.

2. Materials and Methods

2.1. Materials

2.1.1. Instruments

To measure osmolarity, a osmometer was used (the Advanced Micromometer 330, USA). The pH was calculated by a pH meter (Mettler-Toledo, Malaysia). The quantitative determination of electrolytes (Ca, Mg, Na, and K) were done using the single beam atomic absorption spectrometer (AAS) by using atomic absorption spectrophotometer (Bulk Scientific model 7000, Shimadzu, Japan). For analysis of amino acids and carbohydrates, a glass column of 1.5 X 30 cm and a rotavapor was employed for the preparation of the sample with different aged coconut water. Amino acids were quantified with a Waters 2695 Alliance HPLC

system (Waters Inc., Milford, CT, USA) which consisted of a 9012Q pump, 9100 autoinjector and 9075 fluorescence detector. Separation was carried out in a Waters Nova-Pack reversed phase C18 column, 4 µm particle size, 150 X 3.9 mm i.d. A specific Nova-Pack guard column was placed between the autoinjector and column. Waters 2695 Alliance HPLC system (USA) was also used for carbohydrates analysis. A differential refractometer (Waters R-401, USA) was used as detector. All the chromatographic information was reprocessed in Star Work-station (version 4.5) supplied by Varian. All the reagents used were of analytical grades.

2.1.2. Samples

Three coconut trees (all tall variety of green coconuts) in Narinda, Dhaka, Bangladesh were kept in close observation for maturing coconuts in 2018 and samples were collected in different seasons for coconuts of 4 months, 6 month and 8 months of age (n=20 for each). Coconut water were collected and filtered without contamination by the same process as described in Alchoubassi et al., 2020.

2.2. Determination of Electrolytes

The concentration of Na+, K+, Mg2+, Ca2+ were determined by flame AAS analysis. Before analysis blank, standard and sample solution were prepared. For blank solution of Na+, 1000µl (1ml) 10% cesium chloride (CsCl2) was transferred into a 100ml conical flask and then added together with 0.1M nitric acid (HNO3) up to mark. Furthermore, 1ml of each solution of 10% CsCl2, 0.1% KCl, 0.2% KCl were transferred to 100ml conical flask separately for preparing blank solution of K+, Mg2+, Ca2+ respectively and then water for injections (WFI) was added up to mark of each conical flask. The three standard solution for each ion of Na+, K+, Mg2+, Ca2+ were taken into 100 ml conical flask where 0.1, 0.2 and 0.4 ppm (1ml, 2ml, 4ml) for Na+ ion, 0.2, 0.4, 0.8 ppm (2ml,4ml, 8ml) for K+ ion, 0.1, 0.2 and 0.4 ppm (1ml, 2ml and 4ml) for Mg2+ ion and 0.5, 1 and 2 ppm (5ml, 10ml and 20ml) for Ca2+ ion respectively and then added the same ingredients that used to prepare blank solution of each to adjust up to mark. To prepare the sample, 40µl, 20µl, 100µl, 500µl filtered coconut water were taken into 100ml conical flask separately for determination of Na+, K+, Mg2+, Ca2+ respectively and then the same ingredients were added to prepare blank solution of each to adjust up to mark. The dilution factor of Na+, K+, Mg2+, Ca2+ were 2500, 5000, 1000, 200 times simultaneously.

To briefly describe the overall method, the sample first drawn to flame to constitute gaseous phase by ionization process. The calibration curve is obtained from three different concentrations of standard solutions and absorption verses concentration curve is prepared to analyze the samples. Ideal sample injection quantity is 10 to 20ml. After preparations of blank, standard and sample solution using the brands A, B, and C for blank, standard and sample respectively by employing the experimental conditions of Ca, Mg, Na and K ion quantification. In order to evaluate accurately, sample and standard curves were compared.

On the other hand, Cl- concentration were determined by Mohr titrimetric method as described by Sawyer et al., 2003.

2.3. Determination of Amino Acids

Preparation of Samples and Derivatisation. Filtered coconut water was taken into a glass beaker and shaken at a rapid speed. Derivatisation for fluorescence was done by 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate according to a method described earlier (Cohen, 2011).

HPLC analysis. The chromatographic conditions were as follows: flow 0.1 ml/min until 3 minute and then 1.5 ml/min, volume of injection 10 µl for each solvents. Solvents A contained sodium phosphate buffer (10 mM, pH 7.3), methanol, tetrahydrofuran (80:19:1) and solvent B contained sodium phosphate buffer (10 mm, pH 7.3), methanol (20:80). The gradient elution were set up as 100% of solvent A during 3.5 minutes, 0-15% of Solvent B in A for 6 mins, 15% of Solvent B isocratically for 5 mins, 15-30% of solvent B for 5 mins, 30-40%

of solvent B for 4 min and 40-80% of solvent B for 12 mins. Fluorimetric detection is carried out using excitation and emission wavelengths of 340 and 426 nm respectively.

2.4. Determination of Reducing Sugars

Carbohydrate analysis was done by HPLC coupled with a refractometer. The similar chromatographic conditions and method was followed as described in Trani et al., 2017.

3. Results and Discussions

3.1. Osmolarity and pH

The osmolarity and pH of the coconut water is shown in table 1. The osmolarity seemed to decrease from month 4 to month 8 gradually from 486-494 milliosmole/L to 345-353 milliosmole/L. A previous findings reported a decrease in osmolarity from 377.3 to 310.3 milliosmole/L in 8 month and 9 month old coconuts respectively (Neto et al., 1993). Similar results were obtained in other study of coconut from Brazil with median value of 419 milliosmole/L (Vigliar et al., 2006). On the other hand, the pH value of the coconut water decreased with age (table 1).

3.2. Concentration of Electrolytes

Among the cations found in coconut water, potassium ion (K+) is the most abundant regarding any age groups as shown in table 1. The more matured coconut is taken, the more the concentration decreased. Interesting findings were observed in case of Mg2+ which increased from an average of 4.14 milliequivalent/L (mEq/L) of 4 months of age to 12.72 mEq/L and 14.06 mEq/L in 6 months and 8 months aged coconut respectively. Small but significant amount of sodium and calcium ion were present in all samples with concentration rising with maturation. Sodium ion increased almost 4-fold from 4 month and 8 month of sample (1.98 to 8.03 mEq/L). The total cation concentration increased over time. Chlorine was the only anion measured. Its concentration declined from an average of 47.69 mEq/L of 4 month mature sample to 31.83 mEq/L of 6 month (table 1). According to Solangi, et al., 2011, certain genetic varieties of coconut showed similar trend of rise and fall in electrolyte concentrations. For instance, in that study coconut water of Sri Lankan tall variety had potassium concentrations decreasing from 32.94 mEq/L to 31.77 mEq/L in 6-7 month and 11-12 month respectively. In other different studies, potassium and sodium concentration of coconut water oscillated between 35.1-81.8 and 0.7-9.7 mEq/L respectively (Chavalittamrong, et al., 1982; Campbell-Falck et al., 2000). The extended range of concentrations of different observations can be assumed due to soil quality, genetic variation or maturation of the fruit (Jackson, et al., 2004; Uphade, et al., 2008). Coconut water is a good source of citrate and malate ions (Patel et al., 2018) and the anions of the salt form may shift towards those ions rather than chloride ion with increasing age.

Table 1. Variation of osmolarily, pH and electrolytes concentration in waters of differently aged coconuts

Properties	**Values**		
	4 month (n=20)	6 month (n=20)	8 month (n=20)
Osmolarity (milliosmole/L)	486-494	428-446	345-353
pH	4.63-4.65	5.55-5.58	5.64-5.70
K^+ (mEq/L)	67.56±2.19	63.87±1.11	50.88±1.81
Na^+ (mEq/L)	1.98±0.23	2.02±0.18	8.03±0.23
Mg^{2+} (mEq/L)	4.14±0.17	12.72±2.52	14.06±2.11
Ca^{2+} (mEq/L)	4.87±0.30	6.75±0.22	8.78±0.49
Cl^- (mEq/L)	47.69±0.99	42.76±1.31	31.83±0.49

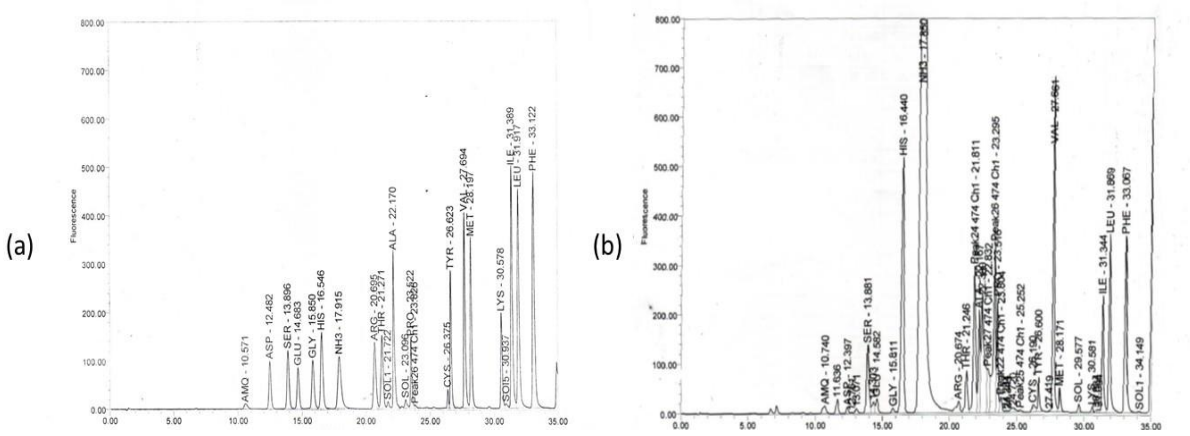
3.3. Concentration of Amino Acids

After HPLC-fluorometric analysis of coconut water, several amino acids in different concentration were observed as shown by the chromatograms of a representative sample in the figure 1.

Overall, amino acid concentration is comparatively higher in more matured coconut with an exception of histidine and leucine (table 2). In 4 month old coconut, histidine (0.430 g/100ml) was found as one of the more prominent amino acid. However, coconuts with 8 months of age contains more serine and glutamic acid 0.266 and 0215 g/100ml respectively. All the amino acids found in the coconut water appeared in previous studies (Young et al., 2009). Moreover, the overall protein content was found to be higher with maturation in a prior study (Jackson, et al., 2004).

Table 2. Variation of different amino acid contents in differently aged coconut water

Amino acids (g/100ml)	Values		
	4 months	6 months	8 months
Serine	0.075	0.129	0.266
Leucine	0.059	0.055	0.033
Glutamic Acid	0.032	0.114	0.215
Aspartic Acid	0.010	0.024	0.033
Glycine	0.005	0.011	0.019
Cysteine	0.030	0.035	0.077
Alanine	0.022	0.027	0.028
Proline	-	0.015	0.096
Valine	0.029	0.088	0.083
Arginine	0.034	0.053	0.053
Histidine	0.430	0.052	0.052
Isoleucine	0.036	0.033	0.062
Lysine	0.007	0.036	0.046



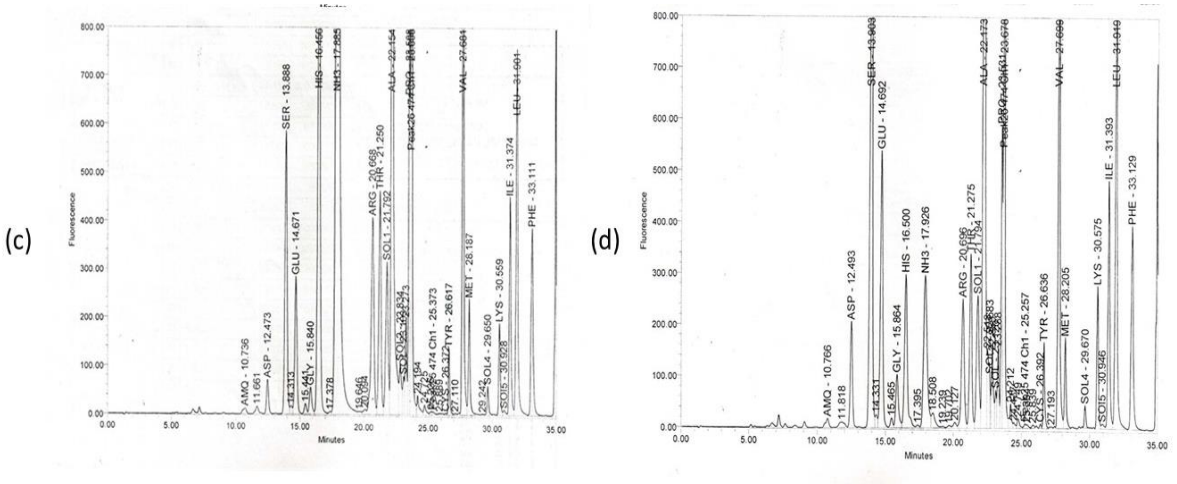
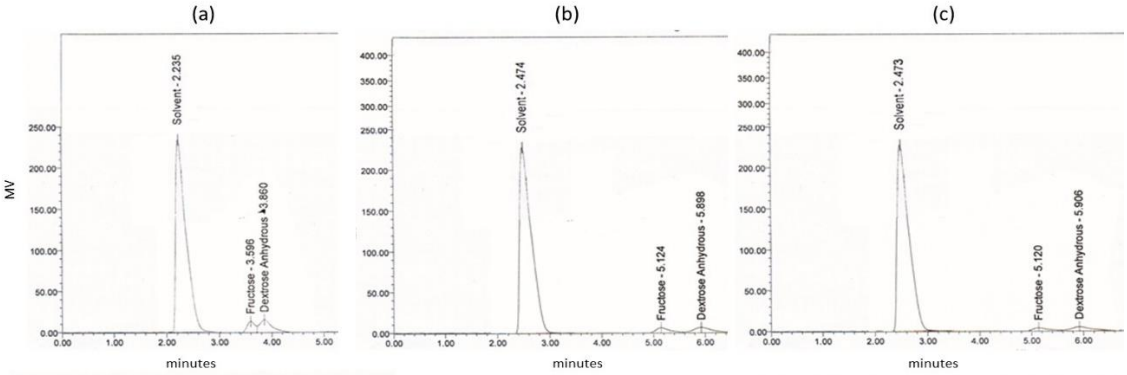


Figure 1. HPLC chromatograms of a representative sample of coconut water: (a) standard chromatogram, (b) chromatogram of 4 months old coconut, (c) chromatogram of 6 months old coconut and (d) chromatogram of 8 months old coconut.itle TNR 12 Normal

3.4. Concentration of Reducing Sugars

The representative chromatograms of HPLC-refractive index analysis for reducing sugars, e.g., dextrose and fructose are represented in the figure 2. Fructose concentration were found 2.30±0.16, 2.98±0.44 and 1.65±0.18 g/100ml in 4, 6 and 8 month old coconuts respectively. Similarly, dextrose concentration were 2.40±0.30, 4.02±0.39 and 2.65±0.08, fluctuating with increasing maturation.

Similar fluctuations of concentrations of total reducing sugar were previously observed by Child et al., 1950; with ultimate drop of sugar concentration in 18 month old coconut's water. Meanwhile, they reported total sugar concentration as higher in 10-14 month old coconut mainly due to increase of non-reducing sugar concentration. In fact, several sugars such as sucrose, glucose, fructose, mannitol, sorbitol, etc. appeared in literatures (del Rosario et al., 1984).



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Solvent	2.235	3215632	236579		
2	Fructose	3.596	138787	13354	2.313	g/100ml
3	Dextrose Anhydrous	3.860	253768	15481	2.403	g/100ml

Peak Results

	Name	RT	Area	Height	Amount	Units
1	Solvent	2.474	5667808	384405		
2	Fructose	5.124	192597	10445	3.061	g/100ml
3	Dextrose Anhydrous	5.898	276059	10808	4.198	g/100ml

Peak Results

	Name	RT	Area	Height	Amount	Units
1	Solvent	2.473	5676728	382477		
2	Fructose	5.120	100427	5476	1.596	g/100ml
3	Dextrose Anhydrous	5.906	175554	7135	2.670	g/100ml

Figure 2. HPLC chromatograms of a representative sample of coconut water for reducing sugar analysis: (a) chromatogram of 4 months old coconut, (b) chromatogram of 6 months old coconut and (c) chromatogram of 8 months old coconut

4. Conclusions

Coconut water is a popular refreshing drinks in the tropical world. There had been several works that explored the chemical and nutritional contents of this drink. Additionally the changes of coconut concentration due to soil quality, maturity and genetic variation was established. However, in Bangladesh, no such studies were found. Moreover, very few researches were available to explore the gradual changes of the coconut water contents from 2-6 months. The current study was aimed to fill this research gap. Samples of differently aged coconut were collected from a specific area of Dhaka and analyzed for electrolytes, amino acids and reducing sugar composition. The result obtained are in harmony with the previous studies in other settings. However, since the total concentration of cations (Na+, K+, Mg2+ and Ca2+) increased over time, gradual decrease of chloride ion concentration indicated an unknown anion-shifting in the salt concentration of the coconut water, probably going towards more citrate and malate ions. The current study had its constraints of not focusing on coconuts older than 6 months. It could neither be streamlined to a specific genetic diversity of coconut tree, nor could it be broadened the analysis to coconut trees in other regions of the country, especially coastal regions. The current authors hope that future studies would try to enlighten those research areas.

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QUALITY CHANGES OF BROILER MEAT FROZEN USING HOUSEHOLD REFRIGERATOR AT -18°C AND THAWED USING DIFFERENT TECHNIQUES

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ABSTRACT

The physico-chemical quality parameters are altered during the freezing and thawing process. The uniform-sized whole broiler chicken samples and cut-up parts samples were individually frozen in the household refrigerator (-18°C) overnight. The frozen meat samples were thawed in the refrigerator (4°C), cool room (20°C), hot air oven (60°C), tap water (27±5°C), and hot water conditions (40°C) until the core temperature of the meat reaches 10°C in all method except refrigerator method. The refrigerator method thawing allowed to reach 4°C core temperature. In this study, the moisture content, water holding capacity, drip loss, cooking loss, and pH were investigated for both the whole and the cut-up parts samples. The moisture content and the drip loss were shown a significant difference ($p<0.05$) between different thawing methods for a whole chicken sample. Since the pH, water holding capacity, and cooking loss were not shown any significant differences ($p>0.05$) between different thawing methods for a whole chicken sample. The overall best quality retention was observed in the cool room thawing method. In cut-up part analysis, pH was shown a significant difference($p<0.05$) between portions in the cool room thawing method but moisture content, water holding capacity, drip loss, and cooking loss were not shown any significant difference ($p>0.05$) between cut-up parts for cool room thawing method. According to this study, the different thawing methods affect the quality parameters such as moisture content and drip loss. The cut-up part analysis results, the cool room thawing method affects the pH of different cut-up parts of the broiler meat.

1.Introduction

Freezing is the most widely used approach in preserving surplus agricultural production. A large proportion of poultry meat sold for cooking purposes in markets is offered as frozen meat. Freezing changes in the quality of meat are direct outcomes of freezing and frozen storage processes. Usually, freezer compartments in household refrigerators are below -18°C (Anderson et al., 2004). The freezing rate influences the meat quality by the size of ice crystals formed during the freezing process (Li and Sun, 2002). According to the rate of freezing, the freezing methods can be classified into slow and fast freezing techniques (Oliveira et al., 2015). The freezer compartment of the refrigerators is categorized under the slow freezing technique, which produces typically larger ice crystals.

Broiler chicken plays a significant part in mitigating the shortage of animal protein (Rokonuzzaman, 2018). According to the World Health Organization, the human required 55g/day of animal protein, but only 15.6 was eaten daily (Rokonuzzaman, 2018). To prolong the shelf life, the broiler meat was kept in freezing conditions. Before further preparation,

such as cutting and heat processing, the frozen meat must be thawed.

The thawing mechanism is slower rather than freezing (Akhtar *et al*., 2013). Quick thawing at low temperatures is desirable in order to ensure food safety by preventing noticeable temperature increases and food dehydration (Akhtar *et al*., 2013). Food is prone to damage by chemical and physical alterations and microbial attacks during the thawing period (Fennema *et al*., 1973). Quality degradation, such as lipid and protein oxidation, protein denaturation, moisture loss, color deterioration, flavor loss, textural changes, and microbial spoilage, occur during the thawing period. (Benjakul and Bauer, 2000). The drawbacks of traditional thawing include food quality loss due to the leaching of soluble proteins, heavy energy consumption, and significant volumes of filled wastewater (Roberts *et al*., 1998).

Room temperature thawing, cold water thawing, steam thawing, and contact thawing are common thawing techniques for frozen foods. High-pressure thawing, microwave thawing, ohmic thawing, and acoustic thawing are now being researched to resolve losses in efficiency and duration of thawing (Li and Sun, 2002). The objective of this study is to evaluate the quality changes of thawed broiler meat using different techniques, which are frozen in the household refrigerator.

2. Materials and methods

2.1. Location

This study was done in the Food Science Laboratory of the Department of Biosystems Technology, Faculty of Technology, South Eastern University of Sri Lanka.

2.2. The samples

The commercially available raw carcass of broiler chicken was used for this study. Approximately 250g of whole broiler chicken carcass within 12 hours after slaughter without excess muscle fat and connective tissue were used as samples. For the cut-up parts study, 150g of each broiler meat part was used.

2.3. The freezing and thawing

The samples were individually frozen in the freezer compartment of the household refrigerator (Eco 251 NF, Sri Lanka) at -18°C. The frozen carcass was thawed in the lower compartment of the refrigerator(4°C), cool room (20°C), hot air oven (60°C), tap water (27±5°C), and hot water (40°C) conditions. The samples were thawed until the core temperature of the meat reaches 10°C in all methods except the refrigerator lower compartment method, where the core was allowed to reach 4°C. The core temperature of the broiler meat measured using a probe thermometer (THM-010-2MX-p, China). Preliminary studies were done to determine the thawing times of each method and to determine the thawing temperature of hot air oven conditions to prevent overheating of the surface of the meat. After thawing, the meat samples were determined for water holding capacity, drip loss, cooking loss, moisture content, pH, and sensory evaluation.

2.4. The drip loss (DL)

The DL was determined by the bag method (Barton-Gade *et al*., 1993). Each meat sample was weighed and put into a net made of cotton, and both meat and net were put into a polyethylene (PE) bag and closed without touching the interior surface of the bag. The prepared samples were placed in a chilling room at 4°C for 24 hours. Afterwards, the sample was gently dabbed with soft tissue, and weight was measured (Barton-Gade *et al*., 1993; Kim *et al*., 2013).

$$\text{Drip loss (\%)} = [(W_1-W_2)/W_1] \times 100 \quad (1)$$

Where,
W_1 = weight before thawing
W_2 = weight after thawing

2.5. Cooking loss (CL)

The sample was heated at 75°C in a water bath (Memmert w350, Germany) until the core temperature reaches 65°C and then cooled, and the weight differences were calculated (Kim *et al*., 2013).

Cooking loss (%) = $[(W_1-W_2)/W_1] \times 100$ (2)

Where,
W_1= weight before cook
W_2= weight after cook

2.6. Water holding capacity (WHC)

Initially, the meat samples were placed in a water bath (Memmert w350, Germany) at 70°C for 30 minutes. Afterwards, samples were minced using a meat mincer (Brice TC12, Australia). The 5g of minced meat was centrifuged for 1000rpm for 10 minutes in a high-speed micro-centrifuge (SCILOGEX SCI24, USA). The centrifugation loss of the meat was calculated as the difference in weight before and after centrifugation (Kristensen and Purslow, 2001).

WHC (%) = $[(M-N) \times 0.951^{*}/M] \times 100$ (3)

Where,
M= Total water content
N= separated water content
*0.951: pure water amount for meat moisture that is separated under 70°C

2.7. Moisture content (MC)

The meat samples were kept in a hot air oven (TLPPL 131, India) at 105°C overnight. The samples were placed in the oven until a constant weight was achieved. The samples were placed in a desiccator before measuring the weight difference. (AOAC, 2000).

MC (%) = $[(W_1-W_2)/W_1] \times 100$ (4)

Where,
W_1 = Initial weight
W_2= Weight after oven drying

2.8. pH

The pH was measured by grinding 15g of the meat sample with 150ml of deionized water for 5 minutes using a meat mixer (Brice TC12, Australia) at high speed (Trout, 1989). The pH of the solution measured using the benchtop pH meter (Bp3001, Singapore).

2.9. Statistical analysis

The results were submitted to analysis of variance (ANOVA), and means were compared by the test of Tukey's HSD at p = 0.05 using SPSS statistical package (SPSS 20.0, IBM, New York, NY, USA).

3. Results and discussions

The freezing and thawing cycles affect the MC of the meat (Leygonie *et al*., 2012). According to this study, MC has shown a significant difference ($p<0.05$) for different thawing methods (Table 1).

Table 1. Moisture content (MC) and Drip loss (DL) values of thawed broiler chicken

	Cool room	Hot water	Oven	Refrigerator	Tap water
MC (%)	76.392±0.957^{a}	76.586±0.446^{a}	77.086±0.261^{a}	77.590±0.838^{ab}	79.641±0.679^{b}
DL (%)	11.051±1.780^{a}	8.455±2.431^{a}	26.207±3.409^{b}	10.107±2.052^{a}	11.917±2.608^{a}

The values are means of triplicates ± standard error mean (SEM)
Within a row, means followed by the same letter are not significantly different by the Tukey's HSD at p=0.05.

The tap water thawing method has shown higher MC, whereas the cool room thawing method shown lower MC. Gonzalez-Sanguinetti *et al*. (1985) reported a significant difference in MC with different thawing techniques. The MC, determined by the WHC of the meat, ultimately affects the final pH of the meat (Rifath and jemziya, 2021). Tenderness, juiciness, firmness,

appearance, quality, and economic values are increased with the moisture content of the meat (Mir *et al*., 2017). Meanwhile, DL has shown a significant difference ($p<0.05$) among different thawing methods (Table 1). The minimum DL was observed in the hot water thawing method, whereas the highest DL was observed in the oven thawing method. Exudate release is influenced by freezing and thawing methods (Leygonie *et al*., 2012). It causes damage to the muscle cells' ultrastructure to release the mitochondrial and lysosomal enzymes, haem iron, and other pro-oxidants (Leygonie *et al*., 2012). The increased rate of thawing contributed to decreased exudate forming (Ambrosiadis *et al*., 1994).

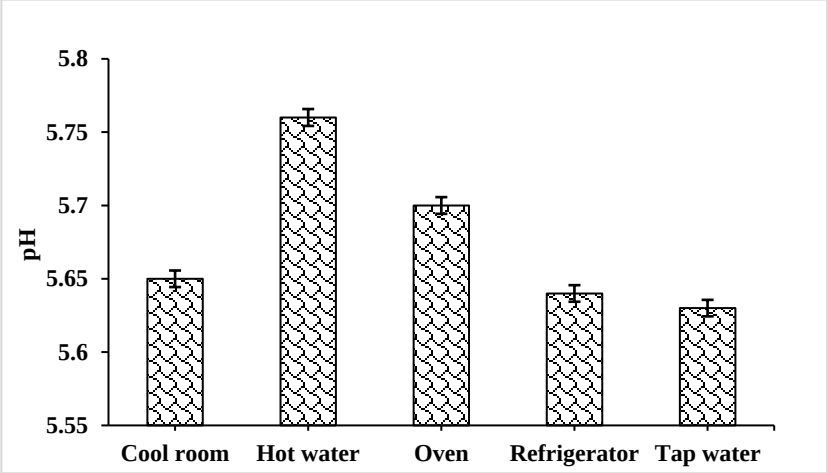

Figure 1. pH of the thawed broiler chicken
The values are means of triplicates ± standard error mean (SEM)

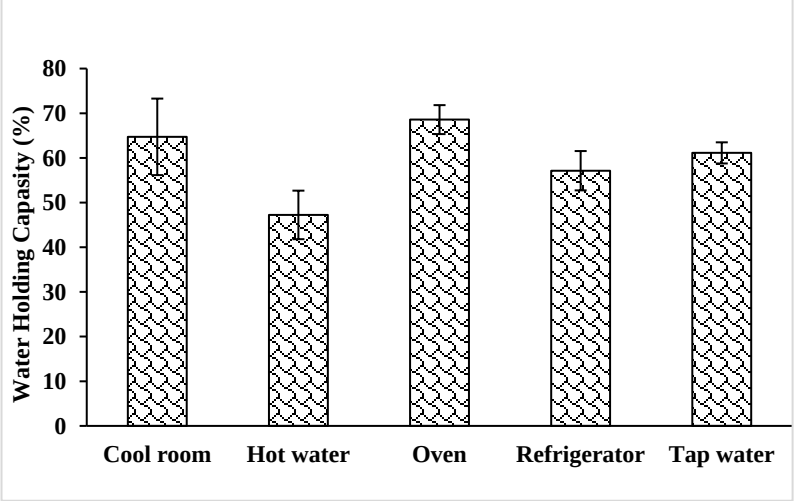

Figure 2. WHC (%) of the thawed broiler chicken
The values are means of triplicates ± standard error mean (SEM)

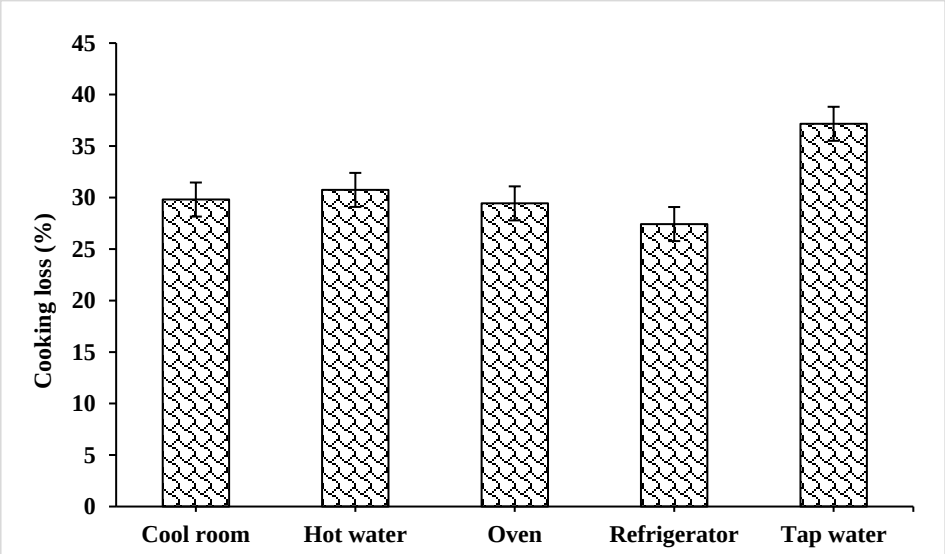

Figure 3. Cooking loss (%) of the thawed broiler chicken
The values are means of triplicates ± standard error mean (SEM)

The pH was not shown a significant difference ($p>0.05$) among different thawing methods (Figure 1). The lowest pH was recorded for the tap water thawing method, and the hot water method was observed with the highest pH value. The thawed meat shows a lower pH than frozen meat (Leygonie *et al*., 2012). It is due to the development of exudate that may denaturate buffer proteins, which triggers the release of hydrogen ions and also raises the concentration of released exudate, which causes pH reduction (Leygonie *et al*., 2012). The ultimate pH of meat depends on initial glycogen content prior to slaughtering and the rate of glycogen conversion (Mir *et al*., 2017).

The WHC was not shown a significant difference ($p>0.05$) among different thawing methods (Figure 2). The WHC for the oven thawing method is highest compared to other thawing methods, whereas the hot water thawing method was observed for a lower value. The WHC of the meat is reduced by the freezing and the thawing cycles (Añón and Cavelo, 1980; Ngapo *et al*., 1999; Vieira *et al*., 2009). The WHC has directly affected color and the tenderness of the raw carcass (Mir *et al*., 2017).

The CL was not shown a significant difference ($p>0.05$) among different thawing methods (Figure 2). The CL of the tap water-thawed broiler meat was considerably higher than other methods, and the refrigerator lower compartment thawing method was recorded with low value. The CL affects the WHC, protein, and fat content of the meat (Aaslyng *et al*., 2003). Protein denaturation and fat-melting during cooking release the chemically binding water from meat during cooking (Vieira *et al*., 2009). When comparing the overall results, the cool room thawing method quality degradation during the thawing period is minimum. The broiler meat thawed using a cool room shows lower values for DL and CL. Meanwhile, it shows higher WHC and moderate pH overall.

Table 2. Physico-chemical quality parameters of cool room thawed cut-up parts of broiler chicken

	MC (%)	pH	CL (%)	WHC (%)	DL (%)
Thigh	76.871±.462[a]	5.61±0.06[a]	29.273±1.722[a]	60.455±2.867[a]	13.844±1.620[a]
Drumstick	76.990±0.497[a]	5.87±0.07[b]	31.432±1.263[a]	58.116±3.008[a]	11.861±2.160[a]
Wing	77.641±0.621[a]	5.60±0.05[a]	32.747±1.607[a]	58.637±4.295[a]	14.544±1.908[a]
Breast	77.655±0.447[a]	5.64±0.06[ab]	29.273±1.497[a]	59.852±1.940[a]	14.805±3.020[a]

The values are means of triplicates ± standard error mean (SEM)
Within a column, means followed by the same letter are not significantly different by the Tukey's HSD at p=0.05.

The quality characteristics analyzed for cut-up parts thawed using the cool room method are given in Table 2. According to the results, pH showed a significant difference ($p<0.05$) between cut-up parts. Zhang *et al*., (2017) also reported that broiler breast cut-up parts thawed using different methods shown a difference in pH. Since MC, CL, WHC, and DL were not shown any significant difference ($p>0.05$) between cut-up parts. The moisture content for the breast part comparably higher than other parts, and the thigh recorded with lower moisture content than other parts. The lowest value for the pH is observed in the wing portion, and higher was observed in the drumstick portion. The wing portion lost a higher amount of exudate during the cooking process. Meanwhile, breast and thigh portions were loosed minimal amount of exudate during the cooking. The highest WHC was recorded in the thigh portion, and the lowest value was recorded in the drumstick. The drip loss was lower in the drumstick portion than other cut-up parts, and the highest DL was observed in the breast portion.

4. Conclusions

This study investigated the changes of quality parameters of broiler meat frozen in the household refrigerator and thawed using different techniques. The broiler meat quality parameters such as moisture content and drip loss depend on the thawing methods. The cool room thawing method shows an overall minimal quality reduction for quality parameters than other methods. The cut-up parts thawed using the cool room method are not showing any difference between portions except pH.

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BIOACTIVE COMPOUNDS, ANTIOXIDANT ACTIVITY AND LIPID CONTENT OF VARIOUS AVOCADO FRUITS

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ABSTRACT

Avocado fruits (*Persea americana*), native to Central America and Mexico, are highly nutritious and grown widely in tropical and subtropical regions. They are rich in fatty acids and other bioactive compounds such as vitamin E, carotenoids and phenolic compounds. Recently, avocado cultivars grown in Vietnam have gained more interest due to their value. This study aimed to investigate variations in lipid contents and bioactive compounds among avocado cultivars grown in Vietnam. Avocado fruits were harvested at mature green and transferred to the laboratory and stored at room temperature for ripening. Lipid content was measured by extraction with hexane. Phenolic content, antioxidant activity and carotenoid content were determined using the Folin-Ciocalteu, DPPH and spectral methods, respectively. The results showed that the lipid content varied from 35.41% for Sap bong cultivar to 61.40% for 034 cultivar. Booth 3 cultivar showed the highest total phenolic content (6.46 mg GAE/g DW), while this value is the lowest for HAC cultivar (2.67 mg GAE/g DW). Flavonoid content also significantly varied between cultivars from 0.70 to 2.01 mg catechin/g DW. Carotenoid content ranged from 38.55 µg/g for HAC to 226.77 µg/g for Nam long. Variations in chlorophyll a and chlorophyll b contents were also observed. Chlorophyll a and chlorophyll b contents varied from 127.60 to 316.30 µg/g and 46.83 to 223.81 µg/g, respectively. Significant variations in the lipid content and the bioactive compounds between the avocado cultivars provide information to select high quality avocado fruits for commercial purposes.

1. Introduction

Avocado (*Persea Americana* Mill.) belonging to the family of *Lauraceae* and is an important fruit native to Mexico and Central America (Tremocoldi *et al.*, 2018). This fruit is worldwide grown in tropical and subtropical regions (Qin and Zhong, 2016). Mexico is the top avocado producer in the world, accounting for 30% of the world production (Tremocoldi *et al.*, 2018). Avocado varieties are very diverse, but only two cultivars, Hass and Fuerte are commonly grown for commercialisation (Rodríguez-Carpena *et al.*, 2011). Avocado fruit

is a nutritious and tasty fruit containing high amount of lipids, vitamins and minerals (Tan *et al.*, 2017).

Avocado fruit is considered a healthy fruit. This fruit is an excellent source of fatty acids in which monounsaturated fatty acids account for 71%, then 13% polyunsaturated fatty acids and 16% saturated fatty acids (Dreher and Davenport, 2013). Lipid content of avocado fruits varies depending on varieties. For instance, Peraza-Magallanes *et al.* (2017) showed the lipid content varied from 7.39 to 36.98% among seven avocado genotypes. Variations in lipid content were also observed in other avocado genotypes: 2.59 to 11.87% fresh weight between seven Taiwan domestic avocado genotypes (Teng *et al.*, 2016) and from 18.28 to 26.77% fresh weight among eight avocado genotypes (Espinosa-Alonso *et al.*, 2017). Lipid content can also be affected by geographic locations (Donetti and Terry, 2014, Ferreyra *et al.*, 2016), harvest time (Wang *et al.*, 2012, Donetti and Terry, 2014) and storage conditions (Vekiari *et al.*, 2004).

Avocado fruit is also rich in bioactive compounds which are potential to use in cosmetics, medical and food industries (Dreher and Davenport, 2013, Rodriguez-Lopez *et al.*, 2017). This fruit contains natural antioxidants such as carotenoids and polyphenols (Lu *et al.*, 2009, Vinha *et al.*, 2013). Avocado fruit is one of few foods containing high levels of antioxidant vitamins (vitamin C and vitamin E) (Dreher and Davenport, 2013). Other pigment bioactive compounds such as chlorophylls and anthocyanins also present in avocado fruits (Ashton *et al.*, 2006). The contents of these bioactive substances vary depending on varieties (Espinosa-Alonso *et al.*, 2017, MARDIGAN *et al.*, 2019), growing locations (Lu *et al.*, 2009), harvest time (Wang *et al.*, 2012) and storage conditions (Donetti and Terry, 2014, Campos *et al.*, 2019).

Vietnam is one of Asian countries where avocados have been grown and this fruit has recently gained more interest for domestic uses as well as for export purposes. In Vietnam, avocados are mainly cultivated in central highlands and South East regions where ecological conditions are suitable for them to produce great yield and fruit quality (Hoang *et al.*, 2013, Hoang *et al.*, 2017). These studies, however, primarily focused on yields, agro-bio parameters and nutritional values. Our study aimed to investigate bioactive compounds and lipid content of 15 selected avocado varieties with the purpose to select avocados with high quality for growth.

2. Materials and methods

2.1. Chemicals and reagents

1,1-diphenyl-2-picryl-hydrazyl (DPPH) and Trolox were analytical-graded and purchased from Sigma (USA). Gallic acid and Folin–Ciocalteu reagent were purchased from Merk (Germany).

2.2. Sample collection

Avocado fruits were collected from avocado trees grown in Phuoc An town, Krong Pak district, Dak Lak province, Vietnam. Five to seven fruits of each avocado variety were picked using specialized tools. Avocado fruits had the same maturity (commercial stage).

Fruits after being harvested were encoded, individually packed with tissue paper, wrapped in foam, individually packed with perforated PE bags, within 24 h transported by air to the laboratory.

Hass avocado grown in Australia were bought at Adelaide Central market and was transported to the Laboratory by air. This avocado was used as a reference.

At the laboratory, the fruit was kept at 20-22 ^{o}C until ripe (about 3-5 days). Three fruits of each cultivar were then cut in half, the flesh, the skin and the seed were separated. The flesh was homogenized by a blender. All samples were stored at -18 ^{o}C and then freeze dried. The freeze-dried flesh was kept in the zip bags and was stored at -18 ^{o}C for further analysis.

2.3. Lipid content determination

Lipid was extracted by n-hexane and the lipid content was calculated based on sample weight reduction after lipid extraction. In brief, the extraction was performed in triplicate. 0.25 g of sample was weighed into a prepared filter bag and lipid was then extracted in three replicates from each avocado cultivar with 45 mL of n-hexane in 10-mL flask for 7 hours for two times. The sample was re-washed with n-hexane and was then dried at 105 ^{o}C for 3 hours before weighing for lipid content calculation.

2.4. Determination of total phenolic content

Fruit extracts for the determination of total phenolic, flavonoid contents and antioxidant activity were prepared using the method adapted from Alothman *et al.* (2009) with some modifications. 0.4 grams of freeze-dried sample were extracted with 8 mL ethanol 70% for 3h at room temperature. The sample was regularly shaken every 10 minutes. The extract was then centrifuged at 6000 rpm for 10 minutes and the supernatant was collected and stored at -20^{o}C. The extraction of each variety was carried out in triplicate.

Total phenolic 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 were added. The reaction was incubated at room temperature in the dark for 2h and the absorbance of the mixture was then measured at 760 nm using a UV–visible spectrophotometer (UV-Vis 1800, Shimadzu, Japan). The results were expressed in mg gallic acid equivalents/g dry weight (mg GAE/g DW) using a gallic acid standard curve. A stock solution of 1 mg/mL gallic acid was prepared and the calibration curve was then established based on working-standard solutions of 0.02, 0.04, 0.06, 0.08 and 0.1 mg/mL.

2.5. Determination of flavonoid content

Flavonoid content was determined by a method described by Baba and Malik (2015). In brief, 1000 µL of crude extract was mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution. After 5 mins of incubation, 0.3 mL of 10% $AlCl_3$ solution was added, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution were added. The mixture was allowed to stand for 15 min, and absorbance was measured at 510 nm. The flavonoid content was calculated from a calibration curve of catechin with working standard solutions of 0, 0.01, 0.02, 0.04, 0.06, 0.08 mg/mL and the result was expressed as mg catechin equivalent per g dry weight.

2.6. Antioxidant capacity measurement

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 300 µL of diluted fruit extract with 2850 µL of the DPPH solution for 30 minutes. A control was also prepared by using 150 µL of methanol instead of fruit extract. The absorbance was then measured at 515 nm using a spectrophotometer (Lambda 25, Perkin Elmer, USA). The results were expressed in Trolox equivalents (µM TE/g DW) using a Trolox standard curve. The standard curve was established based on Trolox standard solutions.

2.7. Determination of chlorophyll and carotenoid contents

The chlorophyll and carotenoid contents were determined by the method of Kotíková *et al.* (2011). 0.3 g of sample was extracted with 6 mL of 100% acetone at 4 ^{o}C for 5 hours. The sample then was centrifuged at 6000 rpm for 10 mins and the supernatant was collected for

absorbance measurement at three wavelengths: 662 nm, 645 nm, 470 nm.

Calculation of chlorophyll and carotenoid contents:

$$C_a = 17{,}75*A_{662} - 2{,}35*A_{645} \quad (1)$$

$$C_b = 18{,}61* A_{645} – 3{,}96* A_{662} \quad (2)$$

$$C_{x+c} = (1000*A_{470} – 2{,}27* C_a – 81{,}4 *C_b)/227 \quad (3)$$

The conversion into µg/g DW

$$\text{Content} = \frac{A*V}{m} \quad (4)$$

In which

C_a: Chlorophyll a concentraion (µg/mL)

C_b: Cholorophyll b concentration (µg/mL)

C_{x+c}: Carotenoid concentration (µg/mL)

A: C_a, C_b or C_{x+c}

m: sample weight (g)

2.8. Statistical analysis

Data and comparisons were analyzed using R version 3.6.2. LSD test was used for the comparison of the means.

3. Results and discussions

3.1. Fruit weight and the proportion of pulp, skin and seed of 15 avocado fruits

The fruit weight varied significantly from 233.6 ± 7.3 to 757.2 g/fruit for Hass cv. and Hong ngoc cv., respectively (Table 1). Similar to Hass cv., Bo mo and bo nuoc cv. had small fruit weight, while Bi xanh cv. showed high fruit weight as Hong ngoc cv. The proportion of pulp, skin and seed also varied widely between the cultivars. Booth 3 cv. had the least pulp proportion of only 56.2% and showed the highest skin and seed proportions of 15.5% and 28.3%, respectively. In contrast, the pulp proportion of Bi xanh cv. was 90.0% since its skin and seed account for only 5.5% and 4.0%, respectively. Cu ba and Sap dai co chai cv. also showed high pulp proportion of 83.5% and 80.0%, respectively.

3.2.Variations in total phenolic, flavonoid contents and antioxidant activity

Phenolics naturally present in fruits and its content varied depending on the type of fruits as well as fruit genotypes. This study showed total phenolics in avocado pulp varied from 2.67 ± 0.10 mg GAE/g DW for HAC cv. to 6.46 ± 0.55 mg GAE/g DW for Booth 3 cv. (Table 2) among avocado fruits grown in Vietnam. Both cultivars belong to the imported group. Sap bong cv. showed the highest total phenolics of 4.84 ± 0.79 mg GAE/g DW, while Sap dai co chai cv. had the lowest total phenolics of 2.77 ± 0.06 mg GAE/g DW among domestic cultivars. Sap thuong, Bi xanh and Trinh 10 cultivars are domestic cultivars with relatively high total phenolics (Table 2). Hass cv. showed the lowest total phenolics in all 15 cultivars with 1.89 ± 0.09 mg GAE/g DW. Genetic differences could result in variation in total phenolics between the avocado cultivars. A previous study also showed two avocado varieties, Hass and Fuerte had different total phenolics of 1.0 and 1.75 mg GAE/g DW, respectively (Rodríguez-Carpena *et al.*, 2011). Husen *et al.* (2014) reported avocado pulp contains 2.36 mg GAE/g DW. A study by Fu *et al.* (2011) indicated phenolic content varied from 11.88 to 585.52 mg GAE/100 g fresh weight (FW) among 62 fruits in which the total phenolics of avocado was 21.86 mg GAE/100 g FW. Booth 3 cv. in our study is a recently imported cultivar of Vietnam and gains interest due to high phenolic content in this avocado fruit can be beneficial for health.

Table 1. Variation in fruit weight, the proportion of pulp, skin and seed among 15 avocado cultivars. Results are means ± SD (n=2-5). Different letters show significant differences (P<0.05).

Cultivar	**Fruit weight (g/fruit)**	**Pulp (%)**	**Skin (%)**	**Seed (%)**
Domestic cultivars(1)				
Bi xanh	738.1 ± 108.1^{a}	90.5 ± 0.2^{a}	5.5 ± 0.3^{e}	4.0 ± 0.1^{h}
Bo mo	241.4 ± 33.7^{hi}	71.8 ± 1.1^{f}	10.8 ± 0.3^{c}	17.4 ± 1.0^{cdef}
Bo nuoc	233.9 ± 34.8^{hi}	68.3 ± 2.6^{g}	11.6 ± 2.0^{bc}	20.2 ± 0.8^{bc}
Nam long	527.2 ± 71.7^{bc}	74.3 ± 2.4^{def}	7.0 ± 0.4^{e}	18.8 ± 2.1^{bcde}
Sap bong	311.9 ± 13.2^{fgh}	77.3 ± 3.7^{cde}	6.9 ± 3.1^{e}	15.9 ± 2.7^{defg}
Sap dai co chai	468.1 ± 52.3^{cd}	80.0 ± 1.6^{bc}	6.2 ± 0.1^{e}	14.1 ± 1.6^{fg}
Sap deo tron	419.9 ± 41.5^{de}	68.0 ± 2.2^{g}	10.6 ± 0.2^{c}	21.5 ± 2.0^{b}
Sap thuong	292.7 ± 9.7^{ghi}	77.8 ± 2.3^{cd}	6.5 ± 0.2^{e}	15.7 ± 2.2^{efg}
Trinh 10	302.9^{fghi}	74.1^{dfe}	11.4^{bc}	14.6^{fg}
034	468.3 ± 78.7^{cd}	77.5 ± 3.2^{cd}	9.5 ± 1.4^{cd}	13.0 ± 2.0^{g}
Imported cultivars(2)				
Booth 3	378.8 ± 29.1^{ef}	56.2 ± 0.5^{h}	15.5 ± 0.4^{a}	28.3 ± 0.2^{a}
Cu Ba	597.5^{b}	83.5^{b}	7.5^{de}	9.1^{i}
HAC	369.6^{efg}	73.2^{ef}	10.5^{c}	16.3^{cdefg}
Hong ngoc	757.2^{a}	70.5^{fg}	7.3^{de}	22.2^{b}
Foreign cultivar(3)				
Hass	233.6 ± 7.3^{i}	67.3 ± 2.5^{g}	13.6 ± 1.2^{ab}	19.1 ± 1.6^{bcd}

(1) Avocado cultivars have been domesticated in Vietnam.
(2) Avocado cultivars have recently been grown in Vietnam.
(3) Avocado fruits were grown in Australia.

Table 2. Variation in polyphenol, flavonoid contents and antioxidant activity among 15 avocado cultivars. Results are means ± SD (n=3). Different letters show significant differences (P<0.05).

Cultivar	**Total phenolics (mg GAE/g DW)**	**Flavonoid (mg catechin/g DW)**	**Antioxidant activity (µM TE/g DW)**
Domestic cultivars(1)			
Bi xanh	4.17 ± 0.37^{bcd}	1.42 ± 0.06^{de}	5.63 ± 0.27^{defg}
Bo mo	3.18 ± 0.57^{efg}	1.48 ± 0.06^{de}	5.74 ± 0.98^{def}

Bo nuoc	3.55 ± 0.82^{cde}	1.35 ± 0.25^{ef}	7.49 ± 0.57^{ab}
Nam long	2.92 ± 0.42^{fg}	1.65 ± 0.08^{bcd}	5.10 ± 0.66^{efg}
Sap bong	4.84 ± 0.79^{b}	2.01 ± 0.18^{a}	4.90 ± 0.56^{gh}
Sap dai co chai	2.77 ± 0.06^{fg}	1.91 ± 0.05^{ab}	5.41 ± 0.36^{efg}
Sap deo tron	3.52 ± 0.26^{def}	1.42 ± 0.18^{de}	6.86 ± 0.69^{bc}
Sap thuong	4.66 ± 0.63^{bc}	1.79 ± 0.36^{abc}	5.92 ± 0.05^{de}
Trinh 10	4.05 ± 0.49^{bcd}	1.31 ± 0.18^{ef}	8.02 ± 0.08^{a}
034	3.46 ± 0.52^{def}	0.95 ± 0.34^{gh}	5.71 ± 0.91^{defg}
Imported cultivars[(2)]			
Booth 3	6.46 ± 0.55^{a}	1.51 ± 0.08^{cde}	4.11 ± 0.16^{hi}
Cu Ba	4.11 ± 0.42^{bcd}	1.26 ± 0.08^{ef}	5.04 ± 0.21^{ef}
HAC	2.67 ± 0.10^{gh}	1.11 ± 0.04^{fg}	2.69 ± 0.06^{j}
Hong ngoc	2.75 ± 0.23^{fg}	0.70 ± 0.06^{h}	6.25 ± 0.06^{cd}
Foreign cultivar[(3)]			
Hass	1.89 ± 0.09^{h}	1.45 ± 0.11^{de}	4.01 ± 7.66^{i}

[(1)] Avocado cultivars have been domesticated in Vietnam.
[(2)] Avocado cultivars have recently been grown in Vietnam.
[(3)] Avocado fruits were grown in Australia.

Flavonoids, a group of phenolics, were also identified in pulp of 15 avocado cultivars. This variable was significantly varied between the cultivars. Within the domestic cultivars, flavonoid content ranged from 0.95 ± 0.34 mg catechin/g DW for 034 cv. to 2.01 ± 0.18 mg catechin/g DW for Sap bong cv. (Table 2). This content of the imported cultivars varied from 0.70 ± 0.06 to 1.51 ± 0.08 mg catechin/g DW for Hong ngoc and Booth 3 cv. respectively. Total flavonoid content of Hass cv. (a foreign cultivar) was 1.45 ± 0.11 mg GAE/g DW. Total flavonoid content significantly varied from 0.08 mg catechin/g DW for avocado to 20.69 mg catechin/g DW for Lolly fruit among 30 selected fruits grown in the Philippines (Recuenco *et al.*, 2016). The difference in extraction methods could result in lower total flavonoid of avocado in the study compared to our study. In fact, the extraction in our study was carried at 30 ^{o}C for 3 hours, while in the study by Recuenco *et al.* (2016) the sample was extracted by homogenizing for 5 minutes before filtering. Another study found significant variation in total flavonoid content of fruit pulp from 14.4 to 714.2 mg catechin/100 g FW among 18 selected tropical fruits grown in Brazil (Barreto *et al.*, 2009). Flavonoids are natural antioxidants which prevent various diseases such as cancer, Alzheimer's disease, atherosclerosis and they are the potential component in nutraceutical, pharmaceutical, medical and cosmetical products (Panche *et al.*, 2016). The natural presence of flavonoids in the edible avocado part is the natural antioxidants for health benefits.

Antioxidants are compounds which are able to gain free radicals and reduce oxidation processes in living organisms. Antioxidants are

very diverse and they can be phenolic compounds, carotenoids or unsaturated fatty acids (Rao and Rao, 2007, Richard *et al.*, 2008, Williamson, 2017). Natural antioxidants exist widely in fruits including avocado and their effect is measured by their activity (Fu *et al.*, 2011, Nguyen *et al.*, 2019). This study found the antioxidant activity in avocado pulp of the 15 selected cultivars significantly varied from 2.69 ± 0.06 µM TE/g DW for HAC cv. to 8.02 ± 0.08 µM TE/g DW for Trinh 10 cv. (Table 2). HAC cv. belongs to the imported group, while Trinh 10 is a domestic cultivar. Other domestic cultivars also showed high antioxidant activities ranging from 4.90 ± 0.56 to 7.49 ± 0.57 µM TE/g DW. To our knowledge, information on total phenolic, total flavonoid and antioxidant activity of avocado grown in Vietnam is limited. Therefore, this study contributes to understanding on bioactive compounds of these avocado cultivars.

3.3.Variations in chlorophyll and carotenoid contents

Avocado pulp shows colour ranging from dark green adjacent to the skin, then pale green and yellow adjacent to the seed and this is the presence of pigments such as chlorophylls and carotenoids (Ashton *et al.*, 2006). The results from Table 3 showed chlorophyll content varied from 137.8 ± 18.0 µg/g DW for Trinh 10 cv. to 259.6 ± 2.46 µg/g DW for Bo nuoc cv. within the domestic cultivars and from 127.6 ± 33.7 µg/g DW for HAC cv. to 316.3 ± 11.3 µg/g DW for Cu ba cv. among the imported cultivars. The chlorophyll b content was lower than the chlorophyll a content and this value varied from 46.8 ± 0.16 µg/g DW for Trinh 10 cv. to 118.6 ± 48.8 µg/g DW for 034 cv. within the domestic cultivars and from 79.2 ± 27.9 µg/g DW for HAC to 223.8 ± 9.6 µg/g DW for Cu ba cv. within the imported cultivars. Hass cv. contained 195.6 ± 11.9 and 128.2 ± 1.8 µg/g DW chlorophyll a and chlorophyll b, respectively. Ashton *et al.* (2006) also found that the chlorophyll a content was higher than the chlorophyll b content in the avocado flesh. Their study showed the chlorophyll content was the highest in dark green flesh and the lowest in yellow flesh.The carotenoid content in avocado pulp significantly varied between avocado cultivars. This content ranged from 63.7 ± 8.1 µg/g DW for Sap thuong cv. to 226.8 ± 10.3 µg/g DW for Nam long cv. among the domestic cultivars, while it varied from 38.6 ± 7.5 µg/g DW for HAC cv. to 192.5 ± 35.5 µg/g DW for Hong ngoc cv. among the imported cultivars. Hass cv. contained the lowest carotenoid content of 32.8 ± 1.2 µg/g DW. Ashton *et al.* (2006) reported the carotenoid content in avocado flesh varied depending on the part of flesh and the ripening state. The total carotenoid content at 2 days after harvest was about 5.1 µg/g FW in dark green flesh, 2.2 µg/g FW in pale green flesh and 3.5 µg/g FW in yellow flesh. Carotenoids also occur in variety of fruits and vegetables such as tomato which has high carotenoid content (8.1 µg/g FW at ripe stage) (Nguyen and Dao, 2018).

Table 3. Variation in chlorophyll a, chlorophyll b and carotenoid contents among 15 avocado cultivars. Results are means ± SD (n=3). Different letters show significant differences (P<0.05).

Cultivar	**Chlorophyll a (µg/g DW)**	**Chlorophyll b (µg/g DW)**	**Carotenoid (µg/g DW)**
Domestic cultivars(1)			
Bi xanh	193.1 ± 18.8^{cde}	76.7 ± 20.1^{fgh}	142.8 ± 2.7^{c}
Bo mo	227.8 ± 50.3^{bcd}	115.3 ± 28^{bcde}	88.7 ± 15.3^{def}

Bo nuoc	259.6 ± 2.46^{abc}	112.1 ± 7.5^{bcdef}	131.2 ± 12.2^{c}
Nam long	232.4 ± 72.8^{bcd}	86.7 ± 18.6^{defgh}	226.8 ± 10.3^{a}
Sap bong	233.0 ± 31.1^{bcd}	98.1 ± 32.2^{cdefg}	93.9 ± 21.7^{de}
Sap dai co chai	224.7 ± 91.2^{bcd}	98.4 ± 22.5^{cdefg}	74.9 ± 28.0^{ef}
Sap deo tron	136.1 ± 25.1^{e}	56.3 ± 14.9^{h}	82.3 ± 16.6^{ef}
Sap thuong	170.0 ± 11.3^{de}	72.4 ± 8.8^{gh}	63.7 ± 8.1^{fg}
Trinh 10	137.8 ± 18.0^{e}	46.8 ± 0.16^{h}	77.0 ± 8.1^{ef}
034	294.2 ± 84.8^{ab}	118.6 ± 48.8^{bcd}	116.0 ± 33.2^{cd}
Imported cultivars[(2)]			
Booth 3	146.7 ± 41.8^{e}	100.7 ± 20.4^{cdefg}	70.3 ± 13.8^{ef}
Cu Ba	316.3 ± 11.3^{a}	223.8 ± 9.6^{a}	141.2 ± 12.1^{c}
HAC	127.6 ± 33.7^{e}	79.2 ± 27.9^{efgh}	38.6 ± 7.5^{gh}
Hong ngoc	258.7 ± 35.1^{abc}	119.6 ± 65.7^{b}	192.5 ± 35.5^{b}
Foreign cultivar[(3)]			
Hass	195.6 ± 11.9^{cde}	128.2 ± 1.8^{bc}	32.8 ± 1.2^{h}

[(1)] Avocado cultivars have been domesticated in Vietnam.
[(2)] Avocado cultivars have recently been grown in Vietnam.
[(3)] Avocado fruits were grown in Australia.

3.2. Variations in total lipid content

The avocado fruits are the good source of lipids which is rich in unsaturated fatty acids (Teng *et al.*, 2016). The total lipid content of avocado pulp varied widely among these avocado cultivars. Within the domestic cultivars, Sap dai co chai cv. had the lowest total lipid content of 35.4 ± 1.2%, while 034 cv. showed the highest content of 61.4 ± 4.1%. Within the imported cultivars, this value ranged from 44.3 ± 2.6 to 59.2 ± 5.6% for HAC cv. and Cu ba cv., respectively. Hass cv. showed the highest lipid content of 72.4 ± 5.4% (Figure 1). Significant difference in total lipid content between the Australian Hass cv. and cultivars planted in Vietnam could be due to genetic variation or geographical effect. Another study in 7 avocado genotypes also found the total lipid content significantly varied from 7.39 to 36.98% and Hass cv. showed the highest total lipid content. Takenaga *et al.* (2008) also reported variation in the total lipid content in pulp of three avocado cultivars grown in Japan. Fuerte and Bacon are domestic cultivars and contained 18.7 and 21.8% total lipid, while the imported Hass cultivar had 18.2% total lipid. The total lipid content in our study showed higher than that in these previous studies since our results were calculated per dry weight, while this were measured per fresh weight in the previous research.

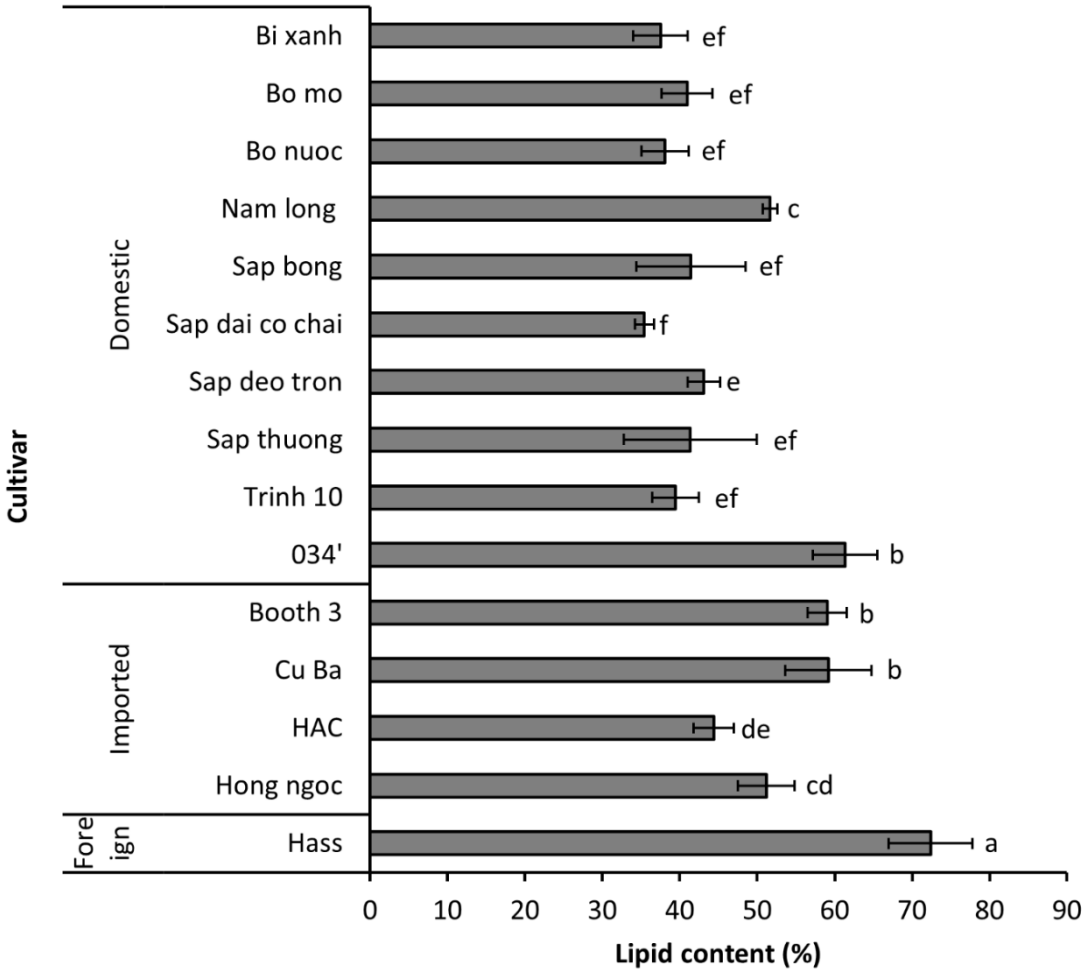

Figure 1. Total lipid content of 15 avocado cultivars. Results are means ± SD (n=3). Different letters show significant differences (P<0.05).

4. Conclusions

Our study showed that the avocado cultivars grown in Vietnam are good sources of bioactive compounds such as phenolics, flavonoids, chlorophyll and carotenoids. Booth 3 cv. showed the highest total phenolics, while Sap bong cv. had the highest flavonoid content. Bo nuoc and Cu ba cv. contain high contents of chlorophyll a and chlorophyll b, respectively and Nam long cv. indicated the highest amount of carotenoid. The total lipid varied widely between avocado cultivars and 034 cv. showed the highest total lipid content among avocado cultivars planted in Vietnam. Variations in bioactive compounds as well as total lipid content among the avocado cultivars indicates genetical diverse between these cultivars and this suggests they can be valuable sources for food as well as other applications.

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EFFECTS OF POMEGRANATE (*PUNICA GRANATUM L.*) FRUIT AND RIND EXTRACTS ON PHYSICO-CHEMICAL, COLOUR, AND OXIDATIVE STABILITY OF RAINBOW TROUT FILLET

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ABSTRACT

Colour changes, oxidation of fat and physicochemical status of rainbow trout fillet were examined after adding water extracts of pomegranate rind (WEPR), ethanolic extracts of pomegranate rind (EEPR), water extracts of pomegranate fruit (WEPF), and ethanolic extracts of pomegranate fruit (EEPF) during four days of refrigerated aerobic storage. These extracts were added in a concentration of 0.01%. Results unveiled that the WEPR group had the highest total phenolic compounds amount and anti-radical activity. However, pH values for the extract treatments did not show a meaningful difference. Analysis of variance of colors showed a remarkable difference ($p<0.05$) about the effects of extracts and storage time. The values of Lightness for both control and EEPF sample at day 0 higher than the other samples. At the end of storage time, total volatile base nitrogen (TVB-N), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) values of the control sample were significantly ($p<0.05$) higher than those of the treated fillets with pomegranate extracts. Overall acceptability scores of water extracts of pomegranate fruit and rind treated fillets were higher than those of ethanolic extracts of pomegranate samples. The results indicated that pomegranate extracts can retard fish spoilage and they may be beneficial as natural antioxidant sources in minimizing the physicochemical changes of fish products during cold storage.

1. Introduction

Fish preservation is a key factor for increasing the shelf life and conserving nutritional value, texture, and flavor which prevents spoilage without affecting the quality (Lakshmanan et al., 2003). Spoilage is the post-harvest change and spoiled fish is typically observed as the change in physical features such as colour, odour, texture, eyes, gills and softness of muscle (Barbosa-Pereira et al., 2013). The process of spoilage of fish shows high complexity in which enzymes, bacteria and chemical components are involved and begins rapidly after the death of fish (Ghaly et al., 2010). The maintenance of freshness and quality of fish fillet as a safe food is important.

Chemical changes can occur in fish fillets during storage. Chemical changes such as lipid oxidation and auto-oxidation are leading culprits for deterioration of the quality of seafood and can reduce its shelf life (Secci et al., 2016). Lipid oxidation may lead to changes in seafood quality-related factors such as color, off-flavor, rancidity, odor, texture, and also nutritional quality (Annamalai et al., 2015). Fresh fish and its products undergoing oxidative changes are tremendously vulnerable products owing to their biological components (Yilmaz et al., 2009). Synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butylhydroquinone (TBHQ) have been successfully utilized to prevent lipid oxidation in fish products (Gai et al., 2014). But, these synthetic antioxidants have possible health effects and toxicity (Devatkal et al., 2010). Therefore, to resolve this problem, natural antioxidants in fish products such as cinnamon (Ojaghi et al., 2010) curry, mint (Biswas et al., 2012), oregano, rosemary (Makri, 2013), thyme, clove (Guran et al., 2015) and green tea (Yerlijaya and Gokoglu, 2010) have been used. Also, recently, Chan-Higuera et al. identified the skin extract of a mollusk called Dosidicus gigas as an antioxidant in Tuna Pâté (Chan-Higuera et al., 2019). However, these products are not as effective as synthetic antioxidants (Qin et al., 2013). Consequently, careful attention has been paid to antioxidants from inexpensive or residual sources in agriculture industries, such as apple peel (Wolfe et al., 2003), peach peel (Rossato et al., 2009), onion peel (Shim et al., 2012) and bamboo leaves (Wenjiao et al., 2013). In a literature review, Pezeshk et al. (2015) discussed the role of some natural antioxidants and anti-bacterial in sustaining the quality and increasing the shelf life of some seafood products.

Pomegranate (*Punica granatum L.*) is cultivated in many tropical and subtropical countries (Mousavinejad et al., 2009). These fruit comrises three parts: seed, juice, and peel (about 30% of the fruit weight). Pomegranate rind is not edible and is obtained upon processing of pomegranate juice. Pomegranate rind and juice are shown to possess considerable antioxidant activities due to tannins and other phenolic compounds (Devatkal et al., 2010). Currently, pomegranate juice, rind powder, and seed powder utilization in chicken, goat, fish patties and pork meat products as sources rich in natural antioxidants has been investigated (Devatkal et al., 2010; Naveena et al., 2008; Qin et al., 2013; Martínez, L et al., 2019). Because there is relatively significant level of polyunsaturated fatty acids (PUFA) in fat and filet of trout fish, oxidation can take place in a higher speed in trout fish in comparison with chicken, goat, or pork. Trout fish filet susceptibility to lipid oxidation is found to be higher than that of other meat products (Gai et al., 2014). As a result, lipid oxidation in trout fish filet must be postponed by adding antioxidants. This study focused on determination of the effectiveness of pomegranate rind and fruit extracts on the physicochemical quality of trout fish filet as measured by pH, total phenolic content, DPPH radical scavenging activity, thiobarbituric acid reactive substances (TBARS), peroxide value (PV), color, total volatile base nitrogen (TVB-N) value, and sensory evaluation in the course of storage at refrigerator temperature.

2. Materials and Methods

2.1. Sample collection

Samples of fresh pomegranate (Punica granatum L.) were purchased from a retail fruit market. The fresh rainbow trout fish samples were purchased from a fishmonger shop and the fillet was removed. The fillets were transferred to the chemistry laboratory and kept at refrigerator temperature until use.

2.2. Preparation of pomegranate rind and fruit extracts

Pomegranate rind (peel) powder was prepared based on a method by Devatkal et al.

(2010). Pomegranates were washed peeled off, and desiccated via air circulatory tray drier at 60°C and duration of 48 h. mixer grinder was used to powder the dried pomegranate peel, followed by sieving by a sieve no. 10 (1.65 mm). Then the dried product was stored at room temperature within high-density polyethylene bags. Similarly powder from pomegranate seeds was prepared by drying the pomegranate fruit seeds in a tray drier and grinding by mixer grinder and sieving by a sieve no. 10 (1.65 mm).

2.3. Ethanolic extract of pomegranate rind (EEPR)

An Ethanolic extract of pomegranate rind powder was prepared with respect to the method by Qin et al. (2013). Briefly, the extraction of 10 g powdered rind of pomegranate was done via adding 100 ml of 80% ethanol in a shaking incubator at 40°C for 24 h. The solutions were passed through the Whatman cellulose filter papers (circles and with a diameter of 110 mm), followed by vacuum evaporation using a rotary evaporator (IKA RV 10 digital). After that, some of dried pomegranate rind powder solved in ethanol with the total volume reaching to 100 ml by adding distilled water. The mixture was then maintained at 4°C until use.

2.4. Water extract of pomegranate rind (WEPR)

Preparation of the Water extract of pomegranate rind powder was performed in accordance with the method suggested by Kamkar et al. (2013), and with the use of a percolator. In this way, the extraction of pomegranate rind powder was achieved by adding distilled water in a percolator apparatus until becoming colorless. Then, the crude extract was passed through the filter and dried in a vacuum.

2.5. Ethanolic extract of pomegranate fruit (EEPF) and water extract of pomegranate fruit (WEPF)

Similarly, ethanolic and aqueous extracts of pomegranate fruit were extracted with 80% ethanol. The freshly prepared extracts (EEPR, EEPF, WEPR, and WEPF) were stored at 4°C until use (for up to 24 h).

2.6. Preparing trout fillet treatments

The rout fillet samples were split equally. After mincing, the trout fillet samples were divided into batches (100 g each), followed by their assignment to the following five groups: control (filet with no antioxidant); WEPF (10 mg WEPF per 100 g fillet); WEPR (10 mg WEPR per 100 g fillet); EEPF (10 mg EEPF per 100 g fillet); and EEPR (10 mg EEPR per 100 g fillet). The fillet samples were formed into 100 gr patties with 10 mg extract. They were smeared with 10 mg extract at aseptic conditions and then gathered in low-density polyethylene bags in the presence of air, and stored at 2-4°C for 4 days. Afterward, analyses were performed every two days (0, 2 and 4).

2.7. pH evaluation

pH of the trout fillet sample was determined with the use of a pH meter (Kent, EIL7020, Kent Industrial Measurement Limited, Surrey, England), using 5 g of the sample blended with 20 ml distilled water. Average of triplicates was reported for each treatment.

2.8. Estimation of total phenolic content

Total phenolic content was evaluated through the Folin-Ciocalteu (F-C) assay (Negi et al, 2003). Diluting 100 µL aliquot of extracts (various concentrations) was performed by adding 5 ml distilled water and 100 µL 10-fold-diluted Folin-Ciocalteu reagent. Following 5 min incubation, 300 µL of 2% sodium carbonate was added. Then, the absorbance was read at 760nm by a UV–VIS spectrophotometer (DR 5000™ UV-Vis Spectrophotometer). Tannic

acid was used as a standard sample. Results were expressed as mg/L Tannic acid equivalents.

2.9. DPPH radical scavenging activity

The scavenging effects of WEPR, WEPF, EEPR, and EEPF against DPPH radicals were measured with respect to the method by Koch et al. (2017). A mixture of 50 µL extracts (various concentrations) + 5 ml DPPH solution (0.004% methanol solution) was prepared and incubated for 30min at room temperature. UV–VIS spectrophotometer was used to measure the absorbance at 517nm. The DPPH radical scavenging activity was estimated by the following equation: Scavenging activity (%) = (Absorbance control-Absorbance sample /Absorbance control) × 100

2.10. Determining peroxide value (PV)

The extraction of Lipid from the trout fillet samples was achieved using the method by Folch et al. (1957). A mixture of extracted lipid and 10 ml chloroform-methanol (7:3 v/v) was prepared in a screw-capped test tube, followed by vortexing for 10-15s. The lipid extracts were evaporated by a rotary evaporator. Then, the PV was analyzed for the 5 g sample of recovered lipids and assessed through the measurement of the iodine released from potassium iodide whose titration was prepared in a standardized 0.01 N sodium thiosulfate solution. The PVs were expressed as the mEq of O2 per kg and calculated as:

Peroxide value (mEq/kg) = 100×(S1 – S2) × N/W, where S1 is consumption volume of sodium thiosulfate of the sample, S2 is consumption volume of sodium thiosulfate of blank, N is normalized sodium thiosulfate (0.01), and W is sample weight.

2.11. Value of thiobarbituric acid reactive substances (TBARS)

TBARS value of the trout fillet sample was determined in accordance with the approach developed by Yousef et al. (2009), with slight modifications. Briefly, Samples were precipitated in chilled 20% trichloroacetic acid (TCA). Then 2 ml extract was homogenized in 2 ml 0.1% thiobarbituric acid (TBA), incubated for 90 min in a water bath with a temperature set on 90ºC, followed by cooling down to room temperature. Then, the absorbance was read at 532 nm. Malonaldehyde was utilized as the standard for TBARS assay. TBARS values were expressed as mg of malonaldehyde per kg of the sample.

2.12. Determining total volatile base nitrogen (TVB-N)

Total volatile basic nitrogen (TVB-N) was measured based on the method by Goulas and Kontominas (2007) with some modifications. A mixture of 10g trout fillet+50ml distilled water was obtained and transferred along with 300ml distilled water to a 500cc round bottom flask. It was then distilled following addition of 2g of magnesium oxide (MgO) and a few drops of paraffin to avoid foaming. The distillate was collected in a 250cc Erlenmeyer flask which contained 25 ml of 3% aqueous solution of boric acid and 0.05 ml of methyl red and bromocresol green. Then, a titration was prepared for boric acid solution with adding 0.1 N sulfuric acid solution. The TVB-N value (mg/100g of fillet) was determined by the following equation: TVB-N(mg/100g)=(Vs-Vb)×14

Vs is the consumption volume of sulfuric acid of the sample and Vb is the consumption volume of sulfuric acid of the blank.

2.13. Instrumental measurement of color

Variations in color for the control and treated trout fillet samples at the time of storage were evaluated by colorimeter (Hunter lab Color Flex, Reston, VA, USA). The colorimeter was adjusted using a standard white tile (L*= 92.23, a*= -1.29, and b*= +1.29). a container was used to locate the trout fillet samples, followed by recording the values of L* (lightness), a* (redness), and b* (yellowness) on the outer and inner surfaces of samples.

2.14. Sensory evaluation

Sensory evaluation was determined with the use of a method presented by Devatkal et al. (2010) and Naveena et al. (2008) with some modifications. Semi-trained panels were 12 people from among the laboratory personnel who evaluated the trout fillet treatments. The panelists rated four characteristics of each sample appearance, juiciness, flavor, and general palatability) on an 8-point descriptive scale. The trout fillets were initially warmed prior to serving and water was served to rinse mouth between sensory evaluations of the samples. The experimental protocol was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1392.14023) and conformed to the ethical principles set forth in the Declaration of Islamic Republic of Iran.

2.15. Statistical analysis

Spss software was employed in this study. All the experiments were performed in triplicate for each of the five groups. Data of total phenolic and DPPH radical scavenging activity were analyzed using a one-way Analysis of Variance (ANOVA). The collected data related to pH, color, TBARS, TVB-N and PV were analyzed using two-way ANOVA considering treatment and storage time as the leading factors. Statistical significance was determined at 95% confidence level ($p < 0.05$).

3. Results and discussion

Figures 1 to 9 demonstrate the results of total phenolic, DPPH, pH, PV, TBARS, TVB-N and color value. Changes in sensory quality of the trout fillet are also shown in Table 1.

3.1. Total phenolic contents and antioxidant activity of pomegranate extracts

Aiming to compare the total phenolic content of extracts, the normality of the observations for each group was evaluated by ANOVA test. All groups showed normalization. The total phenolic contents of WEPR, EEPR, WEPF and EEPF were 5.2±0.23, 4.24±0.21, and 3.02±0.19 and 2.91±0.06 mg/L Tannic acid, respectively (figure 1). Anti-oxidant, antimicrobial and anti-cancer activity has already been observed in Phenolic compounds of pomegranate (Mousavinejad et al., 2009, Afaq F et al., 2009). There are more phenolic and antioxidant compounds in Pomegranate peel than in other parts of the fruit. In this study, the highest and lowest phenolic compounds were observed in WEPR (5.2 ± 0.2 mg/L Tannic acid) and EEPF (2.91 ± 0.6 mg/L Tannic acid) groups respectively. Water and Ethanolic extracts of pomegranate were obtained in similar studies. Devatkal et al. (2010) reported total phenolic of pomegranate rind powder (PRP) and pomegranate seed powder (PSP) to be 4476.2 and 2590.6 (µg/g powder), respectively (Devatkal et al., 2010). Tehranifar et al. (2010) found 295.79-985.37 mg/100g in total phenolic content of twenty Iranian pomegranate juice cultivars (Tehranifar etal, 2010).

3.2. DPPH free radical scavenging activity

The DPPH free radical scavenging activity (% scavenging activity) is depicted in Figure 2. The results revealed that the BHT group had the greatest activity in this regard. WEPR and EEPR groups had the highest capability of neutralizing free radicals, while the EEPF group had the least ability. Results showed an enhanced radical scavenging activity as the extract concentration increased, which was comparable to that of BHT. In this aspect, anti-radical activity of the rind extracts (WEPR and EEPR) were significantly stronger than the fruit extracts (WEPF and EEPF), which was similar to that in BHT ($p<0.05$). The anti-radical function of extracts has been linked to the amount of total phenolic compounds (Mousavinejad et al., 2009).

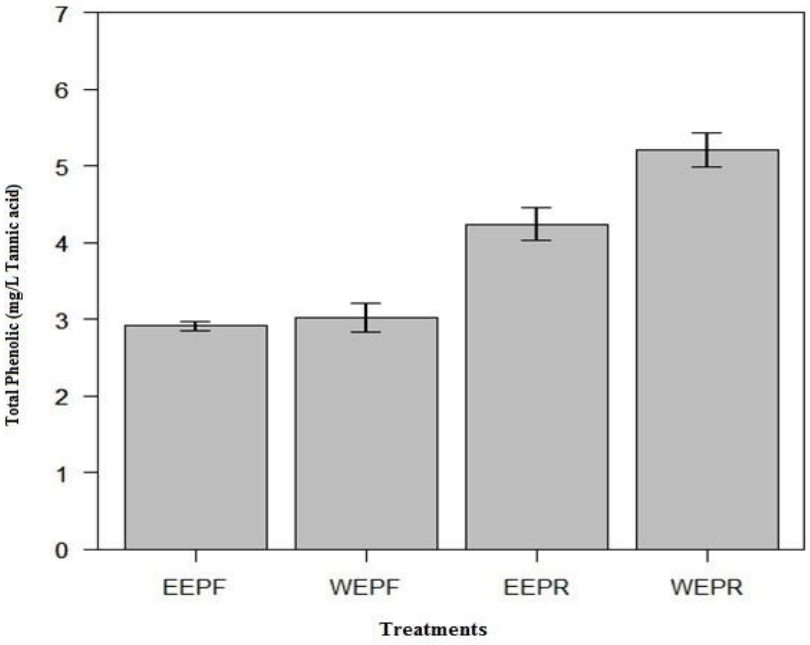

Figure 1. Total phenolic contents in pomegranate extracts.

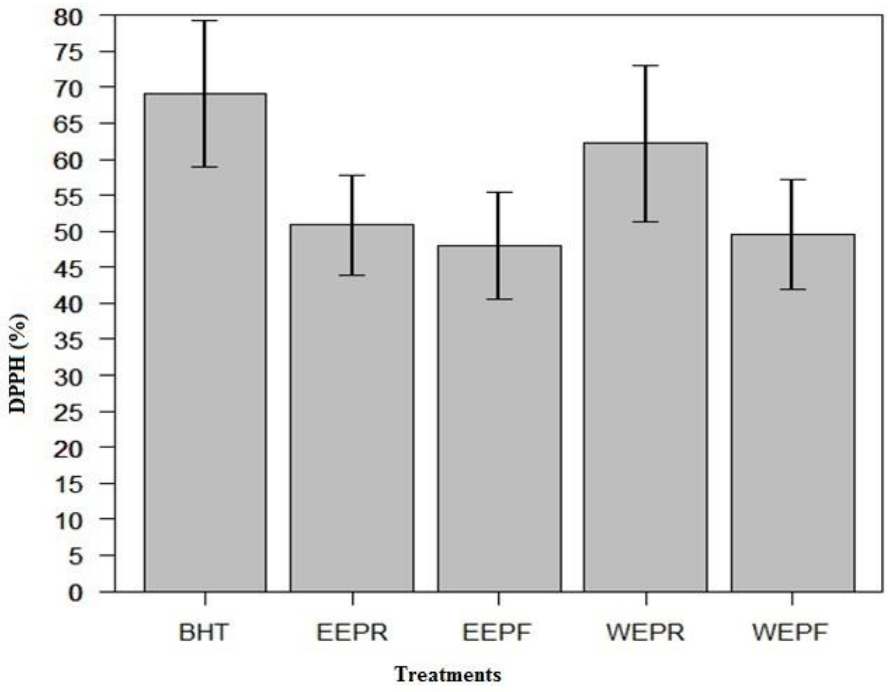

Figure 2. The antioxidant activities of pomegranate extracts and BHT in DPPH assay.

Among four extracts, WEPR had significantly ($p<0.05$) higher phenolic content and anti-radical activities compared to the other extracts. The results were in agreement with what Martínez L et al. (2019) reported, pointing out the potential of pomegranate extract as an antioxidant and antimicrobial compound in vitro (Martínez L et al., 2019). Similarly, Devatkal et al. (2010) and Naveena et al. (2008) indicated free radical scavenging activity in pomegranate juice, rind and seed extracts (Devatkal et al., 2010; Naveena et al., 2008).

3.3. Changes in pH

Figure 3 shows the changes in pH of trout fish fillet mediated by water and ethanolic extraction of rind and fruit pomegranate. pH values of the control group significantly increased to 6.96 during storage ($p<0.05$). Attempting to investigate the effects of the time factor and the interaction between time and extract, Mauchly's sphericity test was used. Results of the current study revealed a significant effect of storage time on pH. However, no meaningful difference was found in pH among different extracts. The pH in fresh fish is almost neutral. Upon death, nitrogenous compounds in fish are decomposed by proteolytic enzymes activity and increasing pH in fish meat during storage (Gokoglu et al., 2004). Increase in pH represents poor quality.

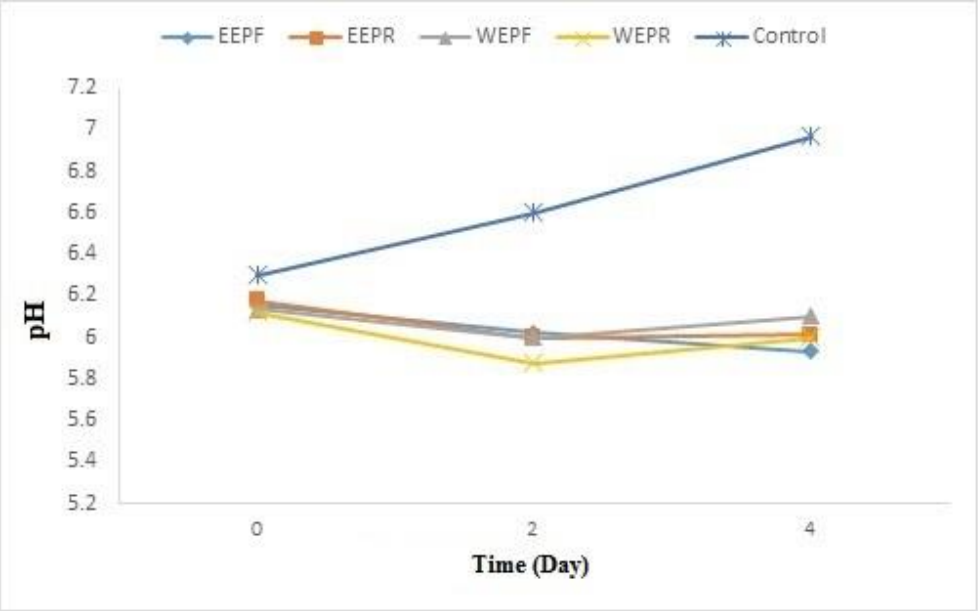

Figure 3. pH values of pomegranate extract samples during refrigerated storage (4°C) in different

A maximum pH of 6.8–7.0 is reported to be desirable during storage at refrigerator (Gai et al., 2014). El Marakchi et al. (1990) found a pH value of 6.1 in raw sardine. During the storage of fish fillet with pomegranate extracts, pH decreased to 5.93. A significant difference ($p<0.05$) in pH was found between the control and treatment groups; pH values of the EEPF treatment were lower than that of other extracts. However, no meaningful changes was found in the pH values of the treatments samples ($p>0.05$) during storage. pH of the trout fish fillet decreased due to the addition of the extracts. Similar values of pH were reported by Gokoglu et al. (2009) in marinated anchovy with pomegranate sauce.

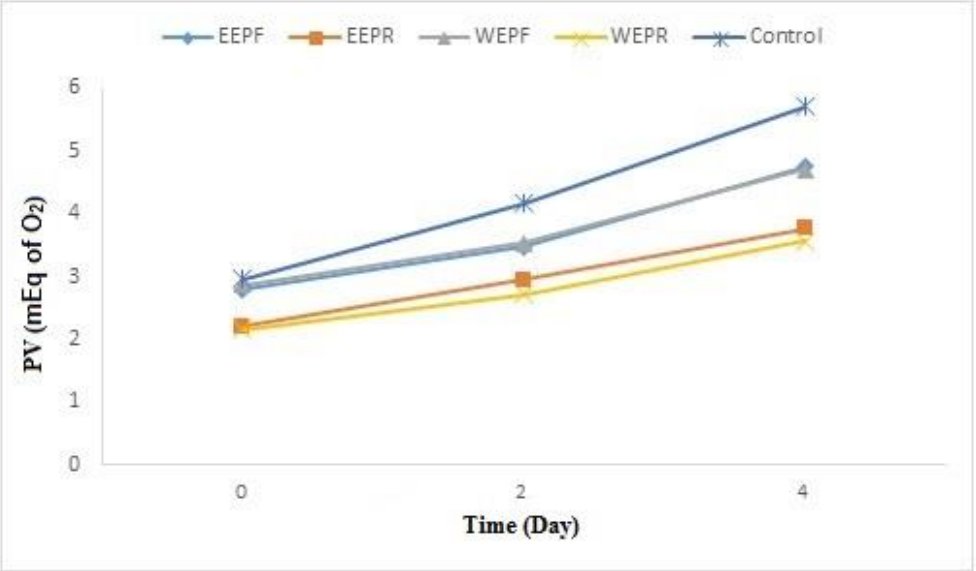

Figure 4. Peroxide value of pomegranate extract samples during refrigerated storage (4°C) on different days.

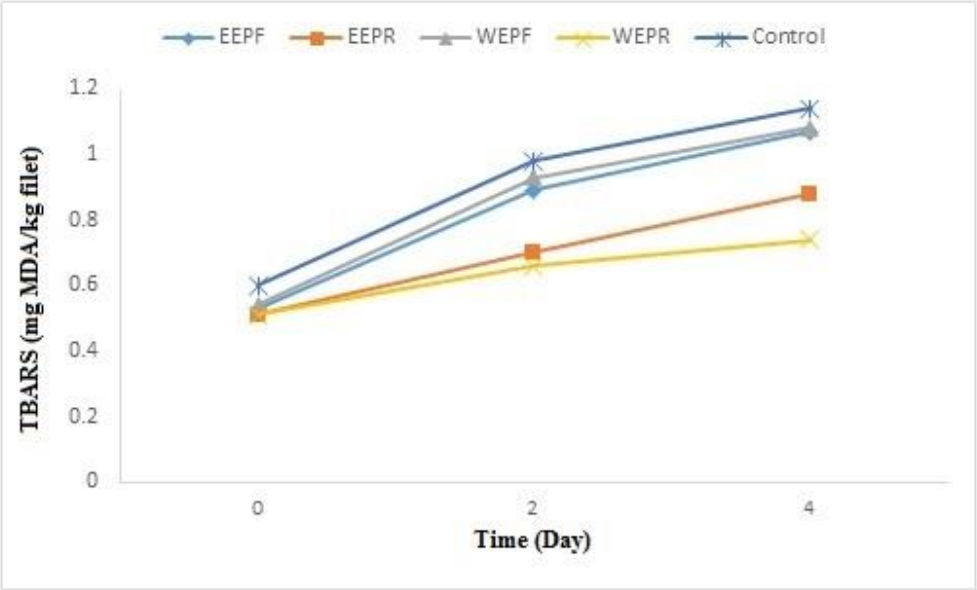

Figure 5. TBARS value of pomegranate extract samples during refrigerated storage (4°C) on different days

3.4. Changes of Peroxide value (PV)

Figure 4 describes the changes in PV value. According to the findings, PV levels of control group were greater than other extraction groups. WEPR and EEPR had the lowest PV value. The results of the Mauchly's sphericity test demonstrated that there was a meaningful difference between storage time and PV value. Polyunsaturated fatty acids are the main Components of fish fillet, making fish fillet extremely susceptible to lipid oxidation (Maqsood and Benjakul, 2010). Peroxide value (PV) is a representative of the primary stages of oxidative change (Shahidi and Zhong, 2005). In this study, a significant increase in PV values was found in both control and treated samples ($p<0.05$) following an increase in storage time, which was due to faster generation of new hydroperoxide which overweighs its degradation. The samples with extractions showed significantly ($p<0.05$) reduced peroxide level in comparison with the control group. WEPR and EEPR reduced the formation of peroxide more efficiently than WEPF and EEPF did, highlighting stronger antioxidant activity of compounds of pomegranate peel. Our results also demonstrated that the pomegranate extracts, especially rind extracts, were capable of PV production in the trout fillet stored in refrigerator (4±10C), which were consistent with those of Pezeshk et al. (2011), and Mexis et al. (2009).

3.5. Thiobarbituric acid reactive substances

Variation in values of TBA during storage are presented in Figure 5. The concentration of TBARS was calculated using the standard curve, obtained by a commercial Malonaldehyde bis (dimethyl acetal) regent (Merck Schuchardt OHG). The following formula was used: $y = 0.0348X + 0.0153$. The highest and lowest TBA values were found in the control and WEPR samples, respectively. Thiobarbituric acid reactive substances (TBARS) have been utilized to measure secondary oxidation products (Shahidi and Zhong, 2005). In the present study, there was a marked increase in TBA values for both control and treated samples ($p<0.05$) as the storage time increased, and control samples showed the highest TBA value. TBARS values were also slightly increased in the WEPR treated sample, being at its minimum rate (<0.74 mg MDA/kg sample) up to 4 days. However, a remarkably hampered TBARS production ($p<0.05$) in the trout fillet treated with WEPR, EEPR, EEPF, and WEPF was found compared to the control group. Reduction in the amount of T-BARS was significantly associated with the total phenolic contents, and he highest content was found in the rind pomegranate extracts (WEPR and EEPR). These result demonstrated that the pomegranate extracts, especially rind extracts, were efficient in slowing down the increase in TBARS levels of trout fillet during refrigeration (4±10C) storage. Similar finding was reported by Yerlikaya et al. (2010) and Ozen et al. (2011).

3.6. Total volatile base nitrogen (TVB-N)

The findings of this study showed that there was a higher level of TVB-N in the control group than the treatment groups. WEPR and then WEPF also had the lowest amount of TVB-N (figure 6).

In this study, the TVB-N values of the control and treated samples significantly increased ($p<0.05$) with storage time ($p<0.05$) Similar trend was also observed in Zhuang, S. et al. (2019) study. However, no significant difference ($p>0.05$) in TVB-N value was observed on day 0 between the control and treated groups. TVB-N is a quality index for fish, which is mainly attributed to trimethylamine, dimethylamine, ammonia, and other volatile basic nitrogenous compounds produced for the activity of spoilage bacteria and endogenous enzymes (Kilinc and Cakli, 2005). Viji et al. (2020) proposed the value of 30 mg N per 100 g fillet as the maximum acceptable level. At the end of the storage period, the lowest amount of TVB-N was found in fish fillet containing WEPR (13.3 ± 1.77), which had a

significant difference with other extracts. Also, the highest TVB-N was observed in control sample (30.1 ± 0.99), which was higher than acceptable limit. The initial TVB-N values in the studied samples were in accordance with results achieved by Gokoglu et al. (2004) and Pezeshk et al. (2011). Our findings also demonstrated that in the end of storage, control sample showed a significantly higher TVB-N values ($p<0.05$) than those of the treated samples (30.1 ± 0.99 mg N per 100g fillet). Minimum TVB-N values were reported in the WEPR samples at the end of storage (13.3 ± 1.77 mg N per 100 g fillet). Low TVB-N in the treated samples can be attributed to the total phenolic content and antioxidant compounds found in rind and fruit pomegranate. Mexis et al. (2009) also reported similar events during the refrigerated storage of oregano essential oil treated rainbow trout fillets.

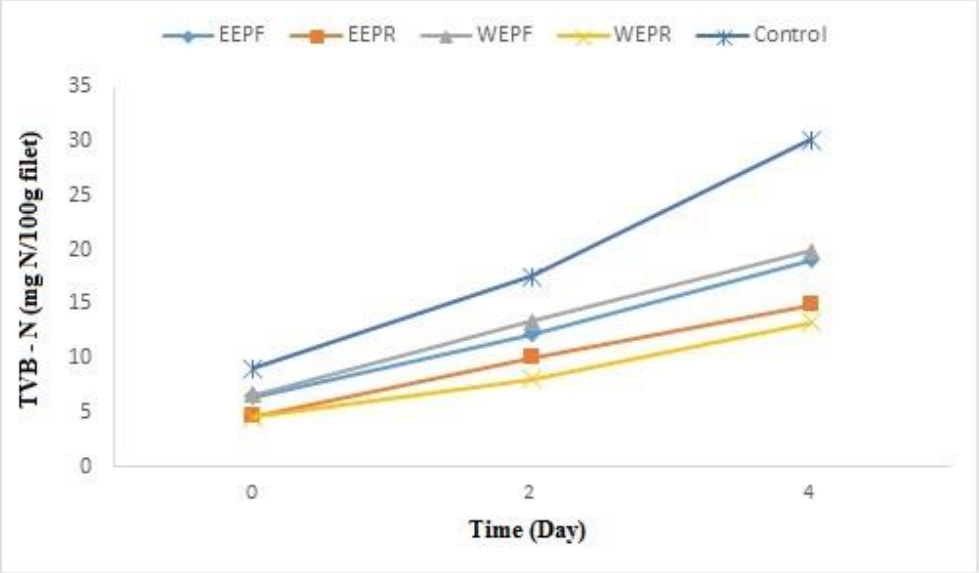

Figure 6. TVB – N value of pomegranate extract samples during refrigerated storage (4°C) on different days.

3.7. Changes of color value

Changes in L*, a*, and b* values of trout fillet with and without antioxidants are presented in Figure 7, 8 and 9, respectively. Analysis of variance of colors showed significant difference ($p<0.05$) in terms of the effects of extracts and storage time.

According to the findings, the amount of Lightness (L* value) on day 0 for the control and EEPF samples was more than that in other samples. The Lightness (L* value) of the control group in the three storage periods (0, 2 and 4 days) was significantly ($p<0.05$) higher than the treated samples with extracts, which was consistent to results of Qin et al.'s (2013) study. Significantly minimum lightness (L* value) was ($p<0.05$) found in the rind pomegranate extracts (WEPR and EEPR). Losses of lightness of the WEPR and EEPR samples during storage might be linked to high turbidity and impurities in the rind of pomegranate. In terms of the Lightness (L* value) , many studies have shown that extracts of plants rich in phenolic compounds (turmeric, mint, black currant, almond) make the meat and food less transparent. (Jia N et al., 2012; Lorente-Mento et al., 2020).

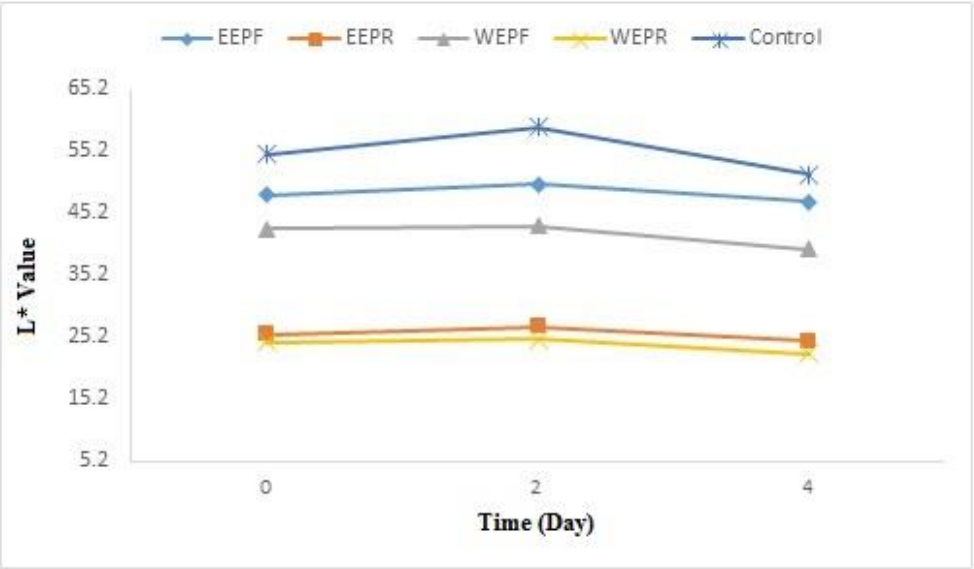

Figure 7. Changes of L* (lightness) value of the trout fillet treatments during refrigerated storage (4°C) on different days.

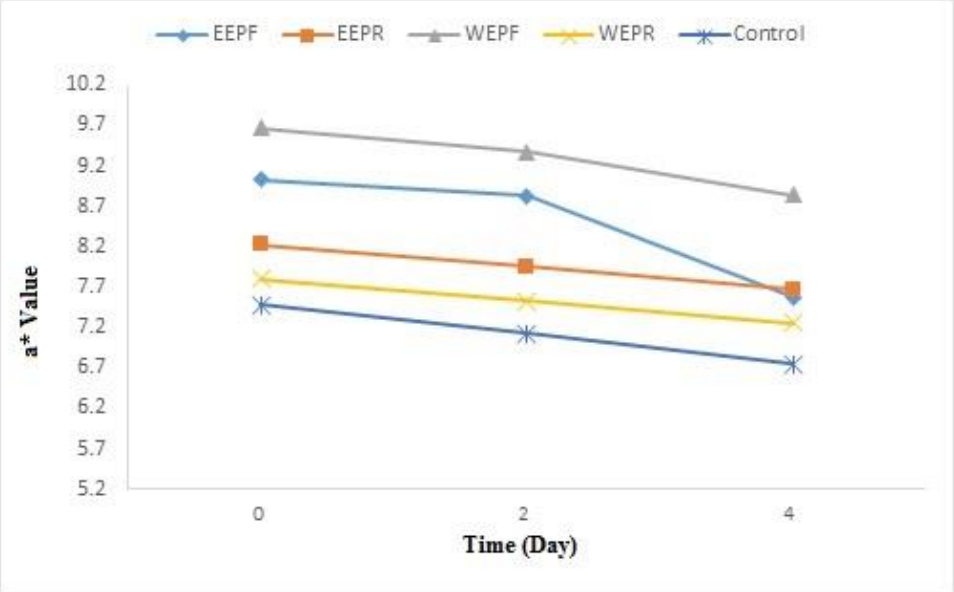

Figure 8. Changes of a* (redness) value of the trout fillet treatments during refrigerated storage (4°C) on different days.

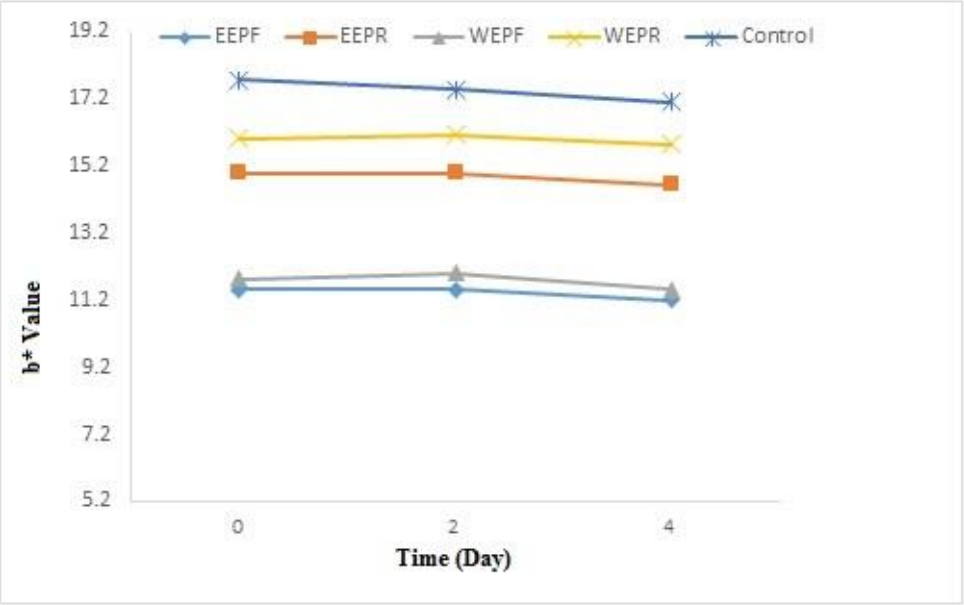

Figure 9. Changes of b* (yellowness) value of the trout fillet treatments during refrigerated storage (4°C) on different days.

According to figure 8, Redness (a* value) was significantly ($p<0.05$) higher in the WEPF sample than the control and other groups, with the minimum level (a* value) being found in the control sample during storage time. In all samples, a * value significantly ($p<0.05$) decreased at the end of storage. Oxidation of lipids could lead to the loss of redness (a* value) of the control sample during storage (Ozen et al., 2011). The main reason for the increased redness (a* value) and reduced lightness (L* value) was attributed to the presence of pomegranate extracts. Increases of the redness of the samples containing pomegranate extracts could be due to the formation of the main pigment (anthocyanins) in pomegranate extracts (Qin et al., 2013).

As shown in figure 9, a significantly higher b* value (yellowness) was found in the control group than the extract groups ($p<0.05$). Minimum b* value (yellowness) ($p<0.05$) was observed in the EEPF group. In all samples, the b * value in the day 1 of storage period increased slightly, and then decreased in the last storage day. Similar results were reported by Biswas et al. (2012) and Naveena et al. (2008). In overall, the degree of color change depended on the extracts and their composition.

3.8. Sensory evaluation

Sensory quality of the trout fillets was evaluated by panelists, as shown in Table 1. The panelists scored appearance, juiciness, flavor, and overall palatability of the trout fillets from 1 to 8. Using Mann Whitney test with Bonferroni correction for data analysis, significant ($p<0.05$) difference was found between the extracts and sensorial quality. Scores of all the parameters were significantly ($p<0.05$) greater in the WEPF group than the others. The flavor and juiciness scores were significantly lower ($p<0.05$) in the control group than that in the sample groups. Also, appearance and overall palatability scores were lowest in the EEPR group. We failed to demonstrate any meaningful difference between the WEPR and WEPF groups in this regard. The highest sensorial quality was observed in the aqueous extracts of pomegranate. Effects of

natural antioxidant such as turmeric, shallot (Pezeshk et al., 2011), and grape seed extract (Moradi et al., 2011) have been also reported in recent studies.

Table 1. Sensory evaluation scores of the trout fillet samples treated with pomegranate extracts

Parameters				
Samples	Appearance[a]	Juiciness[b]	Flavor[c]	Overall palatability[d]
Control group	5.58±1.31	5.25±1.48	5.00±1.82	5.75±0.93
	5.33±0.65	6.00±0.73	5.67±0.42	5.25±0.57
	5.50±1.00	6.50±0.64	6.00±0.54	5.50±0.45
	7.16±0.94	6.92±0.80	6.83±0.51	7.08±0.99
	7.58±0.51	7.41±0.24	7.42±0.26	7.58±0.63

a Appearance: 0 = extremely poor to 8 = excellent.
b Juiciness: 0 = extremely dry to 8 = extremely juicy.
c flavour: 0 = extremely intense odor or flavour to 5 = no flavour or odor.
d Overall palatability: 0= extremely palatable to 8 = extremely unpalatable.

4. Conclusions

The current study demonstrated that utilizing pomegranate extracts as natural antioxidants could delay lipid oxidation in fish fillet during refrigerated storage, with pomegranate rind showing the greatest ability. In the pomegranate extracts-receiving samples, the magnitude of change in TVB-N, TBA, and PV was less than in the control sample. These results indicated that pomegranate extracts could retard the fish spoilage and may be considered as an alternative for synthetic antioxidants in food industry as natural and cheap antioxidant sources to minimize lipid oxidation of fish products.

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ANTIMICROBIAL ACTIVITY OF EGG WHITE PROTEIN-BASED EDIBLE FILMS INCORPORATED WITH THYME AND HOPS LIQUID EXTRACTS ON HAMBURGERS

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ABSTRACT

Antimicrobial activity of egg white protein-based edible films (EWP) incorporated with 5% thyme (TV), 5% hops (HL), and 2.5% thyme + 2.5% hops (TH) liquid extracts was evaluated on hamburgers during refrigerated storage (4 °C). Physical properties such as color and thickness of the films were determined, and according to the results, it was determined that the addition of the extract decreased the *L** value, increased *a** and *b** values, slightly increased the thickness value in the films. The average total viable count, yeast-mold, coliform group bacteria, *Staphylococcus* spp., and *Pseudomonas* spp. numbers of the hamburgers varied between 6.12-8.43 log CFU/g, 2.50-5.46 log CFU/g, 4.87-7.35 log CFU/g, 4.15-6.17 log CFU/g, and 6.10-9.22 log CFU/g, respectively. The groups with hops extract supplements have always had the lowest number of coliforms, indicating that hops extract has higher antimicrobial activity on the coliform group bacteria compared to especially the control group ($p<0.05$). The film application reduced the brightness, and the thyme extract increased the yellowness in the hamburgers. The redness was not affected by storage and treatment factors. While the pH value is very close to each other in the film-packaged groups, it exhibits a constantly increasing slope in the control group.

Abbreviations: C, control group unpackaged with film; EWP, antimicrobial-free film; TV, 5% (v/v) thyme (*Thymus vulgaris* L.) extract added film; HL, 5% (v/v) hops (*Humulus lupulus* L.) extract added film; TH, 2.5% (v/v) thyme extract + 2.5% (v/v) hops extract added film

1.Introduction

Passive packaging techniques, which only have protection functions, have been replaced by new packaging technologies (Delikanli and Ozcan, 2014). Among these technologies, active packaging, which actively protects the product from its environment and adds value to the basic function of packaging, is one of the subjects that researches are concentrated (Suppakul *et al.*, 2003). In general, in active packaging, by maintaining quality an approach based on the relationship between food, packaging material, and environmental atmosphere is applied to extend the shelf life of food, improve food safety, and sensory properties (Cha and Chinnan, 2004). Antimicrobial packaging, which is one of the active packaging types, is a suitable storage method especially for red meat, poultry, and seafood (Suppakul *et al.*, 2003). The basis of the antimicrobial packaging, developed to completely inactivate or limit the growth of existing or reproducible microorganisms in foods, by adding

antimicrobial agents to the packaging material or its environment alone or combination, is a controlled release of the migration of antimicrobial agents. As a result, not only the initial microorganisms are inactivated, but the antimicrobial activity will be longer during the storage and transportation of the product, thus preventing the development of microorganisms that may occur (Cutter, 2002).

There are five basic protein fractions in egg white; ovalbumin, ovotransferrin, lysozyme, ovomucin, and ovomucoid. Ovalbumin, which makes up more than semi of egg white protein by weight, is the just fraction includes free sulfhydryl groups (SH). Other proteins, such as ovotransferrin and lysozyme, include disulphide bonds (S-S) (Mine, 1995). Ovotransferrin is an iron-binding protein. Lysozyme has antimicrobial activity and has been discovered to be effective against Gram-negative bacteria (Dangaran *et al.*, 2009). The film formation mechanism is assumed to include intermolecular and intermolecular S-S bonds. At alkaline pH, S-S bonds are reduced to SH groups, thus simplifying protein dispersal. Heating also opens the protein chains, revealing more hydrophobic and SH groups. During gelation and drying, SH groups are turned into intermolecular and intramolecular S-S covalent cross-links thanks to sulfhydryl-disulfide exchange reactions and oxidation (Mine, 1992; Gennadios *et al.*, 1996). This results in the formation of three-dimensional networks (Lim *et al.*, 2002).

Thymus vulgaris L. is found in the *Lamiaceae* family. Thymus are significant medicinal plants that are known to include antimicrobial substances and are rich in dissimilar active substances such as carvacrol, thymol, γ-terpinene, thymyl methyl ether compounds (Nabavi *et al.*, 2015). Studies have shown that thyme essential oil and extract have powerful antimicrobial (Imelouane *et al.*, 2009; Rota *et al.*, 2008) and antifungal activity (Del Toro-Sánchez *et al.*, 2010; Rasooli and Abyaneh, 2004). Thyme extract can be used as a natural preservative in the preparation of active food packages (Aziz and Almasi, 2018). *Humulus lupulus* L. is found in the *Cannabaceae* family. Between the dissimilar parts of hops, just the female cones and leaves showed antimicrobial properties (Zanoli and Zavatti, 2008; Abram *et al.*, 2015). The antimicrobial activities of multifarious hops extracts are known, as well as the singular hops parts. Two hops bitter acids, humulones (alpha acid) and lupulones (beta acid), showed activity against Gram-positive bacteria such as *Bacillus*, *Clostridium*, *Lactobacillus*, *Listeria*, *Staphylococcus*, *Streptococcus* species (Haas and Barsoumian, 1994; Bhattacharya *et al.*, 2003; Shen *et al.*, 2009; Siragusa *et al.*, 2009; Teuber and Schmalreck, 1973), Gram-negative bacteria such as *Brucella* species and *Helicobacter pylori* (Ohsugi *et al.*, 1997; Shapouri and Rahnema, 2011), and fungi such as *Trichophyton*, *Mucor*, *Fusarium*, *Candida* species (Mizobuchi and Sato, 1985).

In this study, thyme and hops liquid extracts were added alone and combination to edible films based on egg white protein applied to hamburgers. In this way, it is aimed to prolong the shelf life of the product and prevent economic losses with a new active packaging technology by inactivating and/or limiting the growth of microorganisms that may be present in the product or may be occur later in storage.

2. Materials and methods

2.1. Materials

The edible film material used in the research is based on egg white protein and Alfasol brand egg white protein powder was obtained from Kimbiotek Chemical Agents Inc. (Istanbul, Turkey). Thyme (*Thymus vulgaris* L.) liquid extract from antimicrobial agents was obtained according to the method proposed by Xu *et al.* (2008). Another antimicrobial agent, hops (*Humulus lupulus* L.) liquid extract was obtained from Gökçek Şifa Inc. (Istanbul, Turkey).

2.2. Methods

2.2.1. Thyme liquid extract and edible film production

For the production of thyme liquid extract from the antimicrobial substances used in the research, dried thyme was purchased from the herborists in the Konya market, and then ground and ground into powder. 20 g of powdered thyme and 180 ml of distilled water were placed in a flask and kept in a shaking water bath (Wisd, Korea) at 90 ℃ at 150 rpm for 30 minutes. Then incubated in an oven (Nuve EN 120, Turkey) at 37 ℃ for 1 night. Finally, the liquid extract was obtained using Whatman No. 1 filter paper (Xu *et al.*, 2008).

In the production of an edible film based on egg white protein, Gennadios *et al.* (1996) and Kavas (2017) suggested methods were modified and used. For this purpose, 9 g of egg white protein powder, 100 ml of distilled water, and 4.5 ml of glycerol as a plasticizer were homogenized (Wisd HG-15D & HG-15A, Korea) at 700 rpm for 1 minute, and then the pH of the solution was adjusted to 11.25 with 1 N NaOH. Then film solutions were maintained wherein the water bath (Nuve BM 402, Turkey) at 45 ℃ for 20 minutes, and the antimicrobial-free film solution was filtered after cooling to room temperature. Films containing thyme and hops liquid extract were filtered after they were homogenized with a homogenizer (Wisd HG-15D & HG-15A, Korea) at 700 rpm for 1 minute by adding the pre-determined (Karagoz-Emiroglu *et al.*, 2010) 5% (v/v) levels of these substances to the solution. Finally, 15±0.1 g the film solutions were weighed Petri dishes, and were dried wherein the oven (Nuve FN 120, Turkey) at 50 ℃ for 18 hours. The edible films prepared were kept in a vacuum desiccator at room temperature until applied to hamburgers (Figure 1).

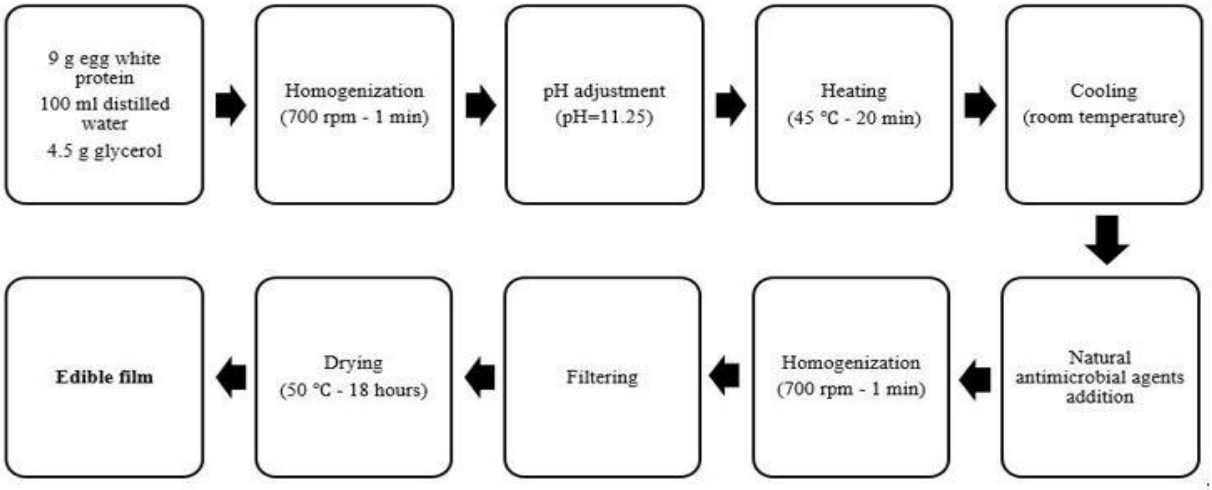

Figure 1. Preparation of edible films

2.2.2. Proximate analysis

Moisture (oven), fat (Soxhlet extraction), protein (Kjeldahl), and ash (ash oven) contents were determined using standard AOAC methods (AOAC, 2000). Moisture (%) was determined by drying 5-10 g of the sample until it reached constant weight at 105 ℃. The fat (%) was determined using the Soxhlet extraction device. Protein (%) was determined according to the Kjeldahl method. The factor 6.25 was used to convert nitrogen to crude protein. Ash (%) was determined by burning 2-2.5 g of the sample until it reached constant weight at 550 ℃.

2.2.3. Physical properties of edible films

The colors of the produced films were determined using a colorimeter device (CR-400, Konica Minolta, Osaka, Japan). The device was calibrated with a white standard plate before the measurement and then *L**, *a**, and *b** values of the films were determined (AMSA, 1991). The

thickness of the produced films was determined using a digital micrometer (Mitutoyo 500-181-30 Digital Caliper, Japan) with a precision of 0.01 mm (Seydim and Sarikus, 2006). Measurements were taken at three random locations of the films.

2.2.4. Preparation of hamburgers

Beef used in the research was obtained from contracted butchers in the Konya market and turned into medium-fat minced meat with a fat content of 15-20%. After adding the additives in the hamburger formulation in certain proportions, they were given the proper shape and film application was carried out immediately. As seen in Figure 2, the films were applied by placing them on the upper and bottom surfaces of the hamburgers. Hamburgers were divided into 5 different treatment groups; control group unpackaged with film (C), antimicrobial-free film (EWP), 5% (v/v) thyme (*Thymus vulgaris* L.) extract added film (TV), 5% (v/v) hops (*Humulus lupulus* L.) extract added film (HL) and to determine the synergistic effect of antimicrobial agents 2.5% (v/v) thyme extract + 2.5% (v/v) hops extract added film (TH). The hamburgers prepared in this way were preserved for 4 ℃ at 7 days and subjected to microbiological, color, and pH analyzes on the 1st, 4th, and 7th days of storage.

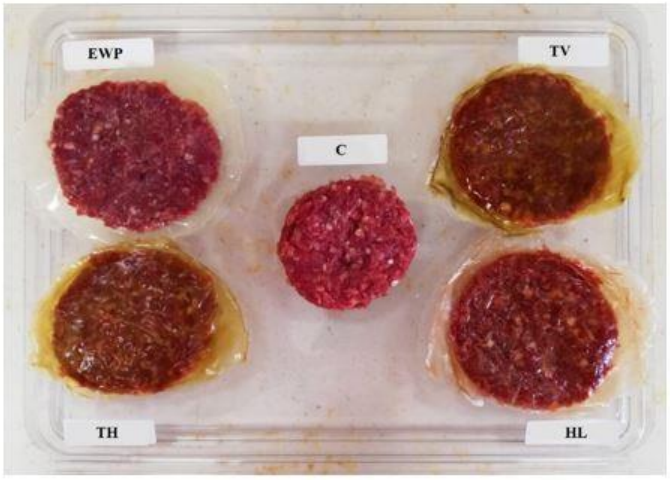

Figure 2. Hamburgers packed with edible films and the control group

Abbreviations: C, control group unpackaged with film; EWP, antimicrobial-free film; TV, 5% (v/v) thyme (*Thymus vulgaris* L.) extract added film; HL, 5% (v/v) hops (*Humulus lupulus* L.) extract added film; TH, 2.5% (v/v) thyme extract + 2.5% (v/v) hops extract added film

2.2.5. Microbiological analysis of hamburgers

In order to perform microbiological analysis, each sample was opened under aseptic conditions and a 10 gram portion was weighed from the center of the hamburgers. In order to make the first dilution, the weighed portion was transferred into the previously sterilized Maximum Recovery Diluent (MRD, Merck) and it was ensured to become homogeneous for 1 minute. Serial dilutions in the range of 10^{-1}-10^{-6} were prepared for all analyses. These prepared dilutions were inoculated on various media by the spread plate method and incubated at certain temperature-time norms. Plate Count Agar (PCA; Merck) medium for total viable count (TVC) and incubation at 37 ℃ for 48 hours, Potato Dextrose Agar (PDA; Merck) medium for yeast-mold and incubation at 28 ℃ for 4-5 days, Violet Red Bile Agar (VRB; Merck) medium for coliforms and incubation at 37 ℃ for 24 hours, Baird Parker Agar (BPA, Merck) + Egg Yolk Tellurite Emulsion medium for *Staphylococcus* spp. and incubation at 37 ℃ for 24 hours, Glutamat Starch Phenol Red Agar (GSP, Merck) + Penicillin G medium for *Pseudomonas* spp. and incubation at 28 ℃ for 3

days conditions were provided. After colony counting, the number of microorganisms as $\log_{10}$colony forming units (CFU)/g is indicated (Halkman, 2005).

2.2.6. Color analysis of hamburgers

The color measurements were performed at days 1, 4, and 7 of refrigerated storage. In this purpose, a colorimeter device (CR-400, Konica Minolta, Osaka, Japan) was used. The device was calibrated with a white standard plate before the measurement and then *L**, *a**, and *b** values of hamburgers were determined (AMSA, 1991).

2.2.7. pH analysis of hamburgers

The pH measurements were performed at days 1, 4, and 7 of refrigerated storage. In this purpose, the pH value of each hamburger was determined by reading from random points with the help of a pH-meter (Testo 205, Germany) (Lambooij *et al.*, 1999).

2.2.8. Statistical analysis

A completely random design was used (two replicates). Data were analyzed using MINITAB software version 16. When a significant ($p<0.05$) main effect was found, differences between means were evaluated using the Tukey's Test.

3. Results and discussions

3.1. Proximate analysis

Moisture, fat, protein, and ash analyzes were conducted to determine the chemical composition of the ground beef used in hamburger making and the results are given in Table 1. In the analyzes performed, the moisture, fat, protein, and ash contents of the ground beef used in making hamburgers was determined as 65.00%, 15.60%, 18.29%, and 1.21%, respectively.

Table 1. Chemical composition of the ground beef (%)

Chemical Composition	%
Moisture	65.00±0.55
Fat	15.60±0.86
Protein	18.29±0.22
Ash	1.21±0.21

Values are means of triplicate samples (±SD)

Gun (2014) was found the amount of moisture, fat, protein, and ash in minced meat samples as 66.19%, 12.67%, 18.70%, and 1.16%, respectively, in his study where he investigated the effect of various dairy by-products on some properties of beef patties. Kececi (2018) was found the amount of moisture, fat, protein, and ash in minced meat samples as 63.99%, 19.02%, 18.10%, and 0.74%, respectively, in his study where he investigated the effect of various vegetable pickle powders on some properties of beef patties. These results and the results we obtained from our study show partial similarity and partial differences. These differences are probably due to the different races, types, ages, and diets of the animals from which the meat was used in the studies.

3.2. Physical properties of edible films

In the study, color and thickness values of edible films based on extracted and non-extracted egg white protein were determined and the results are given in Table 2. When the color values were examined, the brightness-darkness indicator *L** value had the highest value with 87.97 in the edible film based on egg white protein, while the addition of extract caused a decrease in the *L** values of the films ($p<0.05$). The redness-green indicator *a** value had the lowest value with -3.63 in the edible film based on egg white protein, while the addition of extract caused an increase in the *a** values of the films. The liquid extract of hops increased the redness value ($p<0.05$). The yellowness-blue indicator *b** value had the lowest value with 12.61 in the edible film based on egg white

protein, while the addition of extract caused an increase in the *b** values of the films. The liquid extract of thyme increased the yellowness value ($p<0.05$). When the thickness values were examined, it was determined that the thickness of both the extracted and the non-extracted films gave close results, while the addition of the extract increased the thickness in the films somewhat ($p>0.05$).

Table 2. Physical properties of edible films

	EWP	TV	HL	TH
L*	87.97±0.89[a]	74.62±1.42[b]	74.85±2.01[b]	75.90±2.62[b]
a*	-3.63±0.08[c]	0.23±0.86[b]	5.80±1.33[a]	0.25±1.24[b]
b*	12.61±0.96[d]	40.67±0.93[a]	25.47±1.05[c]	36.40±1.97[b]
Thickness (mm)	0.34±0.02[a]	0.35±0.03[a]	0.40±0.09[a]	0.36±0.09[a]

Values are means of triplicate samples (±SD)
[a-d] Means within rows with different superscript letters are significantly different ($p<0.05$)
Abbreviations: EWP, antimicrobial-free film; TV, 5% (v/v) thyme (*Thymus vulgaris* L.) extract added film; HL, 5% (v/v) hops (*Humulus lupulus* L.) extract added film; TH, 2.5% (v/v) thyme extract + 2.5% (v/v) hops extract added film

Kavas *et al.* (2016) examined the application of egg white protein-based films containing orange essential oil on kashar cheese determined the effects of physical, chemical, and antimicrobial properties and reported that the film, which does not contain antimicrobial agents, is brighter and more transparent than films containing essential oil. Similarly, Taqi *et al.* (2011) examined the effects of different olive oil and oleic acid concentrations on the mechanical properties of edible films based on egg white protein and reported that olive oil and oleic acid were added to the film caused a decrease in the films *L** value. They also measured the thickness of the films and they found that the addition of additives caused an increase in the thickness of the films.

3.3. Microbiological analysis of hamburgers

Total viable count (TVC) is a measure to point out the quality of the product that states the non-usability of the product. TVC of the hamburgers packaged with or without edible films during cold storage are presented in Figure 3a. It was determined that the average number of TVC varies between 6.12-8.43 log CFU/g. With increasing time, TVC amounts increased in all treatments ($p<0.05$). The increase in TVC for every treatment during the storage are attached to the first level of microorganism and the level of treatment (Chidanandaiah *et al.*, 2009). Although slightly lower TVC was determined in the hamburgers treated with liquid extracts containing films at day 4 as compared to C and EWP groups ($p<0.05$), no significant differences in TVC was observed between the hamburgers in general ($p>0.05$). This result is similar to the study of Karagoz-Emiroglu *et al.* (2010). Variations in antimicrobial activity between studies may be due to differences in the meat material used. Hamburgers have a complex texture, and it would be not easy to adequately inhibit the growth of microorganisms on hamburgers.

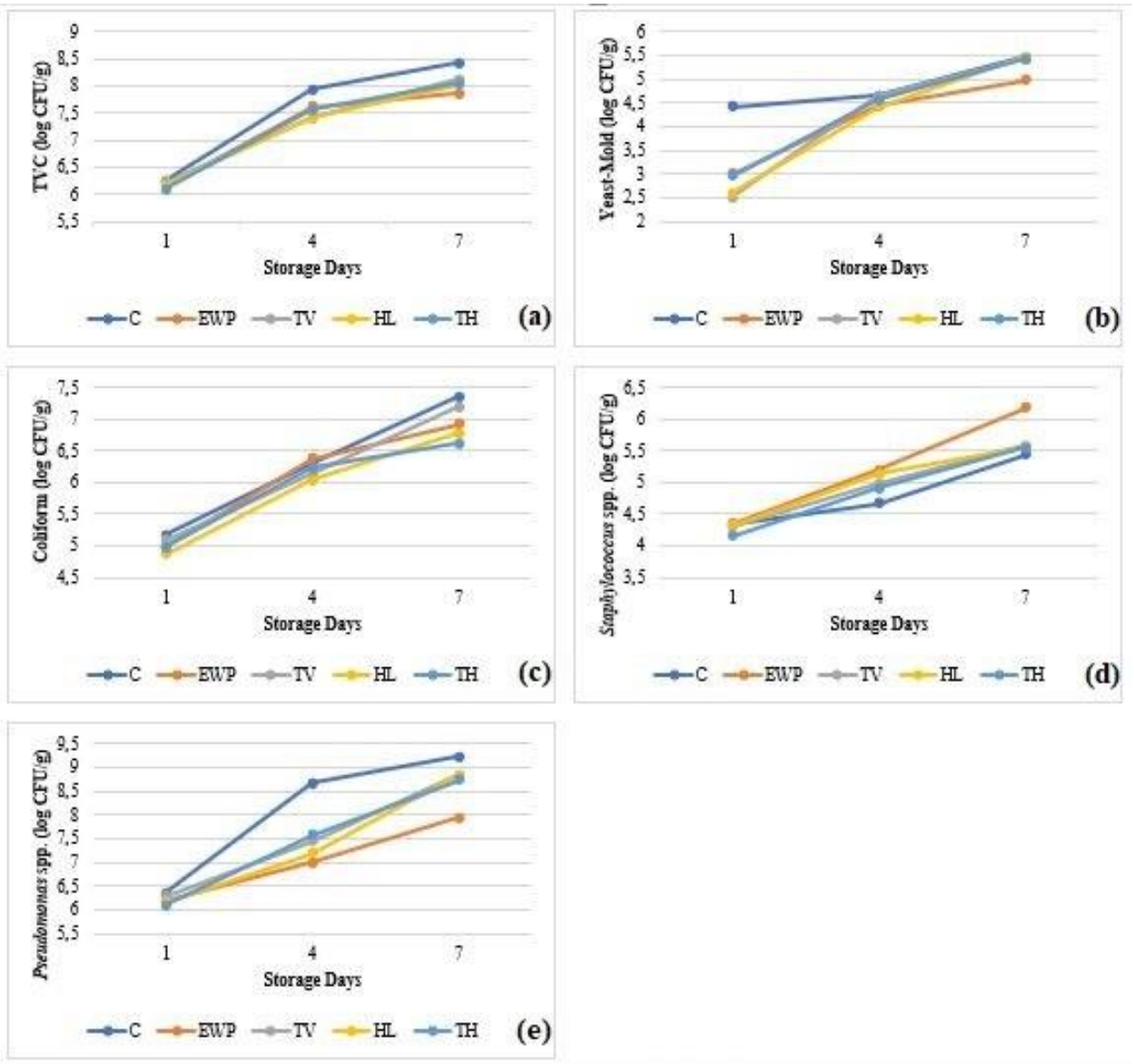

Figure 3. Changes in total viable count (TVC) (a), yeast-mold (b), coliform (c), *Staphylococcus* spp. (d), *Pseudomonas* spp. (e) of different treatment during cold storage (4±1 °C) for 7 days

Abbreviations: C, control group unpackaged with film; EWP, antimicrobial-free film; TV, 5% (v/v) thyme (*Thymus vulgaris* L.) extract added film; HL, 5% (v/v) hops (*Humulus lupulus* L.) extract added film; TH, 2.5% (v/v) thyme extract + 2.5% (v/v) hops extract added film

Yeast-mold counts of the hamburgers packaged with or without edible films during cold storage are presented in Figure 3b. It was determined that the average number of yeast-mold varies between 2.50-5.46 log CFU/g. An increase in yeast-mold counts was observed in parallel with storage ($p<0.05$). The control group has always been the group with the highest yeast-mold count ($p>0.05$). The less count of yeast-mold in liquid extracts treatments can be due to phenolic compounds. Phenolic compounds in the outer membrane of the plant destroy microorganisms, induce the let out of liposaccharides and rise the permeability of the cytoplasmic membrane to ATP. Withdrawal of ATP is concluded in the accomplishment of cellular energy storage and cell death (Burt, 2004).

When coliform group bacteria is mentioned, it is understood that Gram (-), non-spore

forming, rod-shaped bacteria that form acid and gas from lactose within 48 hours at 37 ℃. According to this; *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *Citrobacter freundii*, which are members of the *Enterobacteriaceae* family, are defined as coliform group bacteria (Halkman, 2005). Although the consumption of ground raw meat is one of the most common risks of food-borne pathogenic infections, *E. coli* has been identified in many foods all over the world, confirming this risk (Solomakos *et al.*, 2008). Coliform counts of the hamburgers packaged with or without edible films during cold storage are presented in Figure 3c. It was determined that the average number of coliform varies between 4.87-7.35 log CFU/g. Parallel to the storage, an increase in the number of coliforms was observed and the results were found statistically different from each other ($p<0.05$). The groups with hops extract supplements have always had the lowest number of coliforms, indicating that hops extract has higher antimicrobial activity on the coliform group bacteria compared to especially the control group ($p<0.05$). Arsene *et al.* (2015) investigated the antimicrobial and antioxidant activity and phenolic content of hops ethanol extract. The antimicrobial activity was determined by disc diffusion method against several Gram (+) (*Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*) and Gram (-) (*Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas fluorescens*) bacteria. The results showed that hops extract can be used effectively as a plant-based antimicrobial product.

Staphylococcus spp. counts of the hamburgers packaged with or without edible films during cold storage are presented in Figure 3d. It was determined that the average number of *Staphylococcus* spp. varies between 4.15-6.17 log CFU/g. An increase in *Staphylococcus* spp. counts was observed in parallel with storage ($p<0.05$). While the C group had the lowest *Staphylococcus* spp. numbers, the EWP group had the highest *Staphylococcus* spp. numbers ($p>0.05$). It is thought that this situation is due to the fact that *Staphylococcus* spp. are naturally found in the skin and nose flora of humans, and that they were transferred to the films as a result of carelessness during film preparation. Although the addition of extract slightly reduced this situation, the control group gave the lowest *Staphylococcus* spp. Another study in which higher *Staphylococcus* spp. counts were obtained only in the film-coated samples compared to the control and extract-added films was conducted by Jonaidi Jafari *et al.* (2018).

Pseudomonas spp., one of the common meat spoilage bacteria at refrigerated temperatures, counts of the hamburgers packaged with or without edible films during cold storage are presented in Figure 3e. It was determined that the average number of *Pseudomonas* spp. varies between 6.10-9.22 log CFU/g. An increase in *Pseudomonas* spp. counts was observed in parallel with storage except the EWP group ($p<0.05$). The EWP group was the lowest *Pseudomonas* spp. number compared to other groups ($p>0.05$) and reduced the number of *Pseudomonas* spp. by 1.28 log compared to the control group on the last day of storage. Similarly, Bonilla *et al.* (2014) prepared edible films with and without plant essential oils and aimed to control the microbiota of the pork mincemeat with the films they produce. They reported that the films were effective in controlling microbial growth on pork mincemeat, but the inclusion of essential oils did not contribute to antimicrobial activity.

3.4. Color analysis of hamburgers

Alterations in meat color during storage are significant for consumer admission and purchasing decision. It was determined that the brightness-darkness indicator L^* values of hamburgers varied between 34.85-43.98, the redness-green indicator a^* values of hamburgers varied between 9.71-16.09, the yellowness-blue indicator b^* values of hamburgers varied between 3.95-11.94 on average (Table 3). Film application caused a decrease in the brightness of the samples ($p<0.05$). This impact may be thanks to the oxygen barrier property of edible films, which can postpone oxygen diffusion, and

its reaction with myoglobin (Bojorges *et al.*, 2020). The discoloration in meats is extremely associated with myoglobin content in the muscles; beef includes an oxidative muscle type I with a high myoglobin content (Astruc, 2014). The *a** value was not affected by storage and treatment factors, and the difference between treatments was not statistically significant ($p>0.05$), and data demonstrate the efficacy of using edible films with and without antimicrobial agents to protect color unity. Thyme extract increased a yellowness in the samples ($p<0.05$). This result is compatible with the *b** values in the physical properties of edible films. This change may be caused by the presence of carvacrol (yellow color) in the edible films.

Table 3. Analysis results of color and pH values of hamburgers packed with edible films and control group during cold storage (4±1 °C) for 7 days

	Storage Days	Treatments C	EWP	TV	HL	TH
	1	42.81 ± 1.63^{Aa}	38.97 ± 3.73^{Aabc}	36.26 ± 2.31^{Ac}	37.21 ± 2.20^{Abc}	40.63 ± 2.14^{Aab}
L*	4	41.03 ± 3.93^{Aa}	37.62 ± 1.67^{Aab}	37.92 ± 1.93^{Aab}	36.40 ± 2.14^{Ab}	40.44 ± 1.95^{Aab}
	7	43.98 ± 1.62^{Aa}	38.94 ± 2.54^{Abc}	35.16 ± 1.43^{Ac}	40.11 ± 4.17^{Aab}	34.85 ± 2.09^{Bc}
	1	12.18 ± 0.40^{Bb}	16.09 ± 2.38^{Aa}	15.57 ± 2.40^{Aa}	16.06 ± 1.11^{Aa}	10.27 ± 1.60^{Bb}
a*	4	15.12 ± 1.89^{Aa}	14.33 ± 1.21^{ABa}	9.71 ± 2.60^{Bb}	14.17 ± 1.23^{ABa}	10.82 ± 1.21^{ABb}
	7	12.80 ± 0.56^{Ba}	11.31 ± 2.89^{Ba}	12.13 ± 2.06^{ABa}	11.75 ± 2.34^{Ba}	12.77 ± 1.71^{Aa}
	1	7.90 ± 0.46^{Ab}	5.48 ± 1.51^{Ab}	11.94 ± 4.57^{Aa}	7.14 ± 0.62^{Ab}	7.21 ± 0.58^{Ab}
b*	4	7.31 ± 0.98^{ABa}	4.96 ± 0.81^{Ab}	7.76 ± 1.01^{Aa}	6.04 ± 1.42^{Aab}	6.82 ± 1.03^{Aa}
	7	6.64 ± 0.79^{Bab}	4.45 ± 2.04^{Abc}	8.31 ± 1.67^{Aa}	3.95 ± 1.04^{Bc}	7.83 ± 1.48^{Aa}
	1	5.41 ± 0.02^{Cb}	5.58 ± 0.10^{Aab}	5.57 ± 0.10^{ABab}	5.64 ± 0.11^{Aa}	5.69 ± 0.20^{Aa}
pH	4	5.68 ± 0.18^{Ba}	5.49 ± 0.03^{Ab}	5.46 ± 0.06^{Bb}	5.42 ± 0.02^{Bb}	5.46 ± 0.02^{Bb}
	7	6.80 ± 0.07^{Aa}	5.60 ± 0.14^{Ab}	5.67 ± 0.14^{Ab}	5.52 ± 0.06^{Bb}	5.67 ± 0.17^{ABb}

Values are means of duplicate samples (±SD)

[A–C] Means within columns with different superscript letters are significantly different ($p<0.05$)

[a-c] Means within rows with different superscript letters are significantly different ($p<0.05$)

Abbreviations: C, control group unpackaged with film; EWP, antimicrobial-free film; TV, 5% (v/v) thyme (*Thymus vulgaris* L.) extract added film; HL, 5% (v/v) hops (*Humulus lupulus* L.) extract added film; TH, 2.5% (v/v) thyme extract + 2.5% (v/v) hops extract added film

3.5. pH analysis of hamburgers

It was determined that the pH values of hamburgers varied between 5.41-6.80 on average (Table 3). The pH values of the samples covered with films first decreased, then increased, and gave close results to each other ($p>0.05$). While the pH value of the control group was the lowest on the first day, it gradually increased and reached the highest values on the fourth and seventh days ($p<0.05$). Venkatachalam and Lekjing (2020) were coated pork patties with edible films prepared by adding different antimicrobial substances, and they obtained that the pH values of the film coated patties were almost similar while the pH value of the control group gradually increased. The pH increase with storage time may be due to protein denaturation and collection of alkaline by-products such as amines, ammonia and trimethylamine, all produced during amino acid degradation by microbial or autolytic reactions (Lorenzo *et al.*, 2014). During storage, the pH of hamburgers covered with films had slower rate of increased than with control treatment, throughout the storage time. This was probably due to egg white protein, thyme liquid extract,

and hops extract having antimicrobial activity toward various spoilage bacteria, including volatile basic nitrogen producing microorganism (Ehsani *et al.*, 2014).

4. Conclusions

In this study, the effects of edible films based on egg white protein, containing thyme and hops liquid extract, as natural antimicrobial agents, on microbial inactivation of hamburgers with high production and consumption potential from meat products was investigated. Ground beef used in the study has 65.00% moisture, 15.60% fat, 18.29% protein, and 1.21% ash content. Besides, color and thickness values of edible films were measured before hamburgers were packaged. When the color values were examined, it was determined that the addition of the extract decreases the *L** value and increases the *a** and *b** values in the films. When the thickness value were examined, it was observed that the addition of the extract slightly increased the thickness value in the films. According to the results of the microbiology analysis, it was determined that the average total viable count, yeast-mold, coliform group bacteria, *Staphylococcus* spp., and *Pseudomonas* spp. numbers of the samples varied between 6.12-8.43 log CFU/g, 2.50-5.46 log CFU/g, 4.87-7.35 log CFU/g, 4.15-6.17 log CFU/g, and 6.10-9.22 log CFU/g, respectively. The groups with hops extract supplements have always had the lowest number of coliforms, indicating that hops extract has higher antimicrobial activity on the coliform group bacteria compared to especially the control group. According to the results of the color analysis, it was determined that the film application decreased the brightness in the samples, while the thyme extract increased the yellowness in the samples. The redness was not affected by storage and treatment factors. According to the results of the pH analysis, the pH values of the samples covered with films first decreased, then increased, and gave close results. The pH value of the control group increased continuously and reached the highest level among all samples on the last day of storage. It is thought that more sufficient inhibition effects can be achieved with different film raw materials, different antimicrobial agents, and various combinations of them, and it is suggested that it will be beneficial to continue research on this subject.

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SURFACE DECONTAMINATION OF *SALMONELLA ENTERICA SEROVAR TYPHIMURIUM* ON SHELL EGGS BY VAPORIZED ETHYL PYRUVATE AND PLANT HYDROSOLS

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ABSTRACT

In this study, the decontamination effects of plants hydrosols, and vaporized ethyl pyruvate were investigated against *Salmonella enterica* serovar *Typhimurium* on shell eggs. For hydrosol treatments, the first inoculum level was 6.8 log cfu/g. Inoculated eggs treated with thyme, sage, basil, and their mixed hydrosols and sterilized DI water as a control for 10, 30, 60, and 90 s. Mixed and thyme hydrosols provided the highest log reduction (3 and 3.4 log) in 90 s that can be considered favorable for food decontamination treatments. Inhıbıtıon level was determined in the initial population 6.2 log CFU/g of *S. typhimurium* inactivation at 100µL, 500 µL, and 1000 µL EP concentrations under ambient and refrigerator conditions for 3, 5, and 7 days. Almost complete inhibition was observed with 1000 µL of EP at 7 days of storage at 20°C. At refrigerature temperatures, the maximum log reduction was 4.2 after 7 days of storage. In conclusion, this study showed that vaporized EP applications, which are accepted as GRAS, have high antimicrobial activity against *S. typhimurium* in shell eggs. Hydrosol treatments had lower inhibition levels of *S. typhimurium* compare to EP treatment. Hydrosol treatments had lower inhibition levels of S. *typhimurium* compared to EP treatment.

1.Introduction

Eggs and egg products are often consumed worldwide as a balanced and nutritional protein resource (Alkaya, Erdogdu, Halkman, & Ekiz, 2016; Georgescu, Apostol, & Gherendi, 2017). According to American Egg Board (AEB) per capita egg consumption was 276 per person in 2017. Also, worldwide egg production has been rising to approximately 80 million metric tons of eggs by 2017, up from 37.4 million metric tons in 1990 (Statista, 2017). Centers for Disease Control and Prevention (CDC) reports that Salmonellosis causes about 1.2 million infections, 23,000 hospitalizations, and 450 deaths every year in the United States (CDC, 2019a) and costs the United States $2.8 billion annually (Kim, Moreira, & Castell-Perez, 2011). *Salmonella* poses a great risk in the shell, and when the shell is broken, contamination occurs inside the egg. The product can cause infection when consumed raw or insufficiently cooked. According to the Public Health Agency of Canada (2003), the human oral infectious dose for *Salmonella* species has been determined as 102 – 103 CFU (Humphrey, Whitehead, Gawler, Henley, & Rowe, 1991). Salmonellosis is a zoonotic disease or infection that can be spread between animals and humans directly or indirectly. Eggs and egg products are one of the most common cause of salmonellosis outbreaks

(EFSA, 2009). For humans, salmonellosis is usually linked to infected food consumption (Gabriela Isabel Favier, Estrada, Otero, & Escudero, 2013). The most recent shell egg linked salmonellosis outbreak (*Salmonella enteridis*) has been reported by CDC was on September 8, 2018 in the U.S. which caused 12 hospitalizations (CDC, 2019b). The presence of *Salmonella* on eggshells not only major concern because of contamination risks but also it can penetrate into the egg from pores if egg cuticle has damaged. The cuticle can be damaged at the especially post washing process with usage of chemicals. Therefore, *Salmonella* needs to be inactivated or removed from the egg surface without damaging the cuticule (Keklik, Demirci, Patterson, & Puri, 2010).

The need to guarantee the microbiological safety of eggs without affecting their sensory and nourishing quality has prompted enhancement in the traditional procedures and the improvement of novel non-thermal methods (Lasagabaster, Arboleya, & De Maranon, 2011). Some non-thermal techniques that have been used for eggshell decontamination are pulsed UV light (Keklik et al., 2010; Lasagabaster et al., 2011). UV radiation (Chavez, Knape, Coufal, & Carey, 2002; Gabriela I Favier, Escudero, & de GUZMaÁN, 2001) herbal antimicrobial products ionized water (Davies & Breslin, 2003), atmospheric pressure plasma (Stolz et al., 2015), pulsed electric fields (Sampedro, Rodrigo, Martínez, Barbosa-Cánovas, & Rodrigo, 2006) etc. Each one of them has its own limitations and advantages. Hydrosols as the byproducts of hydro or steam distillation of natural plants may also be used as a natural decontamination method. The advantages of hydrosol use are cost effectiveness and not being harmful to health. Previous studies show that different plant hydrosols were effective on variety of products as a sanitizing agent (Tornuk et al., 2011). Many plant hydrosols that have antimicrobial properties have been used for this purpose. Törnük and Dertli (2015) (Tornuk & Dertli, 2015) have studied on the effectiveness of sage, rosemary, oregano, and thyme hydrosols on parsley samples; Tornuk et al.(2011) (Tornuk et al., 2011) have evaluated black cumin, bay leaf, rosemary, and sage hydrosols on carrots and apples (Tornuk et al., 2011). Both studies demonstrated that hydrosols could be an effective and natural alternative method to improve food safety.

Ethyl pyruvate (EP) is a stable lipophilic therapeutic agent that is also known as an antioxidant. EP and its use in food are classified as generally recognized as safe (GRAS) by US Food and Drug Administration (Tornuk & Durak, 2015). It has been used as a decontamination method for several products especially on fresh fruits and vegetables recently. Törnük and Durak (2014) (Tornuk & Durak, 2015) evaluated the effectiveness of vaporized EP on fresh parsley on specific bacteria and ensured full inhibition of growth. Strawberries and cherries were also evaluated by (Bozkurt et al., 2016) and found that vaporized EP and cold storage together had great potential to delay fungal deterioration and preserve the quality of strawberries and cherries. However, the effect of EP and hydrosols have not been investigated on shell eggs. Hence the study presented here aimed to reduce the *Salmonella typhimurium* load of shell eggs via some plant hydrosol applications and vaporized EP. As hydrosol types, thyme, basil, and sage hydrosols were preferred for their high antimicrobial capacity.

2. MATERIALS AND METHODS

2.1. Bacterial culture and inoculum preperations

As a target pathogen, S. enterica ser. Typhimurium ATCC 140288 was provided from Acıbadem Food Control Lab, İstanbul, Turkey. Stock cultures were preserved at-80 ° C in a 20% glycerol solution. The working culture was grown on nutrient agar (NA; Merck, Darmstadt, Germany) plates at 25 °C for a day, then stored at refrigerator at 4 °C. A colony of *S. typhimurium* was aseptically transferred from the NA into 10 ml of nutrient broth (NB; Merck,

Darmstadt, Germany) and then incubated at 37°C for 24 h.

2.2. Preparation of plant hydrosols

In order to prepare plant hydrosols, dried leaves of thyme (*T. vulgaris L.*), sage (*S. officinalis*) and basil (*O. basilicum*) were provided from a spice warehouse in Istanbul, Turkey. Hydrosols were obtained as the following method of (Sağdıç, 2003). 100 g of each ground plant material was placed into a flask (2 L) with 1 L of distilled water for 2 h with a Clevenger apparatus (Ildam, Turkey). After hydrodistillation, essential oil was separated and obtained hydrosols were kept in sterile bottles at 4 ºC until use.

2.3. Inoculation of egg samples

Grade A shell eggs had been purchased from a nearby Istanbul, Turkey auction. First, surface of the eggs were washed with tap water in order to get rid of dirts. Then, the eggs were soaked in 70% (v/v) of ethanol for disinfection. Before the inoculation, disinfected eggs were dried on sterile filter paper for 30 minutes in laminar flow. 0.1 ml of S. enterica ser. Typhimurium ATCC 140288 was inoculated onto each egg. The eggs were left for drying approximately 50 minutes at room temperature. Inoculated eggs were used for hydrosol and ethyl pyruvate applications.

2.4. Application of ethyl pyruvate (EP)

Inoculated egg samples were placed in previously autoclaved egg boxes. EP was applied to eggs using by Durak (2012) (Durak, Churey, Gates, Sacks, & Worobo, 2012) method with some modifications. EP was pipetted into the boxes that have *Salmonella* inoculated eggs at the amount of 250 ul, 500 ul and 1000 ul. Then, the boxes were closed and wrapped out with strech film. EP-treated and control samples stored at room temperature (~20 °C) and at refrigerator (4°C) for up to 7 days.

2.5. Application of Hydrosol

Inoculated eggs were soaked into thyme, sage, basil and the mix of these three hydrosols. Eggs were put into sterilized beakers containing 200 mL of each sanitizing hydrosol (thyme, sage, basil and mixed) for 0, 10, 30, 60, 90 seconds. Hydrosols were renewed for each trials. *Salmonella* load of hydrosol treated and control samples enumerated. The analysis conducted duplicate.

2.6. Enumeration of *Salmonella* and determination of growth inhibition rate (GIR)

EP and hydrosol treated eggs were seperated from their shells by using spoon and tweezers aseptically and put into stomacher bags. Shells weighed approximately 10 g and incorporated with 90 mL sterile 0.1% pepton water. The sample was homogenized by stomacher for 60 s then serially diluted with 0.1 percent peptone water followed by spread plating. The plates were incubated for 24 h at 37°C then plates were enumarated and the *salmonella* population was expressed as log CFU/g. logarithmic values. In addition to bacterial count, growth inhibition rates (GIRs) of S. enterica ser. Typhimurium was determined caused by hydrosol treatment calculated using the following equation (Eq. 1) as applied by Sagdıc (2003)(Sagdıc, 2003):

%GIR= (PC - PT) / PC x 100

where PC and PT are control populations and the samples being treated at a given time,, respectively.

2.7. Statistical analysis

Each treatment was conducted two times. Windows-based S.A.S. version 8.2 statistical analysis software (SAS Institute Inc., Cary, NC, 1989-2019) was used for analysis. For the statistical analysis of the microbial reductions, two way analysis of variance (ANOVA) was employed. The significance of the differences in mean values was determined using Tukey's multiple range method.

3. Results and discussion

3.1. Application of hydrosol

In this study, antimicrobial activity of thyme, sage, basil and the mix of these three hydrosols and different concentration of EP were evaluated in the inhibition of *S. typhimurium* inoculated shell eggs. The initial population of *Salmonella* on shell eggs confirmed as 6.8 log CFU/g by plating on Nutrient agar for hydrosol study. Inoculated eggs treated with plant hydrosols and sterilized DI water as a control for 10, 30, 60 and 90 s. Water treatment (control) was found to be ineffective ($P>0.05$) at 10 and 30 s treatments, however it was effective after 60 s compared to untreated eggs. This reduction was statistically lower than all the hydrosol treatments ($p<0.05$). There was significant ($P<0.05$) reduction on *Salmonella* population even in 10 s treatments for all type of hydrosols (Table 1). 1.5 to 2 log reductions were observed in 30 s and all hydrosol types showed significant ($P<0.05$) difference from the control. Mixed and thyme hydrosols provided the highest log reduction (3 and 3.4 log) in 90 s that can be considered favorable for food decontamination treatments (Table 1).

Table 1. Antibacterial activity of the plant hydrosols on the *S. typhimurium** inoculated shell eggs.

	Treatment Time (s)			
Hidrosol type	**10**	**30**	**60**	**90**
Control	6.3 ± 0.07^{Aa}	5.9 ± 0.03^{Aa}	$5,5\pm0,1^{Ba}$	5.4 ± 0.2^{Ba}
Thyme	6.1 ± 0.04^{Ab}	5.4 ± 0.01^{Bc}	4.3 ± 0.05^{Cd}	3.9 ± 0.04^{Dd}
Sage	5.9 ± 0.03^{Abc}	5.6 ± 0.08^{Bbc}	4.5 ± 0.01^{Cc}	4.3 ± 0.05^{Cc}
Basil	6.1 ± 0.04^{Ab}	5.8 ± 0.05^{Bab}	5.0 ± 0.08^{Cb}	4.8 ± 0.05^{Cb}
Mixed	5.8 ± 0.03^{Ac}	4.8 ± 0.01^{Bd}	3.7 ± 0.01^{Ce}	3.4 ± 0.01^{De}

*Initial inoculation levels of *Salmonella* was 6.2 log cfu/g.

A–D Different superscript uppercase letters show significant ($P < 0.05$) differences between treatment time within the same hydrosol type.

a-e Different superscript lowercase letters show significant ($P < 0.05$) differences between hydrosol time within the same treatment time.

Tornuk et al. (2011) (Tornuk et al., 2011) studied thyme, sage, black cumin, rosemary and bay leaf hydrosol inhibitory effects against *Salmonella typhimurium* and *Escherichia coli* O157:H7 inoculated on apples and carrots.. They found that sterile tap water was ineffective in reducing *S. typhimurium* and *E. coli* O157:H7, where we found that washing was effective for eggs after 60s. The reason for this may be the surface structure of egg shells. They also demonstrated 1.1 – 1.5 log reduction for thyme hydrosol on *Salmonella* inoculated apple and carrot respectively. The maximum log reductions for sage hydrosol were 0.7 and 0.9 log. Our reduction rates are slightly higher but similarly, we observed a higher decontamination effect in thyme than sage hydrosol. Similar to our findings, Öztürk et al. (2016) (Ozturk, Tornuk, Caliskan-Aydogan, Durak, & Sagdic, 2016) achieved 3 log reduction by thyme hydrosol application for 60 mins on iceberg lettuce samples. Growth inhibition rate (GIR) of *Salmonella* obtained by hydrosols is shown in Fig 1.

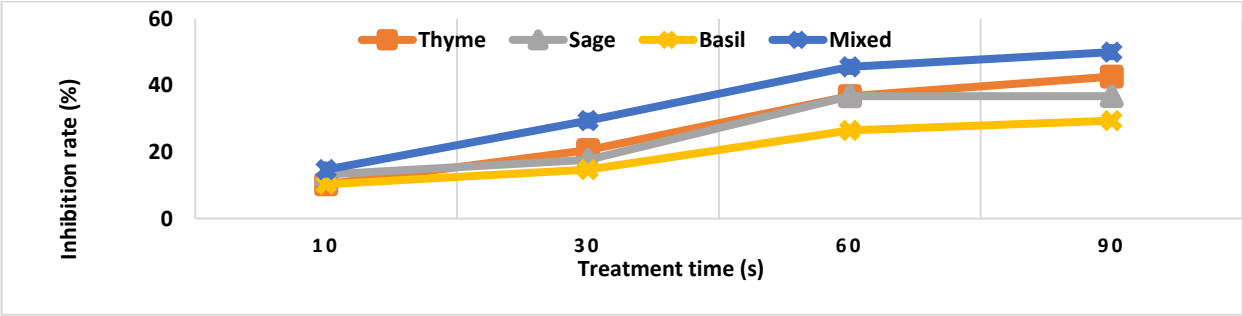

Figure 1. Growth inhibition levels of S.Typhimirum on shell eggs samples by treatments of several plant hydrosols

This figure clearly shows the effect of treatment time and hydrosol types on *Salmonella* inhibition efficiency. Mixed hydrosol reached the highest inhibition rate with 50% at 90 s that is followed by thyme hydrosol by 42%. The lowest inhibition rate belongs to basil hydrosol with 29%. Sage hydrosol inhibited same rate of Salmonella at 60 and 90 s treatments which was around 37%.

3.2. Application of EP

Table 2 shows the effect of different concentrations (100µL, 500 µL and 1000 µL) of EP on Salmonella inactivation for 3, 5 and 7 days at ambient and refrigerator conditions. The initial population of *Salmonella* on shell eggs was 6.2 log CFU/g. Control and EP-treated eggs were monitored over 7 days. Almost the complete inhibition was observed with 1000 µL of EP at 7 days of storage at ambient temperature (Table 2).

Table 2. Antibacterial actıvıty of the EP on the *S. typhimurium** inoculated shell eggs at 20°C and 4°C

20 °C Growth ınhıbıtıon level (log cfu/g)				
Storage Time (Days)				
EP Concentrations (µL)	**1**	**3**	**5**	**7**
Control	0.4 ± 0.08^{ABb}	0.3 ± 0.01^{Bd}	0.6 ± 0.04^{Ad}	0.3 ± 0.01^{Bd}
250µl	0.3 ± 0.01^{Db}	1.0 ± 0.11^{Cc}	1.3 ± 0.09^{Bc}	1.8 ± 0.11^{Ac}
500µl	0.6 ± 0.02^{Db}	1.4 ± 0.01^{Cb}	2.3 ± 0.04^{Bb}	2.8 ± 0.04^{Ab}
1000µl	2.6 ± 0.15^{Da}	3.0 ± 0.11^{Ca}	3.5 ± 0.06^{Ba}	5.4 ± 0.01^{Aa}
4 °C Growth ınhıbıtıon level (log cfu/g)				
Storage Time (Days)				
EP Concentrations (µL)	**1**	**3**	**5**	**7**
Control	0.2 ± 0.01^{Bd}	0.4 ± 0.03^{Ad}	0.5 ± 0.01^{Ad}	0.4 ± 0.03^{Ad}
250µl	0.3 ± 0.01^{Cb}	0.8 ± 0.01^{BCc}	0.9 ± 0.12^{Bc}	1.3 ± 0.05^{Ac}
500µl	0.5 ± 0.01^{Dc}	1 ± 0.01^{Cb}	1.7 ± 0.02^{Bb}	2.3 ± 0.01^{Ab}
1000µl	2.2 ± 0.03^{Da}	2.6 ± 0.03^{Ca}	3 ± 0.42^{Ba}	4.2 ± 0.2^{Aa}

*Initial inoculation levels of *Salmonella* was 6.2 log cfu/g.

A-D Different superscript uppercase letters show significant ($P < 0.05$) differences between treatment time within the same EP concentration.

a-d Different superscript lowercase letters show significant ($P < 0.05$) differences between EP concentration within the same treatment time.

One day storage of eggs with 250 ve 500 µL EP had no statistical difference (P>0.05) from the control sample at 20 °C. On the other hand, 1000 µL of EP treatment was effective even at 1 day storage. Tornuk and Durak (2015) evaluated EP efficiency on high and low inoculation levels of *E.coli* O157:H7 and *S.aureus* inoculated parsleys. Similar to our findings, 1000 µL EP treatments completely inhibited the *E.coli* O157:H7 population and enabled 100% growth inhibition levels at room temperature. At refrigerator temperatures, the maximum log reduction was 4.2 after 7 days of storage. There was a significant (P<0.05) effect of EP concentration for all levels. After 5 days of storage, 3.5 and 2.3 log cfu/g reduction was observed at 1000 and 500 µL EP concentrations, respectively. Durak et al. (2012), assessed the effectiveness of EP on *E. coli* O157:H7 inoculated green onions and spinach at 4 and 10 °C. They observed full inhibition at 7th days for 4 °C and for 5th days for 10 °C at 420 g/L concentration of EP. Similar to our study, they also observed faster decontamination at higher temperatures as expected. GIR of *Salmonella* obtained by EP treatments are shown in Fig 2 and Fig 3.

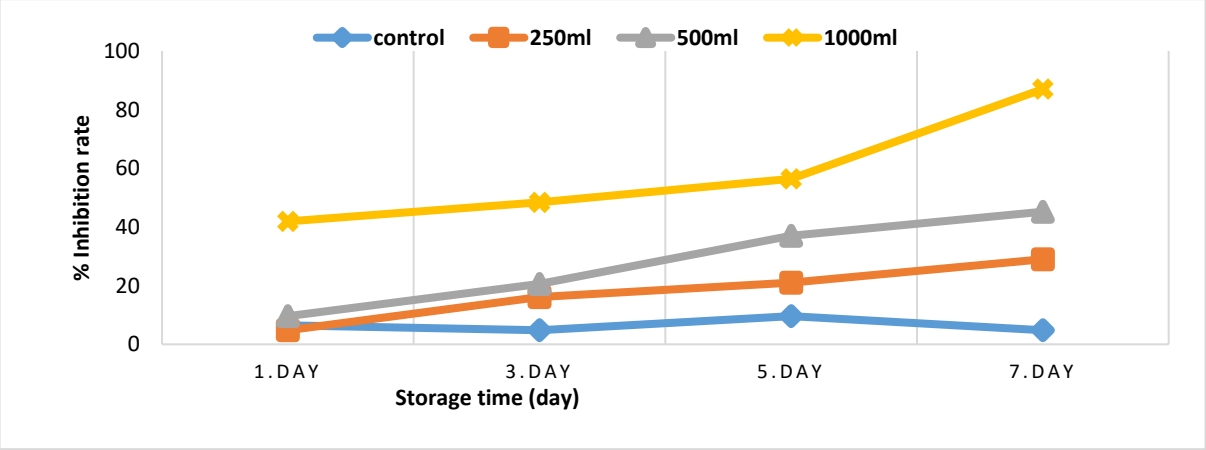

Figure 2. Growth inhibition levels of S.Typhimirum on shell eggs samples by treatments of EP at ambient temperature

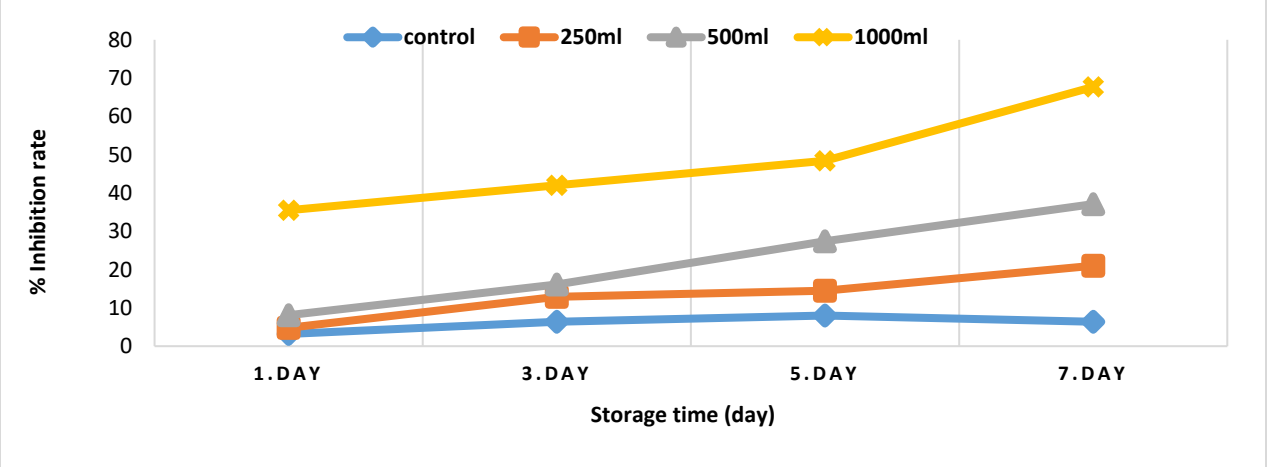

Figure 3. Growth inhibition levels of S.Typhimirum on shell eggs samples by treatments of EP at refrigerator temperature.

The concentration dependence of the inhibition levels can be seen from these figures. While the inhibition rates were under 50% for 100, 250 and 500 μL of EP concentrations; the inhibition rate at 1000 μL of EP was distinctly higher from the others ($P<0.05$), for both temperatures.

4.Conclusions

The results obtained in this study demonstrated that vaporized EP treatments had high antimicrobial activity against *S. typhimurium* on shell eggs, and EP is already recognized as GRAS for human consumption. Plant hydrosol treatment is another natural method that promises surface decontamination of shell eggs. Compare to EP, hydrosol treatments had lower inhibition rates of Salmonella. However, especially mixed and thyme hydrosols reached 50% of inhibition rate. For further studies, different types of plant hydrosols could be used to decontaminate egg shells. Also, since hydrosol treatment maintains limited decontamination levels compared to others, it can be used in combination with another decontamination method.

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IRRADIATION INDUCED CHANGES IN THE *TRANS* FATTY ACID CONTENT AND PHYSICOCHEMICAL PROPERTIES OF SELECTED OILS

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ABSTRACT

Among the existing technologies for food preservation, irradiation of food is recognized as a safe and effective method for a range of specific applications. Although through studies it was found that irradiation may bring biochemical changes in the food commodity. One such potential processing hazard formed due to irradiation is the generation of trans fatty acids. The current study was done to observe the effect of irradiation on trans fatty acid and other physicochemical changes in oils. In the present study, potato fingers were deep-fried separately in 3 different oils primarily consumed in India viz. soybean oil, mustard oil, and palm oil. The fried samples were then irradiated at 2,4 and 6 KGy doses and the effect of different doses of irradiation on the physicochemical properties with special reference to trans fat formation in all the selected oils were studied. Trans fat isomers observed in this study are Palmitelaidic acid (PA) C16:0, Elaidic acid (EA) C18:1t9, Vaccenic acid (VA) (C18:1t11), Linoleic acid (LA) C18:2, and Linolenic acid (LLA) C18:3, other physiochemical properties analyzed are peroxide value, anisidine value, TBA value, free fatty acid, iodine value, and total polar compound. It was observed that irradiation of lipids may lead to the formation of free radicals which may affect its properties. The changes occurring due to irradiation are found to be proportional to the linoleic and linolenic acid content of the oil. Irradiation also found to induce changes in the oxidative parameters of oils tested like peroxide value , anisidine value etc.

1.Introduction

Irradiation is a technology that improves the safety and extends the shelf life of foods by reducing or eliminating microorganisms. Ionizing radiation uses the high energy of gamma rays or accelerated electrons, thereby ionizing molecules. The use of this treatment on food could extend shelf life and protect the host against pathogenic bacteria. Currently, irradiation is permitted by USDA, FSIS, and U.S. FDA at doses up to 4.5 kGy for treating refrigerated, uncooked meat and meat by-products (FSIS 1999). On the other hand, irradiation treatment brings about some biochemical changes that could affect the nutritional adequacy of food (Giroux and Lacroix, 1998). FDA has permitted the irradiation of a number of foods like beef, pork, spices, eggshell, sprouts and fresh fruits and vegetables etc. Although through studies it was found that irradiation may bring biochemical changes in the food commodity. One such potential processing hazard formed due to irradiation is the generation of *trans* fatty acids. *Trans* fatty acids (TFA) are the geometrical isomers of unsaturated fatty acids with at least one non-conjugated, carbon-carbon double bond in the *trans* configuration rather than the more common *cis* configuration (Codex, 1985; EFSA, 2004; Kodali, 2005). Consumption of *trans* fatty

acid in diet may lead to various health issues like cardiovascular disease, obesity, coronary heart diseases, prostrate cancer, breast cancer, etc. It may also affect the cell membrane and auto immune system and can cause damage to brain cells (Zhu *et al.*, 2019). Hence it is required to minimize the intake of *trans* fatty acids, which are not indispensable to humans. Other than *trans* fat formation irradiation may also induce changes in the physiochemical properties of fat generating free radical or causing oxidation. Hence to obtain a comparative study on the extend of degradation in different oils; palm, soybean and mustard/rapeseed oil were selected which together account for 75% of the total edible oil demand, with respective shares of 46%, 16% and 14%.respectively. The effect of irradiation on the physiochemical properties of selected oils was discussed with reference to their fatty acid chain length and the degree of unsaturation.

2.Materials and Methods

Irradiation treatments were given to oil samples for dose of 2, 4, and 6 KGy and the processed samples were then analysed for *trans* fatty acid, peroxide value, anisidine value, TBA value, free fatty acid, iodine value and total polar compound.

*Irradiation of oils were done at DFRL, Mysore. *Trans* fat and other quality parameters were analyzed at CFT, University of Allahabad.

2.1. Reagents and standards

All chemicals, solvents and reagents employed were of analytical grade and purchased from Merck (India). The internal standard (IS) pentadecanoic acid (C15:0) and the individual of five Fatty acids [FA] and Fatty Acid Methyl Esters [FAME] standards: Palmitelaidic acid (PA) C16:0, Elaidic acid (EA) C18:1t9, Vaccenic acid (VA) (C18:1t11), Linoleic acid isomer mix (LA) C18:2, and Linolenic acid isomer mix (LLA) C18:3, were purchased from Sigma-Aldrich (INDIA) (purity; ≥99.99% (GC). The esterifying catalyst Boron Trifloride and solvent heptane were also purchased from Sigma (Sigma–Aldrich, India).

2.2. Irradiation:

Irradiation was conducted using the Co-60 gamma-radiation source and silver dichromate, which was used as a dosimeter, at the Defence Food Research Laboratory, Mysore. The applied radiation doses were 2, 4, and 6 kGy by Gamma rays in γ-chamber 5000 at the dose rate of 3.568 KGy/hr. In order to study the effect of Irradiation on *trans* fat formation 100 ml oil (soybean, mustard and palm oil) was packed and sealed in an HDPE envelop separately and it was then placed inside the irradiation chamber where they were exposed to irradiation at 2, 4 and 6 KGy at ambient temperature.(Yilmaz, 2007)

2.3. Sample preparation

Approximately 0.2 g of oil sample was *trans*ferred to the flask, 10 ml of 1.0 N methanolic NaOH was added and a known concentration of Internal Standard was added to the flask, which was then refluxed for 10 min. About 5 ml of 14 % methanolic boron trifloride (BF3/MeOH) was added and refluxed for an additional 2 min. About 5 ml n-heptane was added to the flask through condenser and then allowed to cool. Organic layer was then separated with centrifugation after adding 10 ml concentrated NaCl solution. About 1.0 ml of the top layer was *trans*ferred into a 10-ml stoppered glass tube using a *trans*fer pipette, and then the sample was diluted to the mark (10 ml) with n-heptane (AOAC , 2001)

2.4. Gas Chromatograph analysis of FAME

FAMEs were analyzed using a GC Clarus 500 Chromatograph (Perkin Elmer, India) equipped with a fused silica capillary column SP-2560 (with column length- 30 m and internal diameter 320 µm) and flame ionization detector [FID]. High-purity nitrogen (99.999 %) was used as the carrier gas with a set flow rate of 1 ml/min and hydrogen and zero air was used as

fuel gas with flow rates 45 ml/min and 450 ml/min respectively. The oven temperature program was as follows: 4 min at 130 ºC, increased/ramped by 2.5 ºC/min up to 240 ºC, and then further ramped at the rate of 5.0 ºC up to 260 ºC held for 20 min. The injector and detector temperatures were 220 and 280 ºC, respectively. The injection volume was 2 µl in split less mode (Modified AOAC, 2001).

For Determination of Peroxide value of oil/fat IS: 548-Part 1, 1964 method was used, while for Free fatty acid [FFA] analysis of oil /fat method used was IS: 548-Part 1, 1964.

2.5. Methods for determination of quality parameters of oil / fat

2.5.1. Determination of Free fatty acid (FFA) of oil /fat :

For analysis of FFA 5 gm sample was taken in a conical flask and to the flask 50 ml of 95% ethyl alcohol (neutralized by adding 0.1N NaOH drop wise) was added. Oil was dissolved in the ethanol and kept on hot plate for boiling. Sample was boiled till all the oil droplets get completely dissolved. 2-3 drops of phenolphthalein indicator was added and the sample was then titrated with 0.1N NaOH (while still hot) till a faint pink color appear in the sample that should last for at least 15 sec in the sample (IS: 548-Part 1a, 1964)

Calculation:

Free fatty acid % = 28.5× V×N /W (1)

Where, V= Volume in ml of standard sodium hydroxide required for titration

N= Normality of standard sodium hydroxide used in titration

W= Weight in ‘g’ of the sample taken for estimation.

2.5.2. Determination of Total polar compound in oil/fat:

2.5g of clean and dry sample was weighed in 50 ml volumetric and 20 ml ether solvent was added to it (87:13). The solution was warmed to dissolve the fat/oil completely and the volume was make up with ether solution. 20 ml of this solution was poured at the top of the glass column slowly with the help of a pipette. The drain was collected in clean and dry conical (pre-weighted). The non-polar components were eluted from the column by draining 150 ml ether solvent slowly. The flow should be slow that it takes 1 hour to elute the solvent completely. After the solvent is eluted completely, the flask was kept in hot air oven to evaporate the solvent. The flask was cooled and the weight of flask was measured (IUPAC, 1992).

*To prepare ether solution for elution, petroleum ether and diethyl ether was mixed in ratio 87:13.

Calculation:

TPC = 1- (weight of flask after evaporation of solvent- initial weight of flask) (2)

2.5.3. Determination of Peroxide value of oil/fat :

Approximately 0.2 ml of sample was taken in a boiling tube. To the sample 9.9 ml of chloroform/methanol (7:3 v/v) was added and swirled to dissolve the sample. Then 50 µl of 10 mM xylenol orange was added and mixed. 50 µl of Fe (II) Chloride solution was added and kept for 5 mins. Absorption was determined at 560 nm wavelength with U.V-Vis spectrophotometer. Standard curve was plotted using standard Fe (III) chloride instead of oil at 0, 1 and 2 ml concentration (IS: 548-Part 1b, 1964)

Calculation:

P.V. = [(A1- A2)×inverse of slope× 55.84 × 2 / weight of sample (3)

Where A1= sample absorbance

A2= blank absorbance

* Xylenol orange solution- 0.019 gm of xylenol orange was dissolved in 25 ml of water.

*Ferrous (Fe II) chloride solution: dissolve 0.5g ferrous sulphate ($FeSO_4.7H_2O$) was dissolved in

50 ml water and 0.4 g barium chloride ($BaCl_2.2H_2O$) in 50 ml water. The 2 solutions were mixed and add 2 ml of 10M HCl was added to it. Now filter out $BaSO_4$ and store the solution in brown bottle.

*Ferric (Fe III) chloride solution (to be used as a standard of 10µg/ml): 0.5g ferric chloride was dissolved in 50ml of 10 M HCl and 2 ml of 30% hydrogen peroxide solution was added to it. The solution is boiled for 5 minutes to remove excess of H_2O_2. Cooled and diluted to 500 ml with distilled water. 1 ml of the prepared reagent was diluted to 100 ml with chloroform/methanol (7:3 v/v) solution.

2.5.4. Determination of Anisidine Value of oil/fat:

Approximately 0.5 gm oil sample was weighed in 25 ml volumetric and volume made up with iso-octane. Then it was mixed thoroughly to dissolve the sample. Absorbance measured at 350nm wavelength against pure iso-octane. After measuring the absorbance 1 ml of anisidine reagent (0.25% w/v) was added to the same sample solution. Tube was then stoppered and kept in dark for 10 min. Absorbance again measured at 350 nm wavelength against pure iso-octane as reference (IS: 548-Part 1b, 1964)

* To prepare anisidine reagent dissolve 0.25gm of para-anisidine was dissolved in 100 ml acetic acid (AR grade)

Calculation:

A.V. = 37.5× [A1- A2) / W. (4)

Where ,

A1 =Absorbance of fat/oil with anisidine

A2= Absorbance of fat/oil without anisidine]

W = Weight of sample

2.5.5. Determination of Conjugated diene value of oil/fat :

Hydroperoxides from PUFAs form conjugated dienes that can be measured quantitatively by spectrophotometric UV measurement at wavelength 232 nm. To measure conjugated diene value, 0.5 gm of sample (melted and filtered to remove suspended impurity) was weighed and then diluted to 50 with iso-octane in volumetric flask. Sample was dissolved completely with shaking and then absorbance was taken at 232-262-268-274 nm wavelengths with pure iso-octane as reference/blank. The absorption value 'indicated by D' read on the spectrophotometer and with K indicated as the specific absorption value (IS: 548-Part 1b, 1964)

Calculation:

The K value is obtained from the equation:

$K = D / C \times S$ (5)

Where, C= solution concentration ing/L (10g/L)

S= cuvette thickness in centimetre.

ΔK value is determined as follows,

ΔK = K268- (K262 + K274 / 2)

* The conjugated diene value is based on the detected absorbance and is expressed as µmol hydroperoxides /g sample.

2.5.6. Determination of Iodine Value of oil /fat

Approximately 0.2 gm of dried and filtered oil sample was weighed into 250 ml stopper conical flask. In the flask 25 ml of carbon tetrachloride was added and agitated to dissolve sample. Further 25 ml of wij's iodine solution was added. The flask was then immediately stoppered, swirled to mix and kept in dark for 1 hr. After 1 hr conical was taken out and 15 ml of 10% potassium iodide solution was added to it. Immediately 100 ml of CO_2 free water (boiled and cooled) was added and the sample was then titrated with 0.1 N standard sodium thiosulphate solution using starch indicator (AOAC 993.20).

Calculation:

I.V. = 12.69 (Blank T.V.– Sample T.V.) × N of $Na_2S_2O_3$ / Weight of sample. (6)

2.6. Statistical analysis:

All values were shown as the means and standard deviations for three replicates. The statistical analyses were performed using the ANOVA function of IBM SPSS Statistics 20.0 for Windows software. Statistical significance was determined as $p<0.05$ using Duncan's multiple range test. Coefficient of variance was calculated for free fatty acid, peroxide value, total polar compound, anisidine value, iodine value and conjugated diene value. All the graphs were plotted on Sigma plot 10.0 software.

3.Results and discussion

3.1.Effect of Irradiation on TFA content in selected oils

Irradiation of samples was done at the Defence Food Research Laboratory, Mysore. The applied radiation doses were 2, 4, and 6 kGy by Gamma rays in γ-chamber 5000 at the dose rate of 3.568 KGy/hr. In order to study the effect of Irradiation on *trans* fat formation soybean, mustard and palm oil and were processed at 2, 4 and 6 KGy at ambient temperature. **Table 1 and 2** shows the content of all *trans* isomers in soybean, mustard and palm oil, respectively, irradiated at doses between 0 and 6 KGy **(Figure 1).** In the present study, the amount of total fatty acids was affected by irradiation, mainly because of the reduction in unsaturated fatty acids (*cis*-UFA). Slight but statistically significant changes ($P<0.05$) of the main polyunsaturated *trans* fatty acids such as C18:2-9c,12c, C18:1-9n were observed in processed samples for each of the 3 oils selected, irradiated at 4 KGy compared with the control sample, at a dose of 2 KGy, no significant change was observed in *trans* fatty acid content. Hence it can be concluded that irradiation induced the formation of TFAs in food when the absorbed dose exceeded 4 KGy. Among the three oils selected for study, maximum increase was observed in soybean oil followed by palm oil and then mustard oil (GC chromatogram of palm oil before and after irradiation treatment is shown in **Figure 2**). Brito et.al (2002) and Yilmaz (2007) also reported that gamma-irradiation leads to an increase in the amount of *trans* fatty acid in food, particularly elaidic acid (C18:1-9t). However in the present study trans isomerization is found more in linolenic acid. Although irradiation caused the increase of *trans* fatty acids, the total amounts of all *trans* isomers were no more than 2%, which is the *trans* fat limit in some countries like Denmark.

Table 1. Effect of irradiation on TFA content in different oils

	Soybean oil	**Mustard oil**	**Palm oil**
Fresh oil	0.63 ± 0.09^{a}	0.88 ± 0.11^{a}	0.93 ± 0.12^{a}
2 KGy	0.67 ± 0.12^{a}	0.91 ± 0.13^{a}	0.94 ± 0.14^{a}
4 KGy	0.78 ± 0.16^{ab}	0.96 ± 0.13^{ab}	0.99 ± 0.13^{b}
6 KGy	0.89 ± 0.11^{b}	1.03 ± 0.14^{bc}	1.07 ± 0.11^{bc}
F-VALUE	2.809	0.847	0.979

*Values with different superscript in the same column differ significantly ($p\leq0.05$)

*All data are expressed as mean±s.d. (n=3)

Table 2. Changes in *trans* fatty acid composition of selected fats/oils during irradiation

Sample	Linoleic acid methyl ester (%)	Linolenic acid methyl ester (%)	Elaidic acid methyl ester (%)	Vaccenic acid methyl ester (%)	Palmitelaidic acid methyl ester (%)	*Trans* fat % in product
Soybean control	0.14±0.02	0.32±0.02	0.15±0.08	ND.	ND.	0.63±0.14[a]
Soybean 2 KGy	0.15±0.07	0.34±0.03	0.16±0.03	ND.	ND.	0.67±0.12[a]
Soybean 4 KGy	0.20±0.09	0.41±0.07	0.27±0.03	ND.	ND.	0.88±0.26[ab]
Soybean 6KGy	0.14±0.04	0.37±0.03	0.16±0.03	ND.	ND.	0.69±0.11[b]
Mustard control	0.13±0.06	0.39±0.05	0.33±0.05	ND.	0.03±0.01	0.88±0.13[a]
Mustard 2 KGy	0.15±0.04	0.41±0.07	0.35±0.01	ND.	0.02±0.01	0.91 ±0.16[a]
Mustard 4 KGy	0.14±0.06	0.44±0.03	0.38±0.02	ND.	0.03±0.03	0.96±0.13[ab]
Mustard 6 KGy	0.15±0.08	0.46±0.02	0.38±0.03	ND.	0.02±0.03	0.98±0.14[bc]
Palm control	0.20±0.4	0.15±0.06	0.45±0.02	ND.	0.13±0.05	0.93±0.21[a]
Palm 2 KGy	0.18±0.1	0.14±0.02	0.48±0.04	ND.	0.13±0.05	0.94±0.19[a]
Palm 4 KGy	0.19±0.2	0.15±0.01	0.49±0.03	ND.	0.15±0.05	0.99±0.17[b]
Palm 6KGy	0.19±0.2	0.16±0.03	0.52±0.05	ND.	0.16±0.05	1.04±0.11[bc]

*All data are expressed as mean±s.d. (n=3)

*Values with different superscript in the same column differ significantly ($p \leq 0.05$)

The formation of *trans* configuration has been confirmed to occur during the irradiation of *cis* fatty acids. Brito et.al (2002) observed an average increase of 80.4 % in TFA amount when they evaluated the effect of TFA content in fresh bovine meat irradiated with doses between 1–5 kGy. Brito et.al (2002) found that although, the gamma radiation has been an excellent method to conserve meat, the molecular structure of this meat were changed during irradiation. Other studies have shown that *cis–trans*-isomerization of unsaturated fatty acids occurs during gamma irradiation of barley grains (Geibler, 2003) and frankfurters (Fan, 2009). More recently, Yilmaz and Gecgel C. (2013) also found significant increases in *trans* fatty acid content of ground beef at doses of 3 kGy or above. At 5 kGy, total *trans* fatty acid content was increased from 7.0%

to 9.4%, a 34% increase. On contrary Chen (2007) found no significant effect of irradiation (0 to 3.2 kGy) on the C18:1 *trans* content of the total lipid fraction of beef semi-tenderness muscle; however, some changes occurred in the much lower concentrations of C16:1 *trans* and C18:2 *cis*, *trans* content of the neutral and polar lipid fractions. Wang *et. al* (2012) reported that irradiation led to a significant increase of total *trans* fatty acid content in ground beef and liquid egg with an absorbed dose range between 6.743 and 11.472 kGy (P\0.05). The change in C18:1-9t content was the most significant compared with other *trans* fatty acids. Irradiation of barley grains (Geibler, 2003) with a dose of 10 kGy resulted in no measurable *trans* isomers.

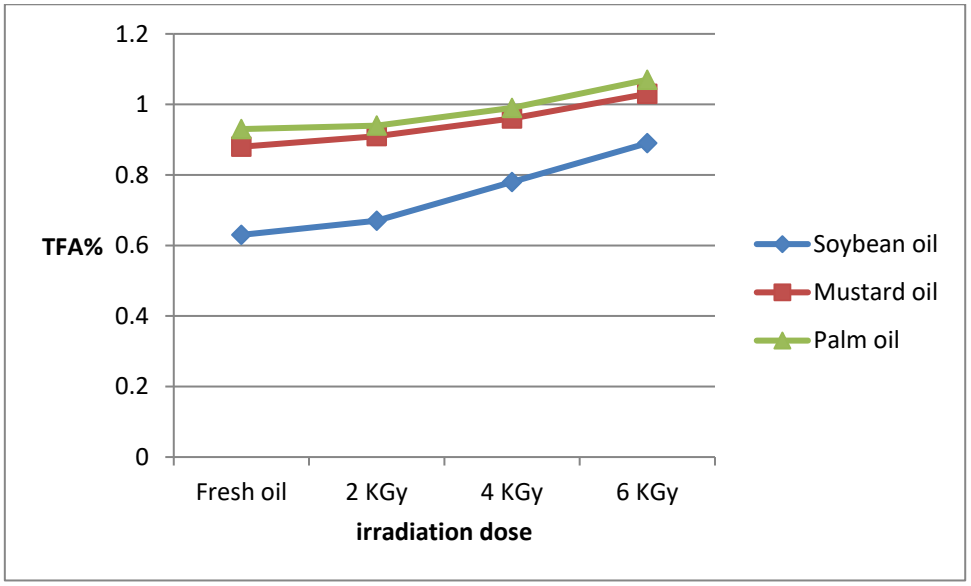


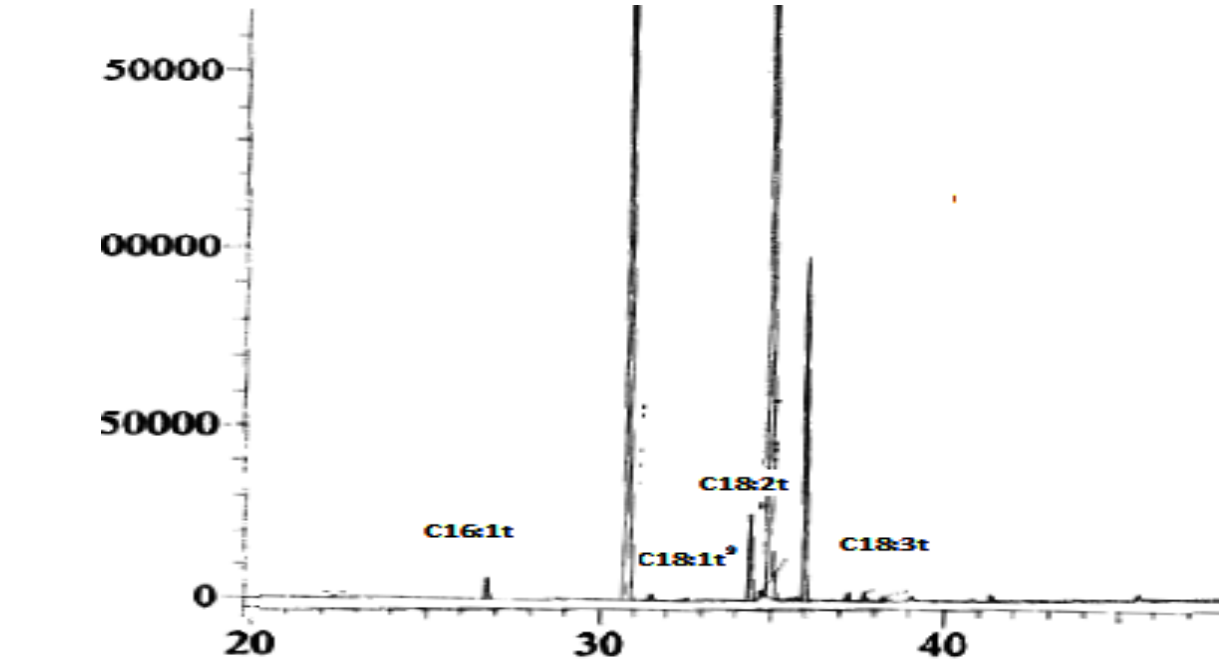
Figure 1. *Trans* fatty acid content in selected oils at different irradiation dose

Food irradiation is a promising technology that has been commercially established because it can effectively extend food shelf-life, control pathogenic bacteria, and delay the ripening or maturation of certain fruits or vegetables (Prakash 2016, Kume *et. al*, 2009). WHO, IAEA, and FAO consider food irradiated with doses of up to 10 kGy safe for consumption (Joint, 1981). The high energy of ionizing radiation may cause chemical modifications in the lipid fraction, which results in radical formation. The *cis*/*trans* isomerization of carbon double bonds has been reported to occur in vivo by a free radical attack against the cell membrane (Ferreri et.al.2004).

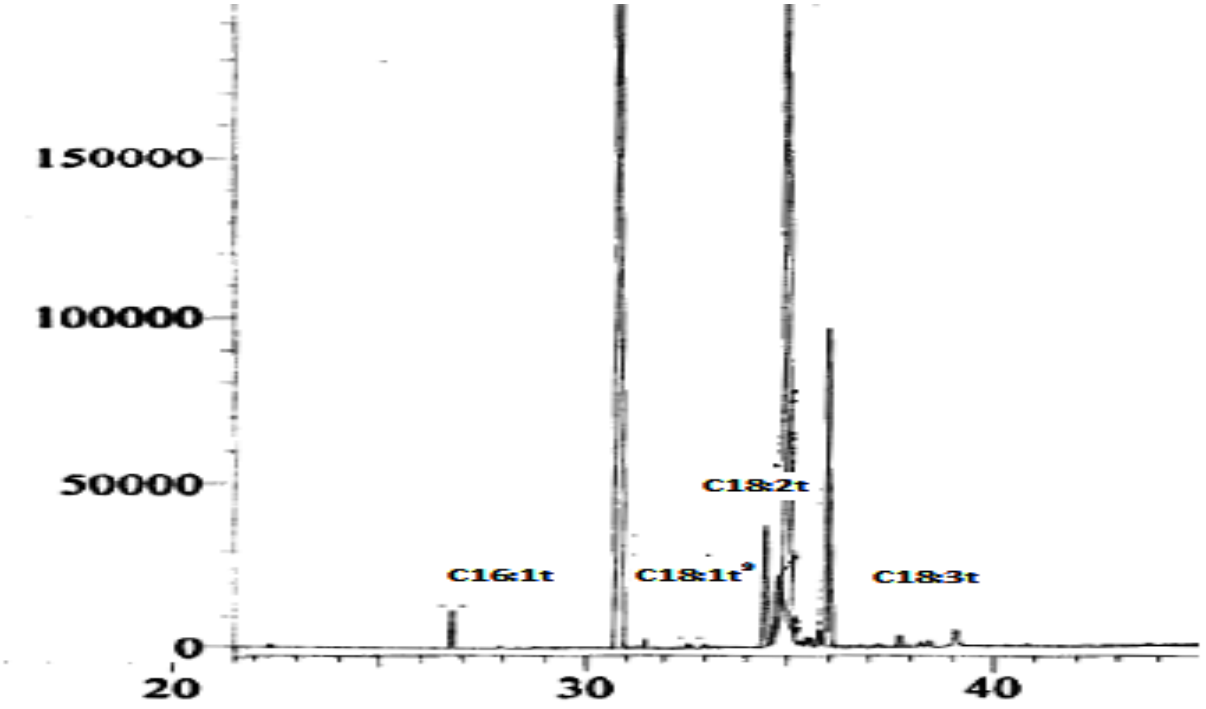
The formation mechanism of TFA due to irradiation was quite different from that during hydrogenation. A radical-catalyzed isomerization of carbon–carbon double bonds in unsaturated fatty acids is reasonable for this case. XH was converted into X via hydrogen abstraction because of gamma-irradiation. The isomerization process occurred between the radical adduct X and a double bond of the fatty acid, and then *trans* isomers were formed via a β-elimination of X (Chatgilialoglu 2002). XH can take the form of compounds such as RSH or RSSH in food, as established in previous studies (Zambonin et.al.2006).

Minami (2012) studied the effect of gamma radiation on soybean and soybean oil. Irradiation at 10 to 80 KGy under anaerobic conditions did not markedly changes the fatty acid composition. While 10 KGy radiation does not affect the fatty acid composition even under aerobic condition while 40 KGy irradiation considerably altered the fatty acid composition under aerobic condition. Moreover 40 KGy irradiation produces significant amount of *trans* fatty acid under aerobic condition, but not under

anaerobic conditions. Irradiating soybean oil induced lipid per-oxidation and reduced the radical scavenging activity under the anaerobic condition.



Palm oil- Fresh



Palm oil- After Irradiation

Figure 2. GC chromatogram of Palm oil before and after irradiation (6 KGy) treatment

3.2.Free fatty acid (FFA)

Free fatty acid is the relative measure of rancidity as FFA is normally formed during the decomposition of oil glycerides due to the action of moisture, temperature and/or lipolytic enzyme lipase. Free fatty acids increase the thermal oxidation of oils, and their unsaturation rather than chain length led to significant effects on thermo-oxidative degeneration. The effect of Irradiation on free fatty acid value is given in **Table 3** and a very slight significant change was observed in the FFA content of oils with maximum increase for soybean oil (with co-efficient of variance 0.0888).

Table 3. Effect of Irradiation on free fatty acid value of selected oils

Processing	SOYBEAN OIL	MUSTARD OIL	PALM OIL
Fresh sample	0.18±0.06	0.35±0.21	0.29±0.12
2 KGy	0.19±0.05	0.3±0.11	0.32±0.08
4 KGy	0.19±0.09	0.34±0.17	0.3±0.09
6 KGy	0.22±0.14	0.36±0.15	0.35±0.05
C.V.	0.088823	0.077925	0.083992

*Free fatty acid expressed as % of oleic acid.

*All data are expressed as mean±s.d. (n=3)

3.3. Total polar compound

Total polar compound of the fresh oil samples selected for the study are 0.11±0.03, 0.08±0.04 and 0.11±0.02 (TPC expressed as % by weight) for soybean oil, mustard oil and palm oil respectively. The effect of Irradiation on total polar compound (TPC) is given in **Table 4.** Most significant increase in TPC was observed for mustard oil followed by palm oil and then soybean oil indicating more degradation of triglycerides in mustard oil.

Table 4. Effect of Irradiation on TPC value of selected oils

Processing	SOYBEAN OIL	MUSTARD OIL	PALM OIL
Fresh sample	0.11±0.03	0.08±0.03	0.11±0.05
2 KGy	0.12±0.02	0.1±0.02	0.12±0.06
4 KGy	0.12±0.05	0.11±0.05	0.14±0.06
6 KGy	0.10±0.02	0.1±0.02	0.12±0.04
C.V.	0.085105	0.129057	0.102719

* TPC expressed as % by weight

*All data are expressed as mean±s.d. (n=3)

3.4. Peroxide value

The peroxide value is defined as the amount of peroxide oxygen per 1 kilogram of fat or oil. It gives a measure of the extent to which an oil sample has undergone primary oxidation, Peroxide value of the fresh oil samples selected for the study are 0.8±0.05 mEq/Kg, 0.92±0.11 mEq/Kg and 0.83±0.08 mEq/Kg for soybean oil, mustard oil and palm oil, respectively. A very significant change was observed in peroxide value of oils after irradiation treatment **(Table 5).** Maximum peroxides were generated in soybean oil followed by mustard oil and palm oil. The result observed in this study is found to be in agreement to other findings[15], showing the oxidation of lipids resulting in formation of peroxides. These peroxides may further undergo secondary oxidation thus forming conjugated dienes.

Table 5. Effect of Irradiation on Peroxide value of selected oils

Processing	**SOYBEAN OIL**	**MUSTARD OIL**	**PALM OIL**
Fresh sample	0.81±0.17	0.92±0.21	0.80±0.18
2 KGy	0.83±0.14	0.94±0.18	0.81±0.16
4 KGy	0.88±0.13	0.97±0.22	0.84±0.14
6 KGy	0.96±0.11	0.99±0.28	0.82±0.09
C.V.	0.061415	0.033422	0.020638

*Peroxide value expressed as mEq/Kg of oil

*All data are expressed as mean±s.d. (n=3)

Table 6. Effect of Irradiation on Anisidine value of selected oils

Processing	**SOYBEAN OIL**	**MUSTARD OIL**	**PALM OIL**
Fresh sample	2.13±0.65	7.68±1.05	4.83±0.97
2 KGy	2.43±0.43	7.65±0.99	4.97±0.05
4 KGy	2.51±0.19	7.78±1.32	5.07±0.65
6 KGy	2.64±0.43	7.97±1.08	5.21±0.82
C.V.	0.089141	0.030284	0.011828

*All data are expressed as mean±s.d. (n=3)

3.5. Anisidine value

p-Anisidine reacts with secondary oxidation products formed by combination of free radicals with O2 generating hydroperoxides such as aldehydes and ketones in fats and oils to form products that absorb at 350 nm wavelength of light. It is particularly good at detecting unsaturated aldehydes, which are the ones that are most likely to generate unacceptable flavors. Different processing treatments lead to a variety of chemical reactions which can be categorized as hydrolysis, oxidation, and polymerization of the triacylglycerol molecule. The decomposition products formed by these processes may be volatile or nonvolatile and undergo further degradation. Hydrogenated soybean oil with 0.1% linolenic acid had more hydrolytic degradation, but lower p-anisidine values and

polymer formation, than the soybean oil with 2.3% linolenic acid (Tompkin, 2004). **Table 6** showing the effect of irradiation on anisidine value indicates most significant increase in soybean oil after processing. The mustard oil is having more complex fatty acids and palm oil have comparatively lesser unsaturated fatty acids hence among the three oils soybean oil is found to undergo oxidative changes significantly more compared to other two oils.

3.6. Iodine value

The Iodine value (IV) of an oil/fat is the number of grams of iodine absorbed by 100 g of the oil/fat, when determined by using wiij's solution. The iodine value is a measure of the amount of unsaturation (number of double bonds) in fat/oil. During heat treatment, a progressive decrease in unsaturation was observed in all oils by measurement of IV. This decrease can be attributed to the destruction of double bonds by oxidation, s*cis*sion, and polymerization (Cowan, 1954; Cuesta, 1993). It was followed that the more saturated the oil is the lesser is the degradation of oil into secondary metabolites**.**

As shown, the highest significant ($p < 0.05$) change in the IV was shown by the mustard oil and soybean oil, thus indicating that the highest decrease in double bonds occurred due to oxidative rancidity in the proposed media **(Table 7).** This observation could be due to the presence of a high amount of PUFAs in oil.The greater the degree of unsaturation (or high IV), the more rapid the oil tends to be oxidized, particularly during deep-fat frying. Abbas (2016), reported that iodine value (IV) for microwave heated palm oil, sunflower oil and blend of palm-sunflower oil gradually decreased with increasing heating times. The reduction in IV was highest in sunflower oil (5.73) and lowest in palm-sunflower oil blend (4.11).

Table 7. Effect of Irradiation on Iodine value of selected oils

Processing	**SOYBEAN OIL**	**MUSTARD OIL**	**PALM OIL**
Fresh sample	126±4.29	102±3.09	48±2.07
2 KGy	125±4.17	101±3.69	48±1.78
4 KGy	125±3.98	101±2.74	47±1.43
6 KGy	125±4.21	100±2.18	47±1.84
C.V.	0.006532	0.009456	0.012155

*All data are expressed as mean±s.d. (n=3)

3.7. Conjugated diene

The ultraviolet spectrophotometric analysis of oil indicates the degree of oxidation, being its value expressed as specific extinction co-efficients. The K_{232} value is indicative of carbonyl compounds present in oil. The maximum permitted value of K_{232} in edible oil is 2.50 (Malheiro 2009). A significantly negative co-relation was found for irradiation treatments for increase in K_{232} value.

Absorbitivity of fresh oil sample for K-232 value are 0.21±0.10, 0.24±0.08, and 0.17±0.05 for soybean oil, mustard oil, palm oil respectively. If the increase in conjugated diene was observed for all the processing treatment, it was found that coefficient of variation for diene value during irradiation treatment ranged from 0.0688 to 0.0404 only with no significant change **(Table 8).**

Table 8. Effect of Irradiation on Diene value of selected oils

Processing	SOYBEAN OIL	MUSTARD OIL	PALM OIL
Fresh sample	0.21±0.07	0.24±0.05	0.16±0.05
2 KGy	0.21±0.04	0.26±0.02	0.18±0.06
4 KGy	0.23±0.09	0.26±0.09	0.20±0.02
6 KGy	0.25±0.05	0.27±0.06	0.21±0.10
C.V.	0.068823	0.053422	0.040421

*All data are expressed as mean±s.d. (n=3)

Gecgel (2013), studied Changes in some physicochemical properties and fatty acid composition of irradiated meatballs irradiated using a ^{60}Co irradiation source (with the dose of 1, 3, 5 and 7 kGy). The physicochemical results showed total acidity, peroxide and thiobarbituric acid (TBA) values increased significantly as a result of irradiation doses. The fatty acid profile in meatball samples also changed with irradiation. Gecgel (2013) also reported an increase in t*rans* fatty acids (C16:1*trans*, C18:1*trans*, C18:2*trans*, C18:3*trans*) with increasing irradiation doses.

4.Conclusions:

Slight but statistically significant changes ($P<0.05$) of the main polyunsaturated *trans* fatty acids such as C18:2-9c,12c, C18:1-9n were observed in the samples for each of the 3 oils selected with the irradiation dose of 4 kGy compared with the control sample, at a dose of 2 KGy, no significant change was observed in *trans* fatty acid content. Hence it can be concluded that irradiation induced the formation of TFAs in food when the absorbed dose exceeded 4 kGy. Among the three oils selected for study, maximum difference was observed in soybean oil followed by palm oil and then mustard oil. Oxidative changes for peroxide and anisidine value was most significant in soybean oil, however no significant change was observed for iodine value and conjugated diene value after irradiation in all the selected samples.

5.References:

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BIOLOGICAL EVALUATION AND APPLICATION OF CORIANDER FRUITS AND ITS ESSENTIAL OIL

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ABSTRACT

Our investigation is currently focusing on the impact of coriander fruits and their extracts (essential oil and aqueous extracts) as natural substances could be used as alternative food preservation as well as functional foods. So, the chemical constituents of coriander essential oil (CEO) and the *in vitro* antimicrobial as well as antioxidant activities were determined, also, the hypoglycemic effectiveness was applied on diabetic rats. Results indicated that Linalool, is found to be the major volatile component (62.2 %). The antimicrobial activity of three concentrations of CEO (1, 3 and 5%) and linalool (0.5,1.0 and 2.0%) against five bacterial and five fungal strains by using Disc –Diffusion technique was examined, compared with the results of Genatmicin and Amphotericin B. Results indicated that CEO showed more effective antimicrobial activity than linalool. Also, Gram-negative bacterial strains were susceptible towards tested materials than Gram- positive ones. CEO demonstrated antioxidant activity by DPPH assay. Also, it was found that supplementation of English rich cake with ground coriander fruits (GCF), succeeded in prolonging the shelf life of cake samples up to 12 weeks at 1 and 3 %, however, it reached 16 weeks in the cake supplemented with 5 % GCF, at room temperature (20± 2° C). The finding evinced the superior effect of GCF compared with CEO. On the other hand, our investigation proved the anti-diebetic activity of coriander fruits and their extracts (aqueous macerated and aqueous decocted) on Streptozotocin-induced diabetic rats *via* oral injection or feeding with pan bread incorporated with GCF or CEO.

1.Introduction

Coriander (*Coriandrum sativum* L.) being an annual herb, is most commonly used for seasoning purpose. The herbal material of corianderis the fruit not the seed. Fresh herb is also used as an aromatic spice (Nurzynska-Wierdak, 2013).Coriander is also well known for its antioxidant, anti-diabetic, anti-mutagenic, antianxiety and antimicrobial activities along with analgesic and hormone balancing effect that promotes its use in foods due to numerous health benefits. Furthermore, it is used to preserve the food for a long timefor its protective effect (Bhat, *et al.*, 2014). For the previous effectiveness, coriander is considered one of miraculous herb that functions as both, spice as well as herbal medicine.

Diabetes mellitus is a chronic, metabolic disease characterized by elevated levels of blood glucose (hyperglycemia), and disturbance of carbohydrate and glycogenolysis as well as gluconeogenesis fat and protein

metabolism, which leads over time to serious damage to the heart, blood vessels, eyes, kidneys and nerves. About 422 million peopleworldwide have diabetes, 1.6 million deaths are directly attributed to diabetes each year. Both the number of cases and the prevalence of diabetes have been steadily increasing over the past few decades (WHO, 2021). It was found that DM is associated with chronicle higher risk including heart attacks, blindness, kidney failure and neuropathy (Hameed, *et al.*, 2015).On this occasion, coriander fruit extract is used as a traditional medicine for diabetic patients. Incorporation of ground coriander fruit extract in diet led to marked decline in blood glucose and rise in levels of insulin in diabetic rats (Bhat, *et al.*, 2014). On this connection, Srinivasan, (2005) ascertained that the hypoglycaemic effect of spices may be used in conjunction with antidiabetic drugs to have better therapeutic potential and to minimize the oral hypoglycaemic drug dosage. Many investigations focused on the impact of herbs and spices, although they do not majorly contribute to nutrient supplementation of diet because of their use in lesser quantities and mostly utilized for the purpose of garnishing and flavoring. However, keeping in view the health-enhancing potential of these food components, they must be employed in designing and formulating functional foods (Eidi, *et al.*, 2009 and Helmy, *et al.*, 2017). It was also reported that activity of 200 mg/kg body weight dose of coriander extract is comparable to the commercially available synthetic drug. They also added that coriander had the ability to ameliorate oxidative stress and protect the liver and renal cell from damage (Sreelatha, *et al.*, 2009). Besides, peroxidative damage inhibition, addition of fruitsextract reactivated antioxidant enzymes and antioxidant levels in diabetic rats (Deepa & Anuradha, 2011). Keeping in view the importance of medicinal, aromatic plants as well as natural products, coriander fruits and their extracts (aqueous macerated, decocted and essential oil) were selected for the present study as biopreservative and anti-diabetic agents.

2.Materials and Methods

2.1.Materials

Coriander fruits (*Coriandrum sativum* L.) were purchased from local market, Giza, Egypt. Streptozotocin (STZ), DPPH (2, 2-diphenyl-1-picrylhydrazyl), BHT (Butylated Hydroxy Toluene) and Linalool ((3R)-3,7-dimethylocta-1,6-dien-3-ol) were purchased from Sigma Chemical Company, St Louis, Missouri, USA. Glucose kits were purchased from Bio-diagnostic (BD), Cairo, Egypt. Kits for determingAlanine amino transferase (ALT), Aspartate amino transferase (AST), triglycerides, total cholesterol, high density lipoprotein(HDL), low density lipoprotein(LDL)Creatinine and Urea were purchased from Bio -diagnostic Company, Cairo, Egypt. Methyl alcohol (99%) and ethyl alcohol (99.5%) were purchased from El-Nasr Pharmaceutical Chemical Co., Egypt. Anhydrous sodium sulphate (99%) was purchased from ElGomhoria Co. Chemicals trade, Cairo, Egypt. Ingredients of English rich cake and pan bread were purchased from local market, Giza, Egypt. All other chemicals and reagents used were of analytical grade.

2.2. Methods

2.2.1. Gross chemical composition of Coriander fruits

Coriander fruits were analyzed for their chemical constituents. All the proximate analysis including; moisture, crude protein, crude fat, crude fibers and total ash were determined according to the methods of AOAC, (2012). However, carbohydrates content of coriander fruits was determined to be a nitrogen free extract (NFE) by the following formula:

$$\text{NFE (\%)} = 100 - (\text{Moisture} + \text{crude fat} + \text{crude protein} + \text{crude fiber} + \text{total ash}). \quad (1)$$

2.2.2.Tested microorganisms

Four different bacterial strains, two Gram positive strains (*Bacillus subtilis* ATCC 33221

and *Staphylococcus aureus* ATCC 20231) and two strains of Gram negative bacterial strains (*Escherechia coli* ATCC 6933 and *Pseudomonas aeruginosa* ATCC 9027) were used in this study. Two strains of yeast including, *Saccharomyces cerevisiae* NRRLY 2034 and *Candida lypolitica* NRRLY 1095 and two strains of moulds were used which included *Aspergillus niger* NRRL 2322 and*Aspergillus flavus* EMCC 100. All previous strains were obtained from the Egyptian Microbial Culture Collection (EMCC), Faculty of Agriculture, Ain Shams University, Egypt, except *Aspergillus niger* NRRL 2322 which was obtained from Northern Regional Research Laboratories (NRRL), Peoria, Illinois, USA. The previous microorganisms were checked for their purities and they were reactivated monthly on the suitable media as reported by Conner and Beauchat (1984).

2.2.3. Tested animals

Forty male albino rats (Wister strain) weighing 200 -250g were supplied by the Animal House of National Research Center, Cairo-Egypt.

2.2.4. Separation and phytochemical analysis of coriander essential oil using GC/MS technique

The dried coriander fruits sample was ground immediately prior to extraction in order to avoid losses of volatiles, and then subjected to hydro- distillation (Clevenger trap) for 4 hours according to the method described in the European Pharmacopoeia (1997). The extracted coriander essential oil (CEO) was collected and desiccated over anhydrous sodium sulphate. Extraction was carried out in triplicates and the mean values of essential oil yield was calculated as follows:

Yield (% v/w) = (volume of essential oil/mass of the dried fruits) x 100 (2)

The chemical constituents of CEOwere fractionated and identified by using Gas Chromatography/Mass Spectrometry (GC/MS) technique, using the GC/MS system (Agilent Technologies), which was equipped with Gas Chromatograph (7890B) and Mass Spectrometer detector (5977A). Oil sample was diluted with hexane (1:19, v/v).The GC was provided with HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness). Helium was the carrier gas at a flow rate of 1.0 ml/min at a split ratio of 1:30, injection volume of 1 µl and the following temperature program: 40 °C for 1 min, rising at 3 °C /min to 160 °C and kept for 6 min, rising at 4 °C/min to 210 °C and kept for 1 min. The injector and detector were controlled at 280 °C and 220 °C, respectively. Mass spectra were obtained by electron ionization at 70 eV and using a spectral range of m/z 50-550. Identification was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and National Institute of Standards and Technology Mass Spectral Library data.

2.2.5. Antimicrobial activity of coriander fruits essential oil

Precursorytrial ascertained that the CEO completely inhibited the tested microorganism, so, CEO was diluted to 1, 3 and 5 % by using ethanol 95 %. Also, Linalool was tested at different concentrations (0.5, 1.0 and 2.0%). Disc diffusion Assay was applied to assess the antimicrobial activity of coriander essential oil according to the method described by Hood *et al*. (2003). Twenty ml of either Mueller Hinton agar medium or Sabouraud agar medium, for antibacterial and antifungal activities assay, respectively, containing 1% Tween 80 as an emulsifier was poured in sterilized plates (90 mm diameter) according to Difco- Manual (1977). Ten µl of each concentration was placed on 6 mm blank antimicrobial susceptibility discs. The essential oil was impregnated the discs, then it was placed onto. The agar plates were incubated overnight at suitable temperature (30 and 37°C for fungal and bacterial strains, respectively). The microbial inhibition zones in millimeters (mm) were recorded.

2.2.6. Antioxidant activity of coriander essential oil

The antioxidant potency of coriander essential oil was determined by using DPPH Scavenging method as described by Choi (2010) and Viuda-Martos, *et al*. (2010). The DPPH analysis was measured as mentioned by Brand Williams, *et al.* (1995). Different concentrations from oil sample were added 10, 25, 50, 100, 150 and 200 µL in test tubes followed by addition 2.8 ml of 0.4 g /L DPPH solution (0.020 ± 0.0001 g DPPH in 50 mlmethanol as a solvent). Both BHT and ascorbic acid solutions at the same concentrations were considered reference antioxidants.

The IC_{50} (50% of inhibition) also was calculated from a graph plotting percentage inhibition against each essential oil concentration.

2.2.7. Preparation of cake

English rich cake samples were processed according to the method described in AACC (2002), by the Bakery Pilot Plant in Food Technology Research Institute (FTRI), Agriculture Research Center (ARC). The formula was (80% fat, 80% eggs, 80% sugars/ 100g flour). Preliminary sensory evaluation was applied to assess the acceptable addition % of ground coriander fruits and coriander essential oil in both cake and pan bread formulations. So cakes were incorporated with ground coriander fruits at 1, 3, 5, 7and 10% or its essential oil at 0.1, 0.3 and 0.5%, compared with BHT at 200 ppm to evaluate the efficiency of coriander fruits or their essential oils to preserve cake samples. Cakes were baked for 25 min at 180 °C, thenthe ten samples were cooled at the room temperature (21 ±3° C) and were packed in polypropylene bags at the room temperature (20± 2° C) for 24 weeks.

2.2.8. Determination of specific volume of cake

Specific volume of cake was determined to assess cake quality, according to the method of Chaiya &Pongsawatmanit (2011). After baking at 180 °C for 25 min, the final cake volume was obtained using the rapeseed displacement method. The cake was cut into 25 × 25 × 25 mm cubes. Then, one piece of cake was weighed (W), and placed in a container and the rest of the container volume was filled with rapeseed (V2). The volume of the empty container (V1) was calculated by filling with rapeseed. Both V1 and V2 were later determined by a graduated cylinder and the difference between V1 and V2 was defined as the cake volume (Vo). The specific volume was then calculated as the ratio of the volume to weight (Vo/W).

2.2.9. Determination of microbial load in cake during storage period

Microbial load was estimated by using pour plate method according to Vanderzant & Splittstoesser (1992). The counts of total bacteria (TBC), mold and yeasts (Fungal count, FC), respectively, were determined and expressed as $CFU.g^{-1}$, during storage period at (20± 2° C).Alsovisible microbial growth (MG) was recorded by visual observation.

2.2.10. Lipid oxidation

Two chemical parameters, Peroxide value (PV) andThiobarbituric acid (TBA) were determined as indications of lipid oxidation in the fat portion extracted from cake samples according to Habib & Brown (1956). PV was determined according to the methods of (AOAC, 2000) and expressed as (mequivalent peroxide/ Kg oil). However, TBA was determined colorimetrically at 538 nm as described by (Pearson & Cox, 1976). The results of (TBA) were expressed as mg malondialdehyde/kg oil sample.The samples of cake were analyzed for these parameters immediately after baking of cake samples (zero time) and 2 weeks interval for 24 weeks of storage at room temperature (20± 2° C).

2.2.11. Preparation of pan bread for biological assay

Pan bread was prepared according to the method described in (AACC, 2000) with some modifications. The coriander oil (0.2, 0.3 and 0.5 %) was added by replacing the oil of control sample, however, ground coriander fruits (3.0, 5.0, 7.5 and 10.0%) as appropriate

%were added by replacing the flour of control sample. The processing of pan bread was applied according to Abdulla & Abdel-Samie (2015). Bread loaves obtained were sensory evaluated to select the most accepted ratios for both coriander fruits and their essential oil to be utilized in the biological experiment.

2.2.12. Sensory evaluation of cake and pan bread

Cakes prepared by adding ground coriander fruits or their essential oil as well as BHT, compared with control sample (without additives), were sensorial evaluated. After cooling of cake samples at room temperature, sensory characteristics were judged by ten members from Experimental Kitchen Res. Unit, Food Tech. Research Institute. Giza, Egypt. Cake samples with a thickness of 1 cm, were evaluated on the basis of acceptance of their crust color (10), texture(10), odor(10), taste(10), appearance(10) and overall acceptability(10). The ten samples were coded differently and served to panelists. The panelists were asked to score the characteristics of cake samples crust color, odor, texture, taste and overall acceptability, according to Ibrahium, *et al.* (2013). Meanwhile, eight Pan Bread samples (prepared by adding ground coriander fruits or their essential oil compared with control sample) were coded and presented to evaluate sensory characteristics. Ten panelists, who are familiar with the product, from the Staff of the Cereal Technology Research Department, Food Technol. Research Institute, Agric. Res. Center, Giza, Egypt were asked to evaluate sensory characteristics of pan bread samples, which including crust color (15), crumb color (15), texture (15), odor (20), taste (20) and appearance (15). The scoring scheme was established according to the method described by AACC (2000). All samples were analyzed in the same session. Water was available for rinsing.

2.2.13. Antidiabetic effect of coriander fruitsand its extracts

2.2.13.1. Preparation of coriander fruit extracts for biological evaluation

Coriander fruits were ground by using an electrical blender and stored at – 18° C until utilized.

Decoction treatment: ground coriander fruits (25 gm) were suspended in distilled water (2.5 L) and heated to boil for 30 min., decoctedcoriander fruits (DC) was filtered after centrifugation.

Maceration treatment: ground coriander fruits (25 gm) were suspended in 2.5 Liters cold distilled water for 30 min, macerated coriander fruits (MC) obtained was filtered after centrifugation.

2.2.13.2. Induction of Diabetes

The experimental animals were kept in an environmentally controlled room (temperature 25 ± 2° C, humidity: > 60%) with regular light-dark cycle. The animals were housed individually in polypropylene aerated cages with screen bottoms and provided diet and water *ad libitum*through the experimental period (28 days).The rats were fed on a basal diet (pellet shaped) consisted of 21.70% casein, 53.30% corn starch, 15% sucrose, 5% corn oil, 4% mineral mixture and 1.00% vitamin mixture (Tebib *et al.*,1997) for 10 day as an adaptation period. After that, the rats were randomly divided into two groups, Group 1 (normal control group): Animals received a basal diet.The second group (35 rats) were kept fasting prior to streptozotocin injection. On the day of administration, Streptozotocin (STZ) dissolved in 0.1 M citrate buffer (pH7.4) was injected intravenously at the dosage of 60 mg/kg b.w. to induce hyperglycemia according to Ozsoy-Sacan *et al*. (2005). Blood glucose concentration was checked by the glucose oxidase method of Trinder, (1969). After 3 days of STZ injection, the animals with glucose concentration exceeding 200mg /dL were considered diabetic, so, they were divided into 7 groups, other than normal control rats group as follows:

G1: Normal control rats group fed on a basal diet according to Reeves et al., (1994).

G2: Diabetic control rats group, fed on a basal diet rats and injection with STZ (60mg/kg b.w).

G3: Diabetic rats group, fed on a basal diet and orally injection with (500mg/kg b.w/daily) of decoctedcoriander fruits (DC) by stomach tube.

G4: Diabetic rats group, fed on a basal diet and orally injection with (500mg/kg b.w/daily) macerated coriander fruits (MC) by stomach tube daily.

G5: Diabetic rats group, fed on a basal diet containing ground coriander fruits (GCF) at 500 mg/kg bw.

G6: Diabetic rats group, which fed on a basal diet and orally injection with coriander essential oil (40 mg/kg b.w/daily) by stomach tube (the dose of essential oil was chosen according to its LD_{50}, where the medium 50 lethal doses after acute toxicity as mentioned by Anon. (2021).

G7: Diabetic rats group, fed on pan bread containing 0.3 % coriander essential oil.

G8: Diabetic rats group, fed on pan bread containing 5 % ground coriander fruits.

The biological evaluation of the different tested was carried out by determination body weight gain percentage and hypoglycemic efficiency according to Chapman et al. (1959).

At the end of the experiment (after 28 days) from the beginning of the experiment, body weight gain (BWG)% was calculated as follows:

Body weight gain (%) = (Final weight – Initial weight / Initial weight) X 100. (3)

2.2.13.3. Blood samples collection and Biochemical analysis

At the end of biological experiment, rats were fasted for 12 hours then blood samples were collected retro-orbitally from the inner canthus of the eye under ether anesthesia using capillary tubes containing sodium EDTA (Schermer,1967).Plasma total lipids (TL) were determined by enzymatic method according to Zollener & Kirsch (1962). Triglycerides (TG) and total cholesterol (TC) were determined by enzymatic colorimetric method according toTrinder (1969). High density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (LDL), were determined according to Assman, (1979), AST and ALTactivities were determined by colorimetric methods according to Reitman & Frankel (1957) as liver function enzymes. Serum glucose was determined by enzymatic colorimetric method, according to Barham &Trined, (1972). Regarding, kidney functions, Creatinineand Urea were determined according to Caraways, (1975). Glucose level was determined in blood serum of experimental rats using enzymatic method ascribed by Young *et al*. (1972).

2.2.14. Statistical Analysis

The collected data for various parameters were expressed as mean ± SD (standard deviation) of the mean of three replications, unless otherwise stated and compared using one way analysis of variance (ANOVA) test. All data were averaged.Values were considered statistically significant when P value was less than 0.05 were done using SPSS for windows version 10.

3. Results and discussions

3.1. Gross chemical composition of Coriander fruits

Results indicated that moisture constituted low content (8.86%) in coriander fruits, however, crude protein and total ash constituted 12.37 and 10.30 %, respectively. Regarding nitrogen free extract (NFE), it represented (8.80 %) and crude fibers (41.90 %). On the other hand, crude fat constituted 17.77%. These results are in accordance with those reported by Shahwar, *etal*. (2012). Also, our findings are highly matched with those found by Bhat, *et al.* (2014), who is reported that coriander fruits is considered a potential source of lipids (rich in petroselinic acid) and an essential oil (high in linalool) as well as high contents of dietary fibers and protein in the fruits make distillation residues suitable for animal feed.

3.2. Coriander essential oil yield and chemical composition of the essential oil

Results showed that the coriander fruits essential oil constituted 0.28 %(v/w, on dry basis), which is in agreement with that reported

by El Soud, *et al*. (2012), Freires *et al.* (2014) and Al-Sanafi (2016). Also, Nurzynska-Wierdak (2013) ascertained that the coriander fruits contained 0.17 – 0.29 %, essential oil, depending on the stage of plant development, which exhibited high variability.

Concerning chemical constituents of coriander essential oil, the GC/MS identified 26 components from total of 32 components which representing 98.5 % of total constituents of CEO (Table, 1). The oxygenated compounds constituted higher fraction. It could be noticed that the major constituent was Linalool with 62.2 %,which is considered as a cyclic monoterpene alcohol, followed by geranyl acetate (10.9%), γ -terpinene (10.2 %) and α-pinene (3.2 %). These results are in harmony with those reported by Abou El Soud, *etal*. (2012) and Sourmaghi, *et al.* (2014). The results also are in the same line with those presented in ES(2007).

3.3. Antimicrobial activity

The antimicrobial activity of CEO (1, 3 and 5%) as well as linalool (0.5, 1.0 and 2.0%), compared with two reference antibiotics (Gentamicin and Amphotericin B) at 10 µg, in similar conditions against pathogenic bacterial strains (*Escherichia coli* ATCC 6933, *Pesudomonasaeroginosa* ATCC 902, *Staphylococcus aureus* ATCC 20231 and *Bacillus subtilis* ATCC 33221) by disc-diffusion method was examined.On this connection, the concentrations of linalool were chosen depending on its percent in the selected concentration of the coriander fruits essential oil. The results illustrated in Table (2) showed that Gram-positive bacteria exhibited more sensitive to the CEO, especially *Bacillus subtilis* ATCC 33221 (inhibition zones being between 7.4 and 19.4 mm). Gram-negative bacteria were more resistant, especially, *Pesudomonas aeroginosa* ATCC 9027. It could be observed that the diameters of inhibition zones were directly proportional to oil and linalool concentrations. The sensitivity differencebetween the two groups of bacteria occurred because Gram-negative bacteria possess an outer membrane and a unique periplasmic space is not presented in gram-positive bacteria (Dorman & Deans, 2000).

It is noteworthy that the linalool exhibited lower antimicrobial activity against the tested strains compared with CEO, however, linalool, didn't show any inhibition at low concentration (0.5%), except on the two tested mold strains. On the other hand, the differences between the antimicrobial activities of oil were significant (Table 2). On the other hand, the results ascertained that CEO has demonstrated a remarkable antifungal effect against *Aspergillus niger* NRRL 2322 and *Aspergillus flavus* EMCC 100, compared to Gentamicin and Amphotericin B,at all concentrations used in this experiment. Similar findings were noticed for *Candida lypolitica* NRRLY 1095, where, the tested agents succeeded in inhibiting the growth of *Candida lypolitica* NRRLY 1095, with the remarked superiority of CEO ($p \geq 0.05$), followed by the rest tested agents.

Our finding means that the antimicrobial efficiency of such oil didn't refer to the main component only, but to the synergistic effect of all oil components as reported by Herman, *et al.* (2016), who ascertained the antimicrobial efficiency of linalool and also reported that a synergistic manner between linalool and some essential oil components enhanced their antimicrobial efficacy against *P. aeruginosa, S. aureus,E. coli* and *C. albicans*. Our results are highly in harmony with those found by Ultee, *et al.,* (2002) ascertained that the antifungal property of the oil was likely due to the eugenol. On the other hand, Nanasombat & Lohasupthawee (2005) proved that the antimicrobial effects of spices and herbs were due to their complex chemical composition, which included compounds such as thymol, carvacrol, methyl eugenol, linalool, α-pinene, 1, 8-cineole and camphor. Other component presented in CEO and has antimicrobial potency is geranyl acetate which has antifungal activity and anti-inflammatory effect (José Goncalvesa, *et al*., 2011). Furthermore, the antimicrobial activity of the essential oil could be contributed to the presence of active

compounds such as α-pinene and ß-pinene (Dorman & Deans 2000and Zardini, *et al*. (2012), ρ-cymene and γ-terpinene (Xianfei, *et al*. 2007). All the previous components were fractionated and identified in CEO (Table 1).Concerning Linalool, it was found to have antimicrobial activity against various microbes and it inhibits the spore germination and fungal growth by the mechanism of respiratory suppression of aerial mycelia (Koutsoudaki *et al.*, 2005).

Table 1. Chemical constituents of coriander EO by using GC/MS

Peak no.	**Compounds**	**RT***	**`Area (%)**
1	Nonane	4.13	0.2
2	Unknown	4.27	0.1
3	α-Pinene	4.38	3.2
4	Decane	4.74	0.2
5	Cis-Ocimene	5.01	0.3
6	β-Pinene	5.29	1.1
7	γ-Terpinene	5.79	10.2
8	Sabinene	6.32	0.4
9	Limonene	6.91	2.1
10	n-Ocatanal	8.12	0.6
11	Unknown	8.17	0.3
12	*p*-Cymene	9.02	0.2
13	Humulene	9.17	0.2
14	Nonnal	10.32	0.2
15	Linalool	13.71	62.2
16	Camphor	14.51	0.2
17	Citronellal	17.28	0.1
18	Decanal	18.79	3.8
19	Terpinen-4-ol	20.11	0.1
20	Borneol	23.27	0.2
21	Unknown	24.10	0.2
22	Unknown	25.37	0.3
23	Unknown	28.21	0.2
24	α-Terpineol	29.07	0.1
25	Bornyl acetate	30.21	0.2
26	Caryophyllene	31.32	0.3
27	Neryl acetate	33.74	0.1
28	Unknown	34.21	0.4
29	Geranyl acetate	37.52	10.9
30	Elemene	39.22	0.2
31	Eugenol	40.29	0.5
32	Octadecanol	42.39	0.7
Total identified (%)			**98.5**
Total non-oxygenated compounds (%)			**18.6**
Total oxygenated compounds (%)			**79.9**

Table 2. Antimicrobial activity of coriander essential oil and linalool using agar disc diffusion method (mm)*

Microbial Strains	Tested materials							
	Coriander essential oil (%)			Linalool (%)			Gentamicin*	Amphotericin B*
	1	3	5	0.5	1.0	2.0		
Gram positive bacteria								
Bacillus subtilis ATCC 33221	$7.4^{aD} \pm 0.13$	$12.3^{aBC} \pm 0.25$	$19.4^{aA} \pm 0.07$	$0.0^{bE} \pm 0.00$	$6.1^{bD} \pm 0.03$	$11.8^{abC} \pm 0.14$	$14.2^{aB} \pm 0.11$	$11.0^{bC} \pm 0.04$
Staphylococcus aureus ATCC 20231	$0.0^{bC**} \pm 0.00$	$7.1^{cB} \pm 0.03$	$14.0^{bA} \pm 0.21$	$0.0^{bC} \pm 0.00$	$0.0^{cC} \pm 0.00$	$8.2^{bB} \pm 0.28$	$13.3^{aA} \pm 0.2$	$0.0^{cC} \pm 0.00$
Gram negative bacteria								
Escherichia coli ATCC 6933	$7.2^{aC} \pm 0.05$	$10.0^{bB} \pm 0.14$	$13.7^{bA} \pm 0.14$	$0.0^{bD} \pm 0.00$	$7.1^{aC} \pm 0.07$	$10.3^{bB} \pm 0.11$	$11.5^{bAB} \pm 0.1$	$9.5^{bB} \pm 0.23$
*Pesudomonasaeroginosa*ATCC 9027	$0.0^{bC} \pm 0.00$	$7.4^{cBC} \pm 0.18$	$11.0^{bcA} \pm 0.00$	$0.0^{bD} \pm 0.00$	$6.8^{abBC} \pm 0.1$	$8.3^{bB} \pm 0.15$	$12.2^{abA} \pm 0.1$	$10.0^{bAB} \pm 0.1$
Molds								
*Aspergillus niger*NRRL 2322	$9.1^{aC} \pm 0.11$	$15.1^{aB} \pm 0.15$	$22.0^{aA} \pm 0.33$	$6.8^{aC} \pm 0.11$	$8.5^{aC} \pm 0.11$	$14.7^{aB} \pm 0.70$	$8.3^{cC} \pm 0.14$	$19.0^{aAB} \pm 0.1$
Aspergillus flavus EMCC 100	$8.8^{aD} \pm 0.71$	$15.9^{aB} \pm 0.22$	$19.8^{aA} \pm 0.13$	$7.1^{aD} \pm 0.16$	$9.3^{aD} \pm 0.33$	$12.3^{aC} \pm 0.28$	$9.0^{cD} \pm 0.21$	$17.3^{abA} \pm 0.2$
Yeasts								
Saccharomyces cerevisiae NRRLY 2034	$0.0^{bC} \pm 0.00$	$0.0^{dC} \pm 0.00$	$0.0^{cC} \pm 0.00$	$0.0^{bC} \pm 0.00$	$0.0^{cC} \pm 0.00$	$0.0^{cC} \pm 0.00$	$8.2^{cB} \pm 0.19$	$17.0^{abA} \pm 0.1$
Candida lypolitica NRRLY 1095	$7.2^{aC} \pm 0.05$	$10.0^{bB} \pm 0.14$	$13.7^{bA} \pm 0.14$	$0.0^{bD} \pm 0.00$	$7.1^{abC} \pm 0.1$	$10.3^{bB} \pm 0.11$	$11.5^{bAB} \pm 0.1$	$9.5^{bB} \pm 0.23$

Values are mean inhibition zones of three replicates ± standard deviation.
Means followed by different capital letters in the same row represents significant difference ($p \leq 0.05$) between tested materials or concentrations.
Means followed by different small letters in the same column represents significant difference ($p \leq 0.05$), for each microorganism.
* Reference antibiotics at concentration (10 μg). ** No observed inhibition zone.

On the contrary, neither CEO nor linalool affected the growth of *Saccharomyces cerevisiae* NRRLY 2034 strain, which was resistant, however, two tested antibiotics inhibited the growth of this strain. This means that CEO could be added to bakery products as a seasoner without negative effect on the growth of baker's yeast and consequently the fermentation process. The results of Freires *et al.* (2014) are matching with ourfindings.

Concerning the results of antimicrobial activity of reference antibiotics (Gentamicin and Amphotericin B), it was reported that Gentamicin is an aminoglycoside antibiotic and is considered as a bactericidal antibiotic that works by binding the 30S subunit of the bacterial ribosome, negatively impacting protein synthesis. However, Gentamicin was found to have antibacterial activity against *Escherichia coli*, *Pseudomonas aerogenosa* and *Staphylococcus aureus* (Anon., 2019).

3.4. Free radical scavenging activity

In vitro antioxidant potential by means of free radical scavenging activity (RSA) of CEO was applied using the DPPH (1,1-diphenyl-2-picryl hydrazyl) radical method. Different concentrations of CEO and Linalool (10, 25, 50, 100, 150 and 200 µl) were tested, compared with BHT and ascorbic acid as control synthetic antioxidant agents at the same previous concentrations. Results in Table (3)indicated that no antioxidant activity was noted at low concentrations (10 and 25 µl) for CEO, linalool and ascorbic acid, however it exhibited RSAat 50 µl and above (except for linalool). Our findings ascertained that at 100 and 200 µl, both CEO and BHT exhibited the highest antiradical scavenging activity with no significant ($p \geq 0.05$)differences. On the other hand, no significant difference was recorded concerning RSAbetween linalool and CEO at 200 µl, where, significant differences were observed among ascorbic acid and the other tested materials ($p \geq 0.05$).

Table 3. The DPPH radical scavenging activity (%) of coriander fruits essential oil and linalool at different concentrations.

Tested materials	Concentrations (µl)					
	10	**25**	**50**	**100**	**150**	**200**
CEO*	$0.00^{a} \pm 0.00$	$0.00^{b} \pm 0.00$	$31.95^{c} \pm 2.12$	$51.28^{a} \pm 0.86$	$69.92^{b} \pm 0.77$	$88.63^{ab} \pm 1.64$
Linalool	$0.00^{a} \pm 0.00$	$0.00^{b} \pm 0.00$	$0.00^{d} \pm 0.00$	$46.22^{b} \pm 0.65$	$62.44^{c} \pm 2.81$	$83.18^{b} \pm 0.58$
BHT	$0.00^{a} \pm 0.00$	$50.80^{a} \pm 1.16$	$53.80^{a} \pm 1.95$	$58.22^{a} \pm 0.19$	$87.32^{a} \pm 1.01$	$99.16^{a} \pm 0.61$
Ascorbic acid	$0.00^{a} \pm 0.00$	$0.00^{b} \pm 0.00$	$40.03^{b} \pm 2.11$	$46.33^{b} \pm 2.83$	$63.12^{c} \pm 1.58$	$70.81^{c} \pm 1.03$
	IC_{50} (µg/ml)					
CEO*	$78.3^{b} \pm 1.91$					
Linalool	$82.1^{b} \pm 2.19$					
BHT	$76.5^{b} \pm 1.11$					
Ascorbic acid	$112.2^{a} \pm 2.87$					

* Coriander Essential oil.BHT and ascorbic acid were used as control antioxidant agents.
The mean values (n=3) with different letters in the same column are significantly different($p<0.05$).

Concerning the IC_{50} value (the concentration with scavenging activity of 50%), our findings illustrated in Table (3) indicated that the highest scavenging efficient was noticed for BHT (76.5µg/ml), however, no significant ($p \geq 0.05$) differences were found between BHT, CEO and linalool. On the contrary, ascorbic acid showed the lowest RSA, where IC_{50} value was significantly higher ($p \geq 0.05$)than those recorded by the other tested materials (112.2µg/ml).

The antioxidant potential of CEO could be referred to the presence of certain components such as thymol, eugenol and carvacrol

(phenolic compounds) which are indeed responsible for the antioxidant activity of many essential oils that contain the previous components (CEO contains 0.4 % eugenol) and a scant antioxidant activity is given to monoterpene and sesquiterpene hydrocarbons, three monocyclic components, γ-terpinene (10.2%), terpinen-4-ol (0.1%) and α-terpinene (0.1%), in addition to a lesser degree, a bicyclic sabinene (0.4%), which show considerable activity (Ruberto & Baratta, 2000). Zhang, *et al*. (2016), also ascertained the strong antioxidant activity of clove essential oil refers to the presence of eugenol, the main constituent of cloves.On the other hand, Jabir, *et al.* (2018) demonstrated the high antioxidant activity of linalool on in comparison with ascorbic acid as a standard reference. They also confirmed that linalool could donate hydrogen atoms and remove the electron from DPPH, and as a result, they suggested that linalool could be useful for the management of numerous deleterious diseases and cancer because of their scavenging activity. Also, Paarakh (2017) indicated that linalool has been traditionally used for medicinal purposes because of its potent antioxidative activity; hence, it could be used in the synthesis of several types of compounds with ability to act as antioxidant and could be used as a medicine drug.

3.5. Sensory evaluation of cake and pan bread samples

Sensory characteristics of cake samples supplemented with ground coriander fruits (1, 3, 5 and 7 and 10%) or coriander fruits essential oil (0.1, 0.3 and 0.5%) were evaluated and the results are illustrated in Table 4. It could be noticed that crust color, odor and taste parameters didn't affect with the substitution withGCF or their essential oil at the previous concentrations compared with a control cake sample. Meanwhile, texture, appearance and overall acceptability significantly ($p \geq 0.05$) decreased at 7 % GCF. However, no significant($p \geq 0.05$) differences were noted for cake samples supplemented with CEO at all tested concentrations, compared with control cake sample. On the contrary, supplementation with GCF at 10% reduced all sensory parameters, with significant ($p \geq 0.05$)differences except crust color. Also, adding BHT at 200ppm had no significant ($p \geq 0.05$) difference on evaluated sensory parameters. It is noteworthy that utilizing CEO at 0.1, 0.3 and 0.5% exhibited good sensorial properties. However, GCF at 3 and 5% gave approximately similar effect, besides increasing the nutritional value of cake due to its high contents of crude protein, crude fibers and ash.

Table 4. Sensory characteristics of cake supplemented with ground coriander fruits and their essential oil.

Sensory criteria	Control	Cake supplementedwith ground coriander fruits (%)					Cake supplemented with coriander essential oil (%)			BHT (200 ppm)
		1	3	5	7	10	0.1	0.3	0.5	
Crust Color (10)	9.60^{a} ±0.87	9.55^{a} ±0.17	9.50^{a} ±0.12	9.20^{a} ±0.11	9.15^{a} ±0.02	9.00^{a} ±0.07	9.50^{a} ±0.32	9.80^{a} ±0.72	9.08^{a} ±0.02	9.10^{a} ±0.04
Texture (10)	9.51^{a} ±0.56	9.37^{a} ±0.27	9.25^{a} ±0.01	9.50^{a} ±0.03	9.10^{b} ±0.01	7.30^{c} ±0.09	9.40^{a} ±0.01	9.50^{a} ±0.05	9.25^{a} ±0.13	9.23^{a} ±0.52
Odor (10)	9.52^{a} ±0.63	9.45^{a} ±0.38	9.15^{a} ±0.15	9.30^{a} ±0.04	9.00^{a} ±0.04	8.20^{b} ±0.06	9.50^{a} ±0.02	9.90^{a} ±0.80	9.12^{a} ±0.02	9.20^{a} ±0.13
Taste (10)	9.53^{a} ±0.73	9.51^{a} ±0.52	9.40^{a} ±0.12	9.50^{a} ±0.10	9.00^{a} ±0.12	6.10^{b} ±0.18	9.50^{a} ±0.11	9.65^{a} ±0.62	9.10^{a} ±0.01	9.12^{a} ±0.03
Appearance (10)	9.60^{a} ±0.81	9.58^{a} ±0.12	9.50^{a} ±0.02	9.55^{a} ±0.07	8.60^{b} ±0.23	6.00^{c} ±0.12	9.50^{a} ±0.02	9.55^{a} ±0.07	9.15^{a} ±0.23	9.33^{a} ±0.06
Overall acceptability (10)	9.38^{a} ±1.02	9.46^{a} ±0.33	9.36^{a} ±0.22	9.41^{a} ±0.50	8.85^{b} ±0.75	7.00^{c} ±0.10	9.48^{a} ±0.22	9.55^{a} ±0.50	8.83^{b} ±0.75	9.30^{a} ±1.43

Values are means (n= 10 ± SD).

Each value within the same row, followed by the same letter is not significantly different at 0.05 level.

Values of crust color, crumb color, odor, texture, taste, appearance and overall acceptability of pan bread samples are shown in Fig. 1. Analysis of variance was done for all sensory parameters of pan breads and panelists accepted all pan bread samples with different acceptability ranks. Crust color and taste parameters didn't affect with any addition, where, no significant differences($p \geq 0.05$) were noticed. With respect to sensory criteria, no significant ($p \geq 0.05$) differences were noticed with addition of up to 7.5 % GCF and 0.3% coriander essential oil (CEO) compared with control sample of pan bread, where all organoleptic parameters were higher than 95.33% from the upper limit.

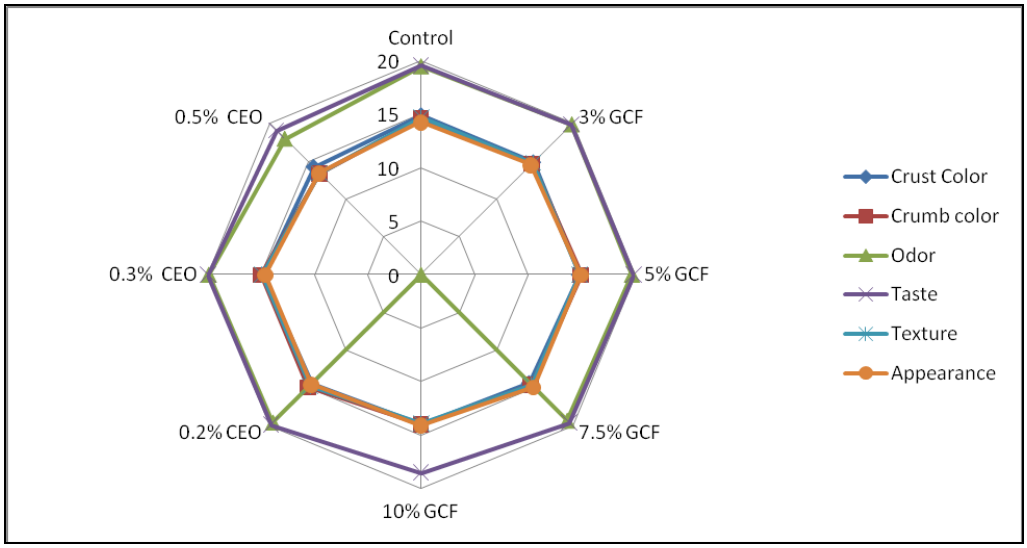

Figure 1. Sensory evaluation of pan bread incorporated with different % of coriander fruits and essential oils.

3.6. Microbial load in cake during storage period

To determine the efficiency of coriander fruits or its essential oil to prolong the shelf life of bakery products, cake was supplemented with coriander fruits (1, 3, 5, 7 and 10 %) and CEO (0.1, 0.3 and 0.5 %), compared with BHT at 200ppm and a control sample (without any additives).Storage was applied at room temperature (20± 2° C) for 24 weeks. Cake samples were prepared according to ES (2005). Data presented in Table (5) illustrated that no TBC or fungal counts were observed up to 6 weeks of storage in a control cake sample, this finding could be referred to the proper hygienic considerations applied during preparation. However, a slight growth (non visible growth) was detected 39 and 28 $CFU.g^{-1}$, respectively, and the numbers were approximately doubled, after 8 weeks, then clear microbial growth was noted (after 8 weeks). On the other hand, supplementation of cake with GCF succeeded in prolong the shelf life of cake samples up to 12 weeks in supplementation ratios of 1 and 3 %, however, it reached 16 weeks in the cake supplemented with 5 % coriander fruits.

Also, the perusal of data ascertained that supplementation with CEO could preserve the cake samples to 14, 16 and 22 weeks, at supplementation with 0.1, 0.3 and 0.5 % CEO, respectively. Furthermore, BHT (200ppm) led to prolong the shelf life of cake samples up to 14 weeks. These results are in harmony with those reported by Darughe, *et al.* (2012), who ascertained that CEO also showed better antifungal activity in cakesamplesat 0.15%. Such effect could be due to the presence of

terpenes and terpenoids compounds in the CEO. They also added that overall acceptability of cakes containing 0.05% CEO was almost equal to that of BHA.

3.7. Chemical quality evaluation during storage

The oxidation degradation of lipids is one of the main factors limiting the shelf life of food products. So, both peroxide value (PV) and thiobarbituric acid content (TBA) of the cake samples were determined during storage at room temperature (20± 2° C). Data presented in Table (6) indicated that the PV of control cake sample was higher than all supplemented cake samples at different storage periods and it increased gradually during storage time, which indicated that cakes were oxidized to lipid hydroperoxides, where, PV measures primary products of lipid oxidation. These unstable, primary oxidation products were consequently broken down by a free radical mechanism in which the O-O bond was cleaved on either side of the carbon atom bearing the oxygen atom to give the hydroxyl free radical and many types of secondary products such as alcohols, aldehydes, ketones and malonaldehydes which cause off-flavors (Lean & Mohamed, 1999). On the other hand, it was verified thatsupplementation with coriander on both forms (fruits or essential oil) gave slower increment rate of PV. It was noticed that the lowest PV was recorded for cakes prepared with 0.5 %CEO and BHT (200 ppm) compared with the respective initial mean value as well as control sample at zero time and during the storage period.

It is noteworthy that TBA measures the formation of secondary oxidation products, mainly malonadialdehyde, which may contribute off- flavors to Theoxidized oil (Rossel, 2005). Our findings indicated that TBA of control sample increased with storage time owing to the simultaneous increase in PV (Table, 6), wheresoever's, there were increments in the control sample that could be noticed during storage time at 20 ± 2°C up to 6 weeks, where the value reached 0.22 mg malondialdehyde/kg oil (initial TBA value was 0.11). This finding is highly in conformity with that reported by Darughe, *et al*. (2012), who mentioned that CEO exhibited good antioxidant activity in butter cake and its effect was comparable with BHA at 0.02%. These effects could be due to the presence of terpenes and terpenoids compounds in the CEO. Also, the finding of essential oil is due to its radical scavenging activity could be used as natural antioxidant to enhance the shelf stability of many foods (Ramadan *et al*., 2003). Moreover, addition of CEO at different levels also had TBA value almost equal to BHT at 0.02%. It indicates that CEO inhibited the formation rate of primary and secondary oxidation products in cake and their effects were almost equal to BHT at 0.02%. These findings could be due to Linalool, which has antioxidant activity as previously mentioned in Tables (3 and 4).

3.8.Specific volume of baked cake

It is noteworthy that the specific volume of baked cake indicated the amount of air that can remain in the final product, where a higher gas retention and higher expansion of the product leads to a higher specific volume. Furthermore, high voluminous cakes are desirable for the consumers. According to specific volume results (Table 7), the highest values were obtained by BHT (200 ppm) and 0.5% CEO (2.37 and 2.36 cm^3/g, respectively), compared to control cake, 2.34 cm^3/g (without additives) as shown in Table (7), which reflects the highest porosity and specific volume. On the contrary, cake supplemented with coriander fruits above 1%, led to decrement in specific volume. The explanation of this phenomenon is referred to nature and amount of dietary fiber are known to affect the specific volume of cakes (Aydogdu, *et al.,* 2018). Also, Jahanbakhshi & Ansari (2020) ascertained the reduction in specific volume after adding dietary fibers to cakes, which, probably happens because of a disruption in the gluten network which leads to the decrease in the gas retention capacity.

Table (5): Bacterial and fungal counts (CFU.g^{-1}) of English rich cake supplemented with coriander fruits and its essential oil during storage at room temperature (20 ± 2° C)

Microbial count	Control	Ground coriander fruits (%)					Coriander ess. oil (%)			BHT
		1	3	5	7	10	0.1	0.3	0.5	
At zero time										
TBC	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fungal	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
After 2 weeks										
TBC	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fungal	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
After 4 weeks										
TBC	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fungal	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
After 6 weeks										
TBC	3.9 X 10	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fungal	2.8 X 10	ND	ND	ND	ND	ND	ND	ND	ND	ND
After 8 weeks										
TBC	3.7 X 10^2	ND	ND	ND	3.1 X 10	ND	ND	ND	ND	ND
Fungal	2.4 X 10^2	ND	ND	ND	3.0 X 10	ND	ND	ND	ND	ND
After 10 weeks										
TBC	6.7 X 10^4	ND	ND	ND	3.9 X 10	3.7 X 10	ND	ND	ND	ND
Fungal	4.1 X 10^3	ND	ND	ND	3.1 X 10	3.0 X 10	ND	ND	ND	ND
After 12 weeks										
TBC	**MG	ND	ND	ND	7.7 X 10	4.9 X 10	ND	ND	ND	ND
Fungal	MG	ND	ND	ND	5.1 X 10	3.7 X 10	ND	ND	ND	ND
After 14 weeks										
TBC	MG	3.4 X 10	3.7 X 10	ND	ND	ND	1.3 X 10	ND	ND	3.1 X 10
Fungal	MG	3.1 X 10	3.7 X 10	ND	ND	ND	0.7 X 10	ND	ND	2.1 X 10
After 16 weeks										
TBC	MG	7.9 X 10	5.9 X 10	4.1 X 10	MG	1.7 X 10^2	3.0 X 10	1.2 X 10	ND	1.1 X 10^2
Fungal	MG	5.9 X 10	4.2 X 10	3.1 X 10	MG	1.1 X 10^2	1.2 X 10	0.5 X 10	ND	0.9 X 10^2
After 18 weeks										
TBC	MG	2.3 X 10^2	2.1 X 10^2	9.1 X 10	MG	MG	2.4 X 10^2	2.7 X 10	ND	MG

Fungal	MG	1.7×10^2	1.1×10^2	4.1 X 10	MG	MG	2.7 X 10	1.3 X 10	ND	MG
After 20 weeks										
TBC	MG	MG	MG	MG	MG	MG	MG	6.3 X 10	ND	MG
Fungal	MG	MG	MG	MG	MG	MG	MG	5.3 X 10	ND	MG
After 22 weeks										
TBC	MG	MG	MG	MG	MG	MG	MG	1.3×10^2	2.0 X 10	MG
Fungal	MG	MG	MG	MG	MG	MG	MG	0.6×10^2	1.3 X 10	MG
After 24 weeks										
TBC	MG	MG	MG	MG	MG	MG	MG	MG	1.7×10^2	MG
Fungal	MG	MG	MG	MG	MG	MG	MG	MG	0.8×10^2	MG

* TBC: Total Bacterial Count Values are means of three replicates. ** ND: No observed microbial growth.
[a] MG: Visible microbial growth. BHT was added at 200 ppm

Table (6): Effect of supplemented with GCF and CEO on lipid profile of cake during storage (20 ± 2° C).

Oxidative stability	Control	GCF (%)					CEO (%)			BHT (200ppm)
		1	3	5	7	10	0.1	0.3	0.5	
At zero time										
PV*	1.74	1.61	1.61	1.61	1.61	1.61	1.61	1.61	1.61	1.61
TBA**	0.11	0.11	0.11	0.10	0.10	0.12	0.08	0.08	0.09	0.08
After 2 weeks										
PV	2.84	1.57	1.59	1.56	1.59	1.59	1.54	1.52	1.52	1.48
TBA	0.13	0.11	0.11	0.11	0.11	0.10	0.08	0.08	0.09	0.12
After 4 weeks										
PV	3.12	1.55	1.59	1.59	1.59	1.59	1.54	1.53	1.55	1.41
TBA	0.17	0.13	0.12	0.11	0.11	0.11	0.08	0.09	0.09	0.11
After 6 weeks										
PV	3.37	1.40	1.54	1.61	1.60	1.61	1.53	1.53	1.49	1.40
TBA	0. 22	0.14	0.12	0.13	0.12	0.13	0.09	0.10	0.09	0.09
After 8 weeks										
PV	4.11	1.42	1.54	1.61	1.60	1.61	1.53	1.50	1.42	1.42
TBA	0.34	0.15	0.12	0.13	0.12	0.16	0.09	0.10	0.10	0.08

After 10 weeks										
PV	4.17	1.44	1.52	1.64	1.55	1.59	1.52	1.49	1.40	1.44
TBA	0.44	1.15	0.13	0.14	0.12	0.19	0.09	0.11	0.10	0.07
After 12 weeks										
PV	**MG	1.48	1.50	1.62	1.52	1.56	1.51	1.48	1.40	1.41
TBA	MG	1.14	0.15	0.14	0.13	0.22	0.12	0.11	0.10	0.07
After 14 weeks										
PV	MG	1.49	1.50	1.63	1.50	1.53	1.48	1.47	1.32	1.45
TBA	MG	0.16	0.17	0.14	0.13	0.28	0.12	0.11	0.10	0.07
After 16 weeks										
PV	MG	1.52	1.50	1.63	MG	1.52	1.46	1.40	1.29	1.49
TBA	MG	1.18	0.18	0.17	MG	0.33	0.13	0.11	0.09	0.09
After 18 weeks										
TBC	MG	1.55	1.49	1.60	MG	MG	1.43	1.40	1.29	MG
TBA	MG	0.18	0.18	0.18	MG	MG	0.13	0.11	0.11	MG
After 20 weeks										
PV	MG	MG	MG	MG	MG	MG	MG	1.39	1.29	MG
TBA	MG	MG	MG	MG	MG	MG	MG	0.14	0.12	MG
After 22 weeks										
PV	MG	MG	MG	MG	MG	MG	MG	1.38	1.31	MG
TBA	MG	MG	MG	MG	MG	MG	MG	0.15	0.13	MG
After 24 weeks										
PV	MG	MG	MG	MG	MG	MG	MG	MG	1.37	MG
TBA	MG	MG	MG	MG	MG	MG	MG	MG	0.15	MG

Values are means of three replicates. Samples spoiled by microbial growth didn't subject to the tests.

* PV is expressed as mequivalent peroxide / Kg oil. ** TBA is expressed as mg malondialdehyde/kg oil

3.9. Anti-diebetic activity

The produced bread loaves with 0.3 and 5%, CEO and GCF, respectively, were selected to be utilized *in vivo* anti-diebetic activity on experimental rats, (according to sensory evaluated), beside aqueous macerated as well as aqueous decocted coriander extracts.

Table (7). Specific volume of cake supplemented with ground coriander fruits and their essential oil at different concentrations.

Cake sample formulation	Weight (g)	Volume (cm^3)	Specific volume(cm^3/g)
Control	110.88	260	2.34
BHT (200 ppm)	100.9	240	2.37
Coriander essential oil (0.1 %)	97.82	230	2.35
Coriander essential oil (0.3 %)	101.53	236	2.32
Coriander essential oil (0.5 %)	98.85	235	2.36
Ground coriander fruits (1%)	126.22	290	2.35
Ground coriander fruits (3%)	154.6	350	2.26
Ground coriander fruits (5%)	145.81	305	2.04
Ground coriander fruits (7%)	145.14	290	1.99
Ground coriander fruits (10%)	130.89	256	1.95

Table 8. Body weight gain and serum glucose level of rats given with coriander fruits and their extracts.

Groups	Body weight				Serum Glucose (mg/dl)
	Initial	final	WG	BWG (%)	
G1 (Normal Control)	220.0	250.8	30.8	14.00	84.27^{e}±2.02
G2 (Diabetic Control)	220.0	240.3	20.3	9.23	256.47^{a} ±4.57
G3	220.0	280.5	60.5	27.50	109.74^{d}±2.60
G4	221.0	288.0	67.0	30.32	104.40^{d}±1.52
G5	220.0	277.0	57.0	25.91	115.31^{c}±2.09
G6	220.0	277.0	57.0	25.91	119.00^{c}±1.83
G7	220.0	275.0	55.0	25.00	205.32^{b}±2.17
G8	219.0	273.0	54.0	24.66	209.10^{b}±5.18

The mean values (n=5)± SD with different letters in the same column are significantly ($p \geq 0.05$) different. * WG: Weight gain BWG: Body weight gain

G3: Diabetic rats group orally injection decoction coriander extract.

G4: Diabetic rats group orally injection macerated coriander extract.

G5: Diabetic rats group, fed on coriander powder (2gm /100gm diet).

G6: Diabetic rats group orally injection coriander essential oil (40 mg/kg bw daily).

G7: Diabetic rats group, fed on a basal diet with pan bread containing 0.3% oil.

G8: Diabetic rats group, fed on a basal diet with pan bread (5 % coriander fruits).

By referring to the findings in Table (8), it could be noticed that all groups received GCFor their extracts recorded higher body weight gain % (24.66 – 30.32%), compared with that of normal control group (G1, 14.00%) and diabetic control group (G2, 9.23%). The highest values pertained to those groups given aqueous extracts of coriander fruits (G4 & G3, decocted and macerated extracts, respectively). Our findings are supported by Khubeiz&Shirif (2020), who found that final body weight was ($p<0.01$) higher in 2% coriander fruits and feed conversion ratio was significantly ($p<0.05$) better for birds compared with other groups.

The results presented in Table (8) also illustrated that there are significant ($p \geq 0.05$) differences in serum glucose level among all tested groups of rats, however, the normal control group recorded the lowest glucose level (84.27mg/dl), followed by rats received aqueous macerated coriander and aqueous decocted coriander extracts, with non significancy ($p \geq 0.05$) between the last two groups. Similar findings were observed in rat groups received ground coriander fruits (115.31mg/dl) and coriander essential oil (119.00mg/dl). Although, high serum glucose levels noticed in groups received pan bread incorporated with either ground coriander fruits or their essential oil (209.10 and 205.32mg/dl, respectively), they are still significantly lower than that of diabetic control group (256.47mg/dl).

Our findings are in harmony with those found by Gray & Flatt (1999), who ascertained the antidiabetic potential of coriander fruits in streptozotocin-induced diabetic mice. They also observed that consumption of coriander fruits aqueous extract evoked 1.3-5.7 fold stimulation of insulin secretion from colon β-cell line and increased the 2-deoxyglucose transport by 1.6 folds, glucose oxidation by 1.4 folds and incorporation of glucose into glycogen of isolated abdominal muscle by 1.7 folds. They purported also ascertained that insulin releasing and insulin like activity of coriander. Similar findings were also reported by Waheed, et al. (2006), for an aqueous extract of coriander.

Concerning lipid profile of rats as affecting by incorporated with coriander fruits and their extracts, results presented in Table (9) showed that triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL) reduced in all tested groups, with different rates compared to diabetic rats group, however, the parameters of a group incorporated with aqueous extract (decoction one) is close to normal control group in most parameters; no significant ($p \geq 0.05$) differences (TG& LDL), followed by a group orally injected with aqueous extract (maceration one). Also, both coriander fruits and its essential oil could reduce the lipid profile parameters.

Table 9. Blood Lipid profile of rats incorporated with coriander fruits and their extracts

Groups	Lipid profile of rats			
	TG (mg/dL)	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)
G1 (Normal Control)	$118.15^{d}\pm5.03$	$120.53^{g}\pm7.21$	$41.90^{e}\pm4.37$	$38.60^{e}\pm1.83$
G2 (Diabetic Control)	$260.80^{a}\pm9.51$	$276.34^{a}\pm4.49$	$226.40^{a}\pm6.79$	$24.56^{f}\pm4.90$
G3	$128.90^{c}\pm0.10$	$158.90^{e}\pm5.63$	$105.64^{d}\pm2.40$	$41.92^{de}\pm4.72$
G4	$114.70^{d}\pm6.95$	$150.33^{f}\pm5.55$	$100.00^{de}\pm4.30$	$37.27^{e}\pm3.90$
G5	$129.00^{c}\pm5.01$	$185.00^{c}\pm7.50$	$111.60^{c}\pm2.55$	$44.82^{d}\pm2.79$
G6	$140.00^{b}\pm4.23$	$180.50^{cd}\pm3.76$	$109.50^{c}\pm4.90$	$60.52^{b}\pm7.55$
G7	$133.00^{bc}\pm3.76$	$176.64^{d}\pm6.79$	$121.71^{bc}\pm3.45$	$55.53^{c}\pm4.87$
G8	$122.00^{cd}\pm2.93$	$196.40^{b}\pm4.01$	$124.20^{b}\pm2.66$	$63.50^{a}\pm2.66$

The mean values (n=5) ± SD with different letters in the same column are significantly different($p<0.05$).

On the other hand, high density lipoprotein cholesterol (HDL) increased as a result of incorporation with coriander fruits and their extracts in a similar pattern. Our findings are supported with the results of Naquvi, et al. (2012), where they showed that aqueous extract of fruits of coriander (obtained by maceration) at two doses 250 and 500 mg/kg, decreased significantly blood glucose level, with the superiority to higher dose. Also, it also significantly decreased total cholesterol level and increased high density lipid cholesterol.They also ascertained that the aqueous extract of coriander had antidiabetic activity. Numerous investigations proved the hypoglycemic efficiency of coriander essential

oil, Paarakh, (2017) showed that 75 % methanol extract showed significant decrease in blood glucose level at a dose of 100 mg/kg and 200 mg/kg. It also decreased the lipid parameters such as total cholesterol, total triglycerides ASTand ALT when compared with diabetic control. However, Al-Jaff(2011) reported that 2% coriander fruits lower serum glucose ($p<0.05$), serum total cholesterol and LDL, while HDL increased when compared with the control. On this connection, Khubeiz & Shirif (2020) ascertained that the serum TG of broiler chickens was significantly reduced at 1.5%, while the HDL was significantly increased at level 1.5% when compared with the control.

Table 10. Liver and kidney functions of rats incorporated with coriander fruits and their extracts

Groups	Liver function enzymes		Kidney functions	
	AST(U/L)	ALT (U/L)	Creatinine(mg/dl)	Urea (mg/dl)
G1 (Normal Control)	29.55±0.11	24.55±0.56	36.80±0.83	36.82±0.32
G2 (Diabetic Control)	80.56±0.45	73.56±0.44	60.00±0.23	59.53±0.53
G3	44.65±0.67	33.52±0.51	48.50±0.31	47.96±0.66
G4	40.54±0.73	30.75±0.67	40.84±0.23	43.57±0.72
G5	55.38±0.45	40.75±0.53	52.92±0.34	50.80±0.79
G6	51.92±0.11	44.06±0.32	50.00±0.44	52.58±0.33
G7	49.59±0.22	42.56±0-87	53.94±0.32	55.82±0.67
G8	48.51±0.36	43.72±0.34	49.30±0.45	51.59±0.48

The mean values (n=5) ± SD with different letters in the same column are significantly different($p<0.05$).

Regarding liver and kidney functions, results in Table (10), ascertained that all tested groups incorporated with coriander fruits and their extracts recorded the lowest liver function enzymes (AST&ALT), compared with diabetic group. On the other hand, groups of rats orally injected with aqueous extracts by decoction and maceration are closed to those of normal control group. Similar findings were observed for creatinine and urea as kidney functions. Concerning liver function enzymes, our findings are in the same line with those found by Moustafa, *et al.*(2012). Their conclusion depends on the histological observations basically and supported the results obtained from serum enzyme assays. Also, these results are in harmony with those found by Sreelatha, *et al.* (2009) and El- Masry, *et al*. (2016). They added that active components which present in coriander fruits extracts, including flavonoids, polyphenols and carotenoids had antioxidant, anti-inflammatory and free radicals scavenging activities.

4.Conclusions

The obtained results ascertained the potency of coriander fruits and their essential oils as biopreservative agent, for bakery products. Also, on the basis of aforementioned facts, the antidiabetic ability of coriander fruits and their extracts.

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EFFECT OF ULTRAVIOLET (UV-C) LIGHT AND GASEOUS OZONE ON MICROBIAL AND COLOR QUALITIES OF WHOLE BLACK PEPPER SEEDS (*PIPER NIGRUM* L.)

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ABSTRACT

Microbial contamination of spices, especially black pepper, may sometimes reach as high as 8 log cfu/g, which can cause a major problem in the quality and safety of foods they are added. This study aimed to explore the potential of ultraviolet C (UV-C) light and ozone for decontamination of black pepper seeds.

Ozone (15 ppm for 1 h) and UV-C (28.8 J/cm^2) treatments were applied alone, in succession, or simultaneously to whole black pepper seed with a laboratory scale fluidized bed UV-C system. Total aerobic mesophilic bacteria (TAMB) count (in uninoculated seeds), *Escherichia coli* (*E. coli*) count (in inoculated seeds) and color (L*, a* and b*) of black pepper were evaluated.

Ozone and UV-C treatments alone caused 0.41- and 0.77-log reduction in the initial TAMB count (6.97 log cfu/g), respectively. TAMB decreased by 0.74- and 0.66-log upon successive and simultaneous treatments, respectively. Thus, the combined treatments did not have any additive or synergistic effects on TAMB. Both ozone and UV-C treatments alone resulted a 0.8-log reduction in the initial *E. coli* count of 6.3-log. The combined treatments caused an additive effect on inactivation of *E. coli* in black pepper seeds. The successive and simultaneous treatments caused 1.7- and 1.4-log reductions in the *E. coli*, respectively. None of the treatments affected the color (L*, a* and b* values) significantly. In conclusion, the individual treatment has potential for reducing the natural contamination level on the seeds, and the combined treatments may have further potential towards reducing specific microbial contaminations.

1.Introduction

Black pepper (*Piper nigrum* L.) is one of the most widely used spice to enhance the flavour of foods. It is a tropical, perennial climbing plant belonging to the family Piperaceae. The black pepper seeds are produced from berries that have just started to yellow. The berries are generally blanched by immersing them in hot water at 90 ºC for up to 10 minutes. Then, they are dried to reduce moisture content below 12% and to develop the dark, wrinkled layer (Zachariah, 2000). Although these steps reduce microbial load, black pepper, like other herbs and spices, generally contain a high level of microbial load up to 6-8 log cfu/g, which may include pathogenic and/or spoilage organisms. Spices may be contaminated by microorganisms because of poor hygienic conditions under which they are cultivated, harvested, transported and stored. High microbial load of spices may

cause a risk to consumer health if they are added to high moisture foods which are eaten raw or not further processed (Schweiggert et al., 2007; Farkas and Mohácsi-Farkas, 2014). Therefore, appropriate decontamination methods should be applied to spices to food quality and safety.

Fumigation, steam sterilization and irradiation are the most common methods for microbial inactivation in spices (Tainter and Grenis, 2001). Fumigation with ethylene oxide is an oldest decontamination method applied to spices. However, it is not allowed in many countries due its carcinogenic effects (Fowles et al., 2001). Steam sterilization is a chemical-free process but it is associated with color degradation and a reduction in volatile oil content. Additionally, steam sterilization increases the moisture content of spices and requires additional drying step to avoid microbial growth (Schweiggert et al., 2007). Irradiation is another commercial decontamination method, which is approved by many authorities such as Food and Drug Administration, World Health Organisation, and the Codex Alimentarius Commission (Sadecka, 2007). But it has some drawbacks such as formation of oxidative compounds, high costs of installation, and consumer's negative perception (Sharma and Demirci, 2003). Hence, there is a need for finding effective, easily applicable and nontoxic alternative methods to decontaminate spices. Several research studies investigating various technologies to reduce the microbial load of herbs and spices have been reported (Keith et al., 1997; Staack et al., 2008; Eliasson et al., 2014; Hertwig et al., 2015; Nicorescu et al., 2013; Kim et al., 2014; Erdoğdu and Ekiz 2011; Erdoğdu and Ekiz 2013; Cheon et al., 2015).

Ozone is a potent, broad-spectrum antimicrobial agent against a wide variety of microorganisms (Khadre et al., 2001). Because of its high reactivity, high penetration power, short contact time and generally recognized as safe (GRAS) status it can be employed under various forms for decontaminating foods (Patil et al., 2014). Potential of gaseous ozone has been shown for decontamination of herbs and spices such as black pepper (Emer et al., 2008), flaked red peppers (Akbas and Ozdemir, 2008), oregano (Torlak et al., 2013), sumac, cumin and pepper (Hemmati et al., 2017).

UV-C radiation is another effective non-thermal technology generally used for various food surfaces and liquid products. It has lethal effect on microorganisms by forming pyrimidine dimers, disrupting the DNA, which stops its ability to reproduce (Gómez-López et al., 2012). Process parameters, microbial characteristics and product parameters are the main factors affecting microbial resistance to UV-C light (Gayán et al., 2014). The use of UV-C light is limited with food surfaces due to its poor penetrative capacity (Guerrero-Beltrán and Barbosa-Cánovas, 2004). Thus, effective exposure of the surfaces to UV-C is critical for its efficiency in microbial inactivation. This needs new design approaches to make the product surfaces exposed to UV-C effectively. Moreover, hurdle approach to have UV-C combined with other nonthermal methods can be an effective way to enhance microbial inactivation in the products. A lab scale fluidized bed UV-C system was built and tested for decontaminating thyme (*Thymus vulgaris* L.) in our earlier works (Dogu-Baykut et al., 2014; Dogu-Baykut and Gunes, 2019). A limited but significant reduction (up to 1.8-log) in natural microbial load of the samples were observed in these studies. Combination of UV-C and ozone may have improved potential for microbial decontamination of dehyrated herbs and spices. To our knowledge there is no information on effectiveness of such combination on decontamination of powdered spices. The present study was aimed to explore the potentials of combined uses of ozone and UV-C for decontamination of black pepper seeds.

2. Materials and methods

2.1. Chemicals and materials

Unsterilized and dried whole black pepper seeds were obtained from a local company (Kadioglu Spice Co. Inc., Mersin, Turkey). Black pepper seeds, which were separated for use in bacterial inoculation studies, were

weighed to polyethylene (PE) packages and sterilized by gamma rays from a ^{60}Co source at a commercial irradiation facility (Gamma-Pak Sterilization Co., Cerkezkoy-Tekirdag, Turkey) at room temperature.

Sodium chloride was obtained from Riedel-de Haen (Seelze, Germany). Plate count agar (PCA), Chromocult Tryptone Bile X-glucuronide (TBX) Agar, tryptic soy broth (TSB), and buffered peptone water (BPW) was purchased from Merck (Darmstadt, Germany). Peptone was from Oxoid (Basingstoke, Hampshire, UK).

2.2. Preparation of bacterial suspension

A loop of *E. coli* (ATCC 25922) was taken from slant medium and inoculated into TSB broth and incubated at 37℃ for 18 h. Bacterial cells were separated by centrifugation at 5000 rpm for 5 min. The cells were centrifuged for the second time by adding equal volume of TSB and then resuspended in BPW buffer. A stock suspension of *E. coli* with 10^9 cfu/ml in BPW was prepared using McFarland standard (BioMerieux, Durham, NC, USA).

2.3. Inoculation of bacteria

Black pepper samples weighed in a beaker and then the *E. coli* suspension was added into the beaker with a 1:1 ratio (w/w). Intermittent mixing was provided during 5 min dipping period at room temperature to have homogeneous inoculation. The inoculated seeds were separated from the bacterial suspension by filtering through a sterile cheese cloth and placed as a single layer into an open sterile container. The seeds were first incubated at 25 ℃ and 90% relative humidity for 24 h for acclimation of the bacteria on the seeds. Then, the seeds were dried at 30 ℃ and 30% relative humidity for 24 h to achieve their initial moisture and water activity (a_w) levels prior to UV-C and ozone treatments.

2.4. Determination of moisture/relative humidity/water activity

The moisture content of the samples was determined by infrared moisture analyzer (IR35, Denver Instruments, Fisher Scientific, USA). A 2 g. of sample was weighed into the measuring cup and analyzed at 106 ℃.

In the inoculation study, the relative humidity of the environment was measured with a data logger (HOBO U12-013 Temp/RH/2 External Data Logger, Onset Computer Corporation, Bourne, MA, USA) in every 5 minutes.

The a_w of the samples was determined by Lab master a_w device (Novasina, Lachen, Switzerland). After placing the samples into the sample holder, a_w values were measured at 25 ℃.

2.5. UV-C and ozone treatments

The experimental UV-C and ozone system is schematically shown in Figure 1. The UV-C part of the combined system consisted of a quartz glass cylindrical tube (10 cm x 100 cm) with four UV lamps emitting 254 nm light (GHO36T5L/4P, Atlantic Ultraviolet Inc., Hauppauge, NY, USA) installed around it. This system was connected to an air pump with a pipe (100 cm × 10 cm). Air velocity in the treatment area was set approximately to 8m/s to keep the seeds fluidized in the system. Ozone gas was generated using a laboratory scale ozone generator (Model No. H-50, HessMachines International, PA, ABD) and fed through a 6 mm tubing connected to the pipe carrying the air to the system (Fig.1) Ozone concentration was measured continuously by an ozone monitor (Model 106-M, 2B Technologies Co., USA) in the system.

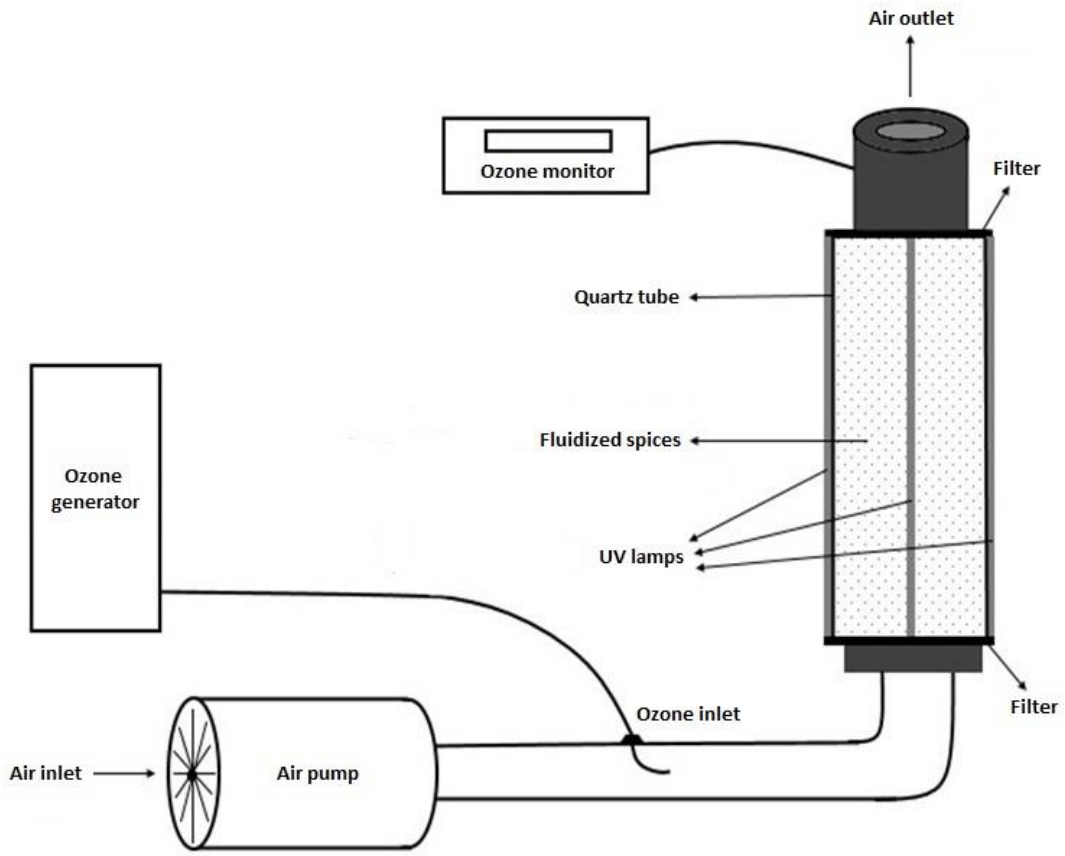

Figure 1. Schematic diagram of the combined UV-C and ozone system.

Each treatment was performed with 10 g sample loaded to the UV system. UV-C and ozone treatments were applied alone (1 h), in succession (1 h ozone after 1 h UV-C) or simultaneously (ozone and UV-C together for 1 h). The ozone application was applied at a concentration of 15 ppm. UV-C intensity was measured with a portable digital radiometer, which measures UV radiation at 254 nm and fitted with a UVX-25 sensor (UVP Inc., Upland, CA, USA), and the average UV intensity was determined as 8 mW/cm^2 in the system. The UV dose was calculated as 28.8 J/cm^2 for 1 h exposure. The control samples were treated for 2 h in the same air flow without ozone and UV-C. The experiment was repeated three times for each treatment.

2.6. Microbial analysis

Pour plate technique was used in microbial analyses (ICMSF 1978). Homogenized samples were prepared by mixing 10 g black pepper seeds with 90 ml of peptone water (0.1%) at medium speed for 2 min using a stomacher (AESAP1068-Easymix, AES Chemunex, Combourg, France). The homogenized samples were diluted in peptone water as needed. The PCA plates used for enumeration of total aerobic mesophilic bacteria were incubated at 37 ℃ for 48 h. TBX plates used for enumeration of *E. coli* were incubated at 44 ℃ for 24 h. The colonies on agar plates were counted and expressed as log cfu/g sample.

2.7. Color analysis

Three color parameters L*, a* and b* of the black pepper samples after UV-C and ozone treatments were measured with a chromameter (Model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The instrument was calibrated using a standard white calibration plate (CR-A43, Konica Minolta Sensing Inc., Tokyo,

Japan) prior to analysis. Color values were measured on each sample (10 g) at three different positions and averaged.

The total color difference (ΔE) with reference to the control samples was calculated using the following equation:

$$\Delta E = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$$

2.8. Statistical analysis

Each treatment was applied in triplicate. Analysis of variance (ANOVA) was performed using SPSS version 21 (SPSS Inc., Chicago, IL, USA). Multiple comparisons of the treatments were done by Duncan's multiple-range test.

3. Results and discussions

3.1. Effect of UV-C and ozone treatments on TAMB

In the preliminary tests, ozone concentration less than 15 ppm did not cause a significant inactivation of total aerobic mesophilic bacteria (TAMB) on black pepper seeds. For this reason only 15 ppm ozone, which was the maximum level achieved in our system, were applied in our study.

Table 1 shows the effect of UV-C and ozone treatments on microbial quality of black pepper samples as assessed by TAMB counts. Initial TAMB count of untreated sample was 6.97 log cfu/g. The UV-C treatment (28.8 J/cm^2) alone decreased the TAMB count of black pepper seeds by 0.77-log ($p<0.05$). The ozone treatment (15 ppm for 1 h) alone caused a 0.41-log reduction in the TAMB count in the samples ($p<0.05$). Although each of the UV-C and ozone treatments resulted in significant reduction in TAMB count of black pepper seeds, inclusion of ozone to UV-C in succession or simultaneously did not have any additive or synergistic effects on inactivation of TAMB ($p>0.05$). TAMB decreased by 0.74- and 0.66-log upon successive and simultaneous treatments, respectively.

We observed a 0.4- and 1.8-log reduction in TAMB count in thyme with 25.7 and 205.6 J/cm^2 UV-C using the same system in our previous work, respectively (Dogu-Baykut and Gunes, 2019). Erdoğdu and Ekiz (2011) reported that UV-C treatment at 37.8 J/cm^2 reduced the TAMB counts of cumin seeds by 0.6-log. Hidaka and Kubata (2006) studied on wheat grain and reported that 6.3 h is required for 1-log reduction in the number of TAMB at a UV-C intensity of 9.7 mW/cm^2 on the recirculating grain sterilization equipment. Different levels of microbial inactivation on dehydrated seeds and herbs by UV-C reported in literature is certainly due to differences in the UV-C system used, food product, and state of microbial flora on the samples.

Limited accessibility of UV-C light to the microorganisms in the lower layers on the surface of foods is the main difficulty in UV light studies. So, hurdle strategies that combine UV light with other novel processing techniques were used in some studies to achieve the necessary microbial reduction. Erdoğdu and Ekiz (2011, 2013) combined UV-C and far infrared radiation (FIR) to decontaminate cumin and black pepper seeds. They obtained additional 1.65-log reduction when cumin seeds exposed to the UV-C light (10.5 mW/cm^2) for 2 h following 5.6 min FIR treatment at 200 ºC. Under the same conditions, combined UV-C and FIR treatments were also used for microbial inactivation of black pepper. However, synergistic effect of UV-C with FIR treatment was not observed. The authors stated that this may be due to surface topography or higher initial microbial count of black pepper seeds.

In the literature, the results of the studies searching decontamination potential of gaseous ozone vary according to the sample type, concentration of ozone gas and exposure time. Surface area is also important factor during ozonation because ground samples require a higher ozone concentration and longer exposure than the whole grain samples to achieve similar microbial inactivation (Akbas and Ozdemir, 2008). Dhillon et al. (2010) designed a fluidized bed system to decontaminate durum wheat grain with gaseous ozone. They found that application of gaseous ozone at 6 ppm for 14 min did not affect aerobic plate count (APC) and yeast and mold count (YMC). In another study conducted by Torlak et al. (2013), gaseous ozone treatment on dried oregano at 2.8 ppm for 30 min did not

significantly reduce initial levels of APC and YMC on oregano, however, ozone treatment at 5.3 ppm for 30 min significantly reduced levels of APC and YMC by 0.5- to 0.4-log, respectively.

In our study, we also obtained a statistically significant decrease of 0.41-log in TAMB count on black pepper after treating with 15 ppm ozone for 1 h. Furthermore, we combined ozone with UV-C treatment. It was expected that ozone-damaged cells could be inactivated by UV-C more effectively, and this combination could have a synergistic effect, but this was not observed in our work. This may be associated with limited penetration of UV-C and ozone to the surface contamination which probably contain biofilm. The inactivated microbial cells by one of the treatments on the most outer surface probably shaded the viable cells in the inner parts against the next treatment.

Table 1. Effect of UV-C and ozone treatments on the TAMB (log cfu/g) of black pepper.

Application method	Application	TAMB	Reduction
Control	2 h air alone	6.97 ± 0.07^{a}	-
Ozone (15 ppm)	1 h	6.56 ± 0.17^{b}	0.41 ± 0.17^{a}
UV-C (28.8 J/cm^2)	1 h	6.20 ± 0.19^{c}	0.77 ± 0.19^{b}
Ozone → UV-C (in succession)	1 h UV-C after 1 h ozone	6.23 ± 0.13^{c}	0.74 ± 0.13^{b}
Ozone + UV-C (in simultaneous)	1 h UV-C and ozone together	6.31 ± 0.02^{c}	0.66 ± 0.02^{ab}

Data represent mean values (n = 3) ± standard deviations
Means within a column having the same letter are not significantly different (p>0.05).

3.2. Effect of UV-C and ozone treatments on *E. coli*

Properties of samples during inoculation steps is shown in Table 2. After inoculation, the samples were stored at 25 ºC and 90% RH for 24 h to stabilize the bacterial population in the samples. In our preliminary studies it was observed that the number of the inoculated bacteria on the sample decreased rapidly when no conditioning at high relative humidity was applied after inoculation. Thus, with the applied inoculation process, a stable *E. coli* count (6.82 log cfu/g) was achieved prior to UV-C/ozone treatments.

Each of the UV-C and ozone treatments caused a 0.8-log reduction in the initial *E. coli* count of 6.3-log, as shown in Table 3. The successive and simultaneous treatments caused 1.67- and 1.38-log reductions in the *E. coli*, respectively. Thus, UV-C and ozone had additive effect on inactivation of inoculated *E. coli* as opposed to TAMB. This may be associated with presence of biofilms of natural flora (TAMB) and lack of biofilms of *E. coli* in freshly inoculated samples. It is known that biofilms make microorganisms more resistant to environmental conditions (Srey et al., 2013).

There are some studies in literature which examined the potential of ultraviolet light and ozone for inactivation of *E. coli* O157:H7 in poultry chiller water and on blueberries (Ngadi et al., 2004; Kim and Hung, 2012). Similar to our results, combined treatments of ozone (4000 ppm for 1 min) and UV-C (0.95 J/cm^2) achieved additional reduction on *E. coli* O157:H7 count on blueberry samples compared to the UV-C or the ozone alone (Kim and Hung, 2012). The UV-C treatment with an intensity of 7.95 mW/cm^2 for 2 min (0.95 J/cm^2) alone caused a 2.2-log reduction in *E. coli* O157:H7 count on the blueberries while the combined treatment caused a 3.7-log reduction on the bacterial count. Similarly, Ngadi et al. (2004) found an additive effect of UV-C (7 J/cm^2) and ozone (1 mg/ml for 30 s) treatments on inactivation of *E. coli* O157:H7 in poultry chiller water when they are applied together.

Table 2. Change of water activity, moisture and *E. coli* count of black pepper seeds during the inoculation with *E. coli* (ATCC 25922)

	Initial sample	**Upon inoculation**	**After 24 h of holding at 25 ºC and 90% RH**	**After drying for 24 h at 30 ºC and 30% RH**
a_w	0.54±0.01	0.94±0.01	0.93±0.01	0.53±0.02
Moisture (%)	3.42±0.13	16.41±0.87	18.02±1.63	3.40±0.12
E. coli count (log cfu/g)	-	7.16±0.12	7.54±0.16	6.82±0.14

Data represent mean values (n = 3) ± standard deviations.

Table 3. Effect of UV-C and ozone treatments on the *E. coli* count (log cfu/g) of inoculated black pepper seeds.

Application method	**Application**	***E. coli***	**Reduction**
Control	2 h air alone	6.35±0.04[a]	-
Ozon (15 ppm)	1 h	5.53±0.09[b]	0.82±0.09[a]
UV-C (28.8 J/cm^2)	1 h	5.52±0.34[b]	0.83±0.34[a]
Ozon → UV-C (in succession)	1 h UV-C after 1 h ozone	4.68±0.22[c]	1.67±0.22[b]
Ozon + UV-C (in simultaneous)	1 h UV-C and ozone together	4.97±0.14[c]	1.38±0.14[b]

Data represent mean values (n = 3) ± standard deviations
Means within a column having the same letter are not significantly different (p>0.05).

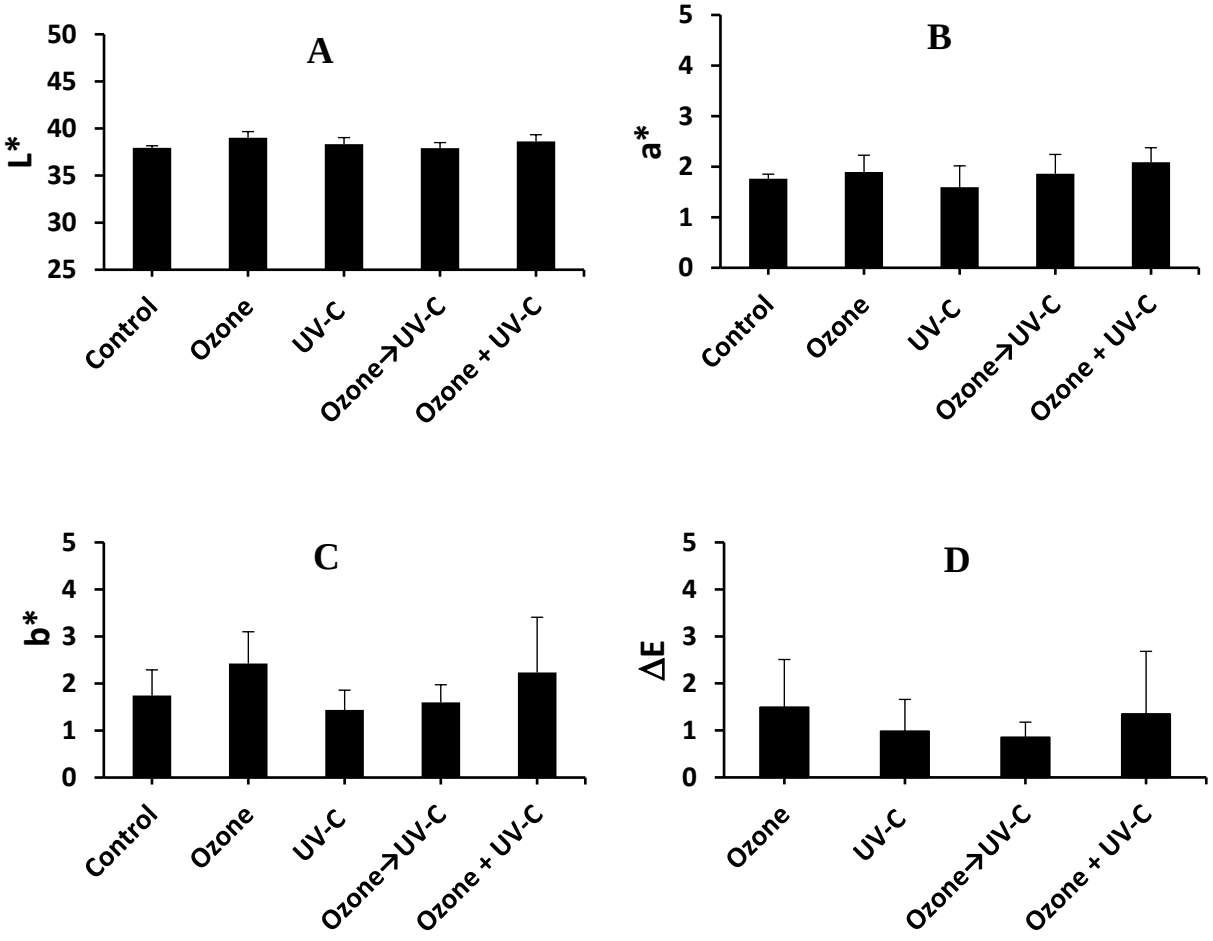

Figure 2. Effect of UV-C and ozone treatments on redness (a), yellowness (b), lightness (c), and total color difference (d) of black pepper. Error bars represent standard deviations of the mean (n = 3). Ozone: 15 ppm for 1 hr; UV-C: 28.8 J/cm^2; Ozone→UV-C: combined treatment applied in succession; Ozone+UV-C: combined treatment applied simultaneously.

3.3. Effect of UV-C and ozone treatments on color values

Black pepper seeds have black, bright color. The loss of color during decontamination processes is important factor for consumer acceptance. The change in L *, a *, b * and total color values of black pepper seeds with UV-C and ozone applications are shown in Figure 2.

The initial L*, a* and b* values of black pepper seeds were measured as 37.96, 1.76 and 1.74, respectively. It was found that the use of the ozone and the UV-C applications individually or together did not cause a significant change in the color values of black pepper ($p>0.05$). Total color changes on the samples were not affected by any of the treatments either ($p>0.05$). The highest total color change (ΔE values) was only 1.49 in the ozone treated samples (Fig. 2). Virtanen et al. (2014) reported that ΔE values below 2 indicates a small difference and cannot be distinguished by an uneducated eye.

Erdoğdu and Ekiz (2013) did not observe a change in Hunter color values of black pepper subsequent to UV-C treatment at 75.6 J/cm^2. Akbas and Ozdemir (2006, 2008) found that flaked red pepper and ground pistachios, which exposed to ozone concentrations at 7 and 9 ppm, had slight, but significantly lower scores for appearance than the samples exposed to ≤5 ppm by panelists on sensory panels. But no significant changes on appearance were obtained by panelists for pistachio kernels. In another study, oregano samples treated with ozone gas at a concentration of 5.3 ppm for 120 min were graded significantly lower than the samples treated at 2.8 ppm for 120 min (Torlak et al., 2013).

4. Conclusions

The UV-C (28.8 J/cm^2) and the ozone treatments (15 ppm for 1 h) caused significant reductions in TAMB and *E. coli* counts (up to 0.8-log) in black pepper seeds. While the combination of these treatments did not have any additive or synergistic effects on TAMB, further inactivation of freshly inoculated *E. coli* on the seeds (up to 1.7-log) was observed. The applied treatments did not adversely affect the color of the samples.

Thus, each of the UV-C (28.8 J/cm^2) and the ozone treatments (15 ppm for 1 h) has some potential for reducing the natural contamination level on black pepper seeds, but the combined treatment may have further potential towards inactivation of specific microorganisms. Increased UV-C and ozone intensities may further increase the inactivation levels and thus should be investigated in further studies.

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TEXTURAL, PHYSICOCHEMICAL AND ORGANOLEPTIC PROPERTIES OF PARTIALLY REPLACED FAT COOKIES INCORPORATED WITH APRICOT KERNEL FLOUR

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ABSTRACT

This study was conducted to respond the current demand of health-conscious consumers towards reduced-fat and fiber enriched foods. Apricot kernel flour (AKF) had significant amount of total phenol contents and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity (83.89±3.53%). Different concentrations (20%, 40%, 60%, 80% and 100%) of AKF were used to replace the shortening in supplemented cookies. In supplemented cookies, fat content reduced from 23.53±0.23% to 3.82±0.03% by increasing level of AKF. Spread value decreased in high percentage AKF supplemented cookies. Total dietary fibers (TDF) and texture values (by Texture Analyzer) increased significantly ($p \leq 0.05$) from 2.63±0.09% to 31.82±1.11% and 3.27±0.11 N/kg to 6.02±0.21 N/kg by enhancing AKF supplementation respectively. Total calories reduced due to partially replaced fat content of supplemented cookies. Cookies (supplemented with 60% and 40% AKF) were recommended better for eating quality on the basis of physico-chemical properties, calories count, texture and organoleptic attributes.

1. Introduction

Functional food helps in specific body functions in addition to supplying basic nutrients (Gul et al. 2017). Trend of functional food increased worldwide due to concern of consumer to healthy food (Das et al. 2011). Apricot kernels can be consumed as roasted and salted contain many different bioactive compounds such as tocopherol and β-carotene (Sharma et al. 2014; Mahloko et al. 2019). In Pakistan, it is native to the inside valley of Baluchistan, Kurram Agency, Hunza, Gilgit and Ladakh (Manzoor et al. 2012). Globally, Turkey is leading country for apricot production that ranges about 9,000 tons per annum and most commonly used as kernels source (Gezer & Dikilitas, 2002). The compositional profile of the apricot shows that it contains good proportion of dietary fibers and minerals like potassium, selenium, zinc, phosphorous, calcium, iron and magnesium. It possesses high nutritional profile due to presence of vitamin A, C, niacin, thiamin and riboflavin (Chandi & Sogi, 2007).

Current cholesterol intake level is increasing risk of cardiovascular disease. According to various studies, excessive consumption of high fat-food leads to health problems as obesity and

heart disease (Kyungwon et al. 2005). It is a challenge for food industry to search new alternatives for fat in foods without losing quality. Commonly fat is replaced by altering the food formulation with carbohydrate-based, protein-based and lipid-based constituents. Even though a variety of fat replacers have been developed, yet no ideal fat replacer completely functions like conventional fat (Lim & Lee, 2014).

Wheat flour is source of carbohydrates as well as it also provide other important nutrients like B-group vitamins, dietary fiber, protein and minerals (Nuttall et al. 2017). All over the world, cookies are one of the most popular, widespread and appealing foodstuffs having higher nutritional, sensorial and textural profile, ready to eat, and cost competitiveness as well. Biscuits with low glycemic index, more protein will increase the dietary fiber intake and decrease in calorie and carbohydrates of baked foods that ultimately improve the health and product quality (Adeola & Ohizua, 2018). Fat content highly influenced the cookies quality. It is recommended that daily fat intake should not more than 30% of total calorie in a diet (Seker et al. 2010).

This study was design to address current demand of reduced-fat and fiber enriched foods. Purposely, apricot based product was developed and assessed for various physicochemical and sensory attributes. The main objective was to develop partially reduced fat cookies supplemented with AKF.

2. Materials and methods

2.1. Sample preparation

Apricot collected in cotton boxes from local market of Rawalpindi and stored at ambient temperature overnight. Next day, pits were removed and washed with tap water. Pits were manually cracked after sundrying and soaked in boiled water for 1 hour to remove kernel coats by hands. Kernels were dried for 2 hour at ambient temperature and ground by coffee grinder for 1 minute to prepare apricot kernel flour. AKF were packed in air tight polythene bags and stored at 10°C. AKF flour was prepared fresh before 1 hour of cookie production to prevent the rancidity and oxidation.

Traditional method was used to prepare cookies as reported in previous study (Singh et al. 2015). Dough was prepared by taking different proportion of ingredients like flour (500 g), grounded sugar (200 g), sodium bicarbonate (2.0 g), sodium chloride (2.0 g), skim milk powder (40 g) and water (as per dough requirement). Shortening was added according to experimental design. Treatments were set by changing the ratio of shortening and AKF percentages, as T_0 (100:0), T_1 (80:20), T_2 (60:40), T_3 (40:60), T_4 (20:80) and T_5 (0:100) respectively. Premix was made by mixing flour, ground sugar, skim milk powder, and sodium bicarbonate. Dough was prepared and converted into 0.5 cm thick sheet. After sheeting next step was to cut the dough sheet by using circular mold. Baking was conducted by baking oven at 170°C for 15 min. Last step was cooling of cookies at room temperature and then packaging in precoded airtight polythene bags for further analysis.

2.2. Proximate analysis of apricot kernel flour

The moisture content of kernel was measured by using an air forced draft oven at a temperature of 100 ± 5°C by following the procedure described in AACC (2000) method No. 44-15A. Moisture (%) = Wt. of original sample – Wt. of dried sample/ Wt. of original sample × 100

The ash content of kernel powder was measured by following the procedure outlined in AACC (2000) method No. 08-01. The samples were taken in pre-weighed crucibles and charred on bunson burner before incinerating in the muffle furnace where a temperature of 550°C was maintained till the sample converted to grayish white residue. Ash (%) = Weight of ash/ Sample weight × 100

The Soxhlet apparatus was used for the determination of crude fat of powder according to AACC (2000) method No. 30-25. Crude fat from 5g of powder was extracted with hexane at up to 5 washing. After distilling excess hexane, the residue of hexane was dried at 100°C for 30

minutes until a constant weight attained. Crude fat (%) = Wt. of extract in sample/ Wt. of sample × 100

Crude fiber contents were determined by following the procedure mentioned in AACC (2000) method No. 32-10. The crude fiber was tested in 2g fat free sample and digested with 200 ml boiling 1.25% H_2SO_4 filtered and washed three time with distill water. Then samples were again digested with 200 ml of boiling 1.25% NaOH for 30 minutes, filtered and washed thrice. The resultant residue was dried at 130°C for 2 hours and weighed. Dried residue was ignited at 550°C ± 15°C, cooled and reweighed. Crude fiber (%) = Wt. of residue left – Wt. of ash/ Wt. of sample × 100

Crude protein was calculated by nitrogen content of sample. The nitrogen contents were determined by Kjeldahl's method as described in AACC (2000) method No. 46-10. Then the recorded observation was used for protein contents calculation in sample. The sample (1 g) was first digested with 25 mL concentrated sulphuric acid in the presence of digestion mixture for 5-6 hours or till light green or transparent color of the sample. The sample was diluted to 250 mL with distilled water. The distillation was done by taking 10 mL of diluted sample and 10 mL of 40% NaOH solution in distillation apparatus. The ammonia thus liberated was collected in 2% boric acid solution containing methyl red as indicator. Finally the sample containing ammonium borate was titrated against 0.1 N H2SO4 solutions till golden brown end point. The nitrogen percentage was determined by the following expression.

N (%) = Vol. of H2 SO4 used × Vol. of dilution × 0.0014/ Wt. of sample × Vol. of sample taken × 100

The protein percentage was calculated by multiplying % nitrogen with a factor 6.25.

2.3. Preparation of extract

10 % (w/v) extract of kernel powder was prepared with 70% ethanol. Then homogenate was centrifuged at 13500 rpm for 30 minutes at 4°C and the resulting supernatants were analyzed for future experiment.

2.4. Total phenol content

Total phenolic contents (TPC) kernel extracts were measured using Folin-Ciocalteu method (Chan et al., 2008) that was based on the reduction of phosphotungstic acid to phosphotungstic blue and as result absorbance increased due to rise in number of aromatic phenolic groups. For the purpose, 50 µL of kernel extract was separately added to test tube containing 250 µL of Folin-Ciocalteu's reagent, 750 µL of 20% sodium carbonate solution and volume was made up to 5mL with distilled water. After two hours, absorbance was measured at 765 nm using UV/visible light Spectrophotometer (CECIL CE7200) against control that has all reaction reagents except sample extract. Total polyphenols was estimated and values were verbalized as gallic acid equivalent (mg gallic acid equivalents/100g). Total phenolic compounds of each extract in gallic acid equivalents (GAE) was calculated by

$$C=c\times V/ m \quad (1)$$

C = Total phenolic contents (mg/g plant extract, in GAE); c = Concentration of gallic acid (mg/mL); V = Volume of extract (mL); m = Weight of kernel extract (g)

2.5. Radical scavenging activity by using DPPH method

DPPH (1,1-diphenyl-1-picrylhydrazyl) highly colored and stable oxidizing radical that result in formation of a yellow colored hydrazine (DPPH-H) associated with abstraction of free hydrogen atoms from phenolic antioxidants. Protocol of Gupta and Prakash (2009) was followed to determine DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity of kernel extract. Sample solution was prepared by dissolving 0.025 mL of sample extract in 10 mL of respective solvent. 3 mL of freshly prepared DPPH solution in respective solvent (6×10-5M) was mixed with 77 µL sample extract. Each sample was kept in dark place for

about 15 minutes at room temperature and decrease in absorbance was measured at 517 nm on UV/visible light spectrophotometer. Note done the blank sample absorbance having the same amount of solvent and DPPH solution except extract was prepared and absorbance was estimated at same wavelength on UV/visible light spectrophotometer. The free radical-scavenging activity of kernel extract can be presented as percentage reduction in DPPH due to given amount of each extract.

Reduction of absorbance (%) = [(AB - AA) / AB] × 100 (2)

AB = Absorbance of blank sample at t = 0 minute; AA = Absorbance of tested extract solution at t = 15 minutes

2.6. Mineral analysis

The composite flour samples were analyzed for K, Mg Fe and Zinc by wet digestion according to method given in AOAC (2006).Flour sample (0.5) was first digested at low temperature (60-70^{o}C) with 10mL of HNO3 for 20 min in 100mL conical flask on hot plate, then it was digested at high temperature (190^{o}C) with 5mL 60% HClO4 till the contents of flask became clear. The mineral contents of the samples were determined by using the respective standard curve prepared for each element. Aliquots were used to estimate Na and K by flame photometer (Flame Photometer Model-EEL). The minerals, such as calcium and magnesium were determined with an atomic absorption spectrophotometer (Perkin–Elmer Model 5000).The samples were quantified against standard solutions of known concentration that were analyzed concurrently.

2.7. Evaluation of Cookies

Prepared cookies were subjected to analysis for following parameters:

2.7.1. Physical analysis of cookies

The width, thickness and spread factor for cookies were estimated according to the method described in AACC (2000).

In order to determine the width (cm) of a cookie, six cookies were placed next to each other horizontally and the total diameter was measured. They were rotated by 90° and the diameter was re-measured. After repeating two more times, the average of four measurements was divided by six to calculate the diameter of a cookie.

The thickness (cm) of cookies was measured by placing six cookies on one another and the total height was measured. After re-stacking them in different order, the height was re-measured and the height of a cookie was calculated from the mean of two height measurements.

Spread factor was calculated according to method no. 10-53 described in AACC, (2000). The spread factor was calculated according to the following formula:

$$SF = \frac{W}{T} \times CF \times 10 \quad (3)$$

Where CF = Correction factor at constant atmospheric pressure (1.0 in this case).

2.7.2. Color analysis

Color value was determined with CIELAB color meter. It was first calibrated with the standards. Random samples of biscuits were ground and filled in the Petri dishes. The surface was made smooth by removing the extra sample material. Optimum reflection of light was obtained by the photo cells of color meter. Reading was noted from the display and compared with the standard. According to Manzoor et al. (2012), L* value indicates (lightness), a* and b* values are chromaticity coordinates (a* from red (+) to green (-) and b*, from yellow (-) to blue (-).

2.7.3. Textural analysis

Texture was analyzed by using texture analyzer (Mod. TA-XT2, stable micro system, Surrey, UK) interfaced with a computer which controls the instrument and records the data. To measure the reading, the texture expert program version 1.21 was used. Probe speed and position of texture analyzer were adjusted to 0.5mm/s and 3mm respectively. Reported values were average of triplicate (Ozbasr et al. 2010).

2.7.4. Chemical analysis

Cookies were analyzed for moisture, ash, fat, protein and crude fiber according to standard respective methods (AACC, 2000) as described above for apricot kernel flour.

2.7.5. Energy value

Energy value of the cookies was determined by using Oxygen Bomb Calorimeter (IKA-WERKE, C2000 Basic, GMBH and CO., Germany) as described by Krishna and Ranjhan (1981). Sample was taken in the metallic decomposition vial. The vial was unscrewed and fastened by a cotton thread onto the middle of the ignition wire with a loop before loading the sample. Then the screw cap was tightened. The decomposition vial was guided into the filler head to the open measuring cell cover until it was in place. The start button was pushed and the measuring cell cover closed. The sample within the vial was burnt through electric spark and heat produced displayed in the form of a graph denoting the temperature against time on digital panel of Bomb Calorimeter. It gave number of calories per gram of a sample.

2.7.6. Total dietary fiber (TDF)

The flour was analyzed for total dietary fiber content, soluble dietary fiber and insoluble dietary fiber according to method No. 32-05, 32-07 and 32-20 respectively as described in (AACC, 2000) by using Megazyme Assay Kit. The samples were dispersed in a buffer solution and incubated with heat-stable α-amylase at 95-100°C for 35 minutes. After cooling, these samples were incubated at 60°C for 30 minutes by adding 100µL protease solution. Furthermore, α-amylase and protease treated samples were incubated with amyloglucosidase at 60°C for 30 min. Fiber contents were precipitated by the addition of alcohol in 1:4 ratios and filtered. Residues were washed with alcohol and acetone. A blank sample was run in a similar manner. TDF was determined by using the following formula:

TDF (%) = Residue wt (g) – protein (g) – ash (g) – blank (g)/ Sample weight (g) x 100

2.7.7. Water activity

An electronic Hygropalm water activity meter (Rotronic Hygropalm Model AW-DIO Huntington NY) was used for estimating the water activity at regular storage intervals following the procedure described in (AOAC, 2006).The cookies samples were placed in the cup and probe was inserted into the sample that connected with digital display unit. Then enter key was pressed and water activity displayed on display unit along with temperature.

2.7.8. Sensory evaluation of biscuits

Each sample was evaluated by 16 judges (8 males and 8 females from 30 to 45 years age group) for sensory properties of cookies. Coded polyethylene pouches of cookies were distributed with score cards. Each assessor evaluated cookies for sensory attributes like color, flavor, taste, texture and overall acceptability at 9-points hedonic scale (Ackbarali & Maharaj, 2014). Results were recorded as average of triplicate readings.

2.8. Statistical analysis

All the tests were conducted in replication (n=3) and mean values stated with standard deviations. Duncan's test was performed to find the significant differences among mean values of different treatments. Statistical interpretation of data was carried out by SPSS software (SPSS version 17, Inc., USA).

3. Results and discussion

3.1. Proximate and mineral analysis

Proximate analysis of AKF indicated considerable amount of moisture content (4.21±0.15%), crude fiber (3.20±0.11%), crude fat (52.47±1.84%), crude protein (17.22±0.60%), ash (2.25±0.08%) and nitrogen-free extract (20.65±0.72%). Apricot kernel flour was subjected to mineral profile analysis. Results revealed that potassium (523±5.23 mg/100g) is present in maximum amount followed by magnesium (273±2.73 mg/100g) whilst, iron (2.52±0.25 mg/100g) and zinc (2.21±0.021 mg/100g) are found only in considerable amounts. Slight variation in the proximate composition and mineral contents might be due to the appropriate cultivar of apricot utilized and the location of that apricot.

Soil profile is key contributor for difference in minerals content of apricot kernel flour.

Present findings are in accordance to previous study of Seker et al. (2010), who reported 4.21% moisture content. Protein, fat and fiber content (21.80, 40.20 and 35.83% respectively) were slightly different from results of present findings. This slight difference may be due to apricot cultivars and area of production. Alpaslan & Hayta (2006) revealed that minerals (mg/100g) of apricot kernel flour are within range: K, 473-570; Mg, 113-290; Fe, 2.14-2.82; Zn, 2.33-3.15. Slight difference exists in Zn value due to variation in apricot variety and location.

Mori et al. (2007), reported apricot kernel composition as moisture content 2.83%, crude protein 24.11%, crude fat 50.90%, crude fiber 2.43% and ash 2.21%. Likewise, Gezer et al. (2000) stated that apricot kernel contains moisture 5.52%, crude protein 21.51%, crude fat 44.23%, crude fiber 16.41% and ash content 2.80%. Ozcan (2000) recorded apricot kernel composition profile including crude protein, ash, crude fiber, moisture and crude fat (20.22, 2.3, 18.01, 3.4 and 44.64%, respectively).

3.2. Phytochemical screening

The assessment of apricot kernel polyphenols is indispensable to determine associated health benefits as they are major antioxidant contributor. Their antioxidant activity is attributed to the free radical quenching or electrons donating potential. Methanolic extract of apricot kernel contained significant amount of total phenolic contents i.e. 43.41±15.41 mg GAE/g on fresh weight basis.

Total phenol content in apricot kernel extract depends on various factors; cultivar, variety, extraction time, temperature etc. Many researchers have elaborated this relationship and measured total phenolic contents using Folin Ciocalteu's method. Present findings are in-accordance with Korekar et al. (2011), who studied the antioxidant profile of apricot kernel and noticed that aqueous ethanol extract of apricot kernel mildly acidified with HCl exhibited 492.01±63 mg GAE/100 g total phenol contents on fresh weight basis. Similarly, Kamiloglu et al. (2014) concluded that aqueous metabolic extract of sweet apricot kernel possess 1.67±0.83 mg GAE/100 g and bitter almond 0.84±0.21 total phenol contents on fresh weight basis.

Yildirim et al. (2010) concluded that black carrots juice exhibited 0.84±0.24 micro g GAE/100 mL of total phenol contents. Health promoting effects of various phenols have necessitated their inclusion in various food products. TPC value of apricot kernel was recorded as 43.41±1.65 mg GAE/100g. Korekar et al. (2011) measured the phenol content of methanol extracts of apricot kernel i.e. 58.31±0.78 mg GAE/100 g. Apricot kernel oil polyphenols impart positive effects on human health as they are highly effective antioxidant agents. Apricot kernel oil is the richest source of natural polyphenols that possess more significant antioxidant potential than vitamin C.

3.3. DPPH scavenging capacity assay

Means for DPPH free radical scavenging activity demonstrated that free radical activity of apricot kernel is 83.89±3.53 % which was comparatively high as compared to other nuts. Higher antioxidant activity of apricot contributed to DPPH free radical scavenging activity. Similar results were obtained by Korekar et al. (2011), who measured the DPPH radical scavenging activity as 43.77 to 123.35 mg/ml. Tian et al. (2011) determined apricot kernel DPPH radical inhibition activity as 0.05–0.8 mg/mL for white apricot kernel oil.

3.4. Moisture content of cookies

Moisture contents play significant role as it has direct impact on shelf life and strongly influence the quality of bakery products. The moisture is one of the most important and commonly recorded characters of food products. It is measured for a number of reasons including legal and label requirements, economic importance of quality and storage stability considerations.

Moisture content of apricot kernel flour supplemented cookies is presented in Table 1. Maximum moisture contents were observed in T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies) respectively. Whereas, the minimum moisture contents were recorded in T_0 followed by T_1 (20% AKF supplemented cookies) and T_2 (40% AKF supplemented cookies). The result indicates that treatments had significant effect on the moisture content of cookies. Fiber content of apricot kernel flour might be responsible for significant difference in moisture percentage of treatments.

Previous study demonstrated similar trend of moisture percentage for various treatments, like T_0 (Wheat flour 100%: Malted barley bran 00%) had 3.34% and T_4 (Wheat flour 50%: Malted barley bran 50%) 3.69% (Ikuomola et al. 2017). The current findings are in harmony with previous work reflecting that during the storage of cookies the moisture contents increases, may be due to hygroscopic nature of some ingredients (Tian et al. 2011). Furthermore same trend was evaluated in cookies for moisture due to the increase level of sweeteners (Ikuomola et al. 2017).

3.5. Ash content of cookies

The statistical results regarding ash contents of cookies supplemented by various concentrations of apricot kernel flour are represented in Table 1. The results indicate that treatments exhibited highly significant effect on ash contents. Minimum ash contents were observed in T_0 (control) followed by T_1 (20% AKF supplemented cookies). Whereas, maximum ash contents were recorded in T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies) respectively. Slight difference in ash content was recorded with treatments due to increase in fiber percentage at higher concentration of apricot kernel flour.

It was shown in past work that ash content significantly varied within treatment. Ash content was increased by addition of bran portion containing mineral contents. Ash percentage was high in T_4 (Wheat flour 50%: Malted barley bran 50%) 1.88% as compare to T_0 (Wheat flour 100%: Malted barley bran 0%) (Ikuomola et al. 2017).

3.6. Fat content of cookies

Apricot kernel flour is a rich source of energy. Fat interact with protein due to surfactant effects thus it is supposed that it will also affect the baking quality of flour. Mean squares regarding fat contents of apricot kernel flour supplemented cookies highly significant results regarding treatments. Maximum fat contents were observed in T_0 (control) followed by T_1 (20% AKF supplemented cookies). While, minimum fat contents were recorded in T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies) as shown in Table 1. Findings revealed that decrease in fat percentage might be due to increase in fiber content.

The trend of this trait is in harmony with the findings of Mushtaq et al. (2010), who recorded a non-significant ($P>0.05$) influence of xylitol replacement on fat contents of cookies. Same results were observed by Pasha et al. (2002), who concluded non-significant effect on fat contents of biscuits during storage.

3.7. Fiber content of cookies

Crude fiber is an organic residue and insoluble which obtained after acid base dilution. The mean squares regarding crude fiber contents of cookies supplemented with apricot kernel flour are presented in Table 1. The results indicate that treatments had significant and their interaction exhibited non-significant impact on crude fiber contents of apricot kernel supplemented cookies. Maximum crude fiber contents were recorded in T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies). Whereas the lowest crude fiber contents were determined in T_0 (Control) followed by T_1 (20% AKF supplemented cookies). Apricot kernel flour is a

good source of fiber, by improving the concentration of apricot kernel flour for cookies resulted in increase of fiber content.

Ajila et al. (2008) recorded the same pattern for crude fiber contents of biscuits with increasing level of mango kernel powder. The current findings are also in harmony with the Mushtaq et al. (2010), who explained a non-significant impact of storage on crude fiber content of biscuit. Furthermore, same results were supported by the Pasha et al. (2002), who recorded that crude fiber content of biscuits ranged from of 0.08 to 0.13%.

3.8. Protein content of cookies

Protein content have important role in baking quality of cookies. It reflects dough strength, elasticity and extensibility. It is considered as a vital quality parameter related to functional and nutritional characteristics of flour. Mean squares regarding protein content of cookies supplemented with apricot kernel flour are shown in Table 1. Treatments had significantly effect on protein content of AKF supplemented cookies. Minimum protein contents were reported in T_0 (Control) followed by T_1 (20% AKF supplemented cookies). Whereas, maximum protein contents were recorded for T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies) respectively.

Table 1. Moisture, ash, fat, fiber, protein, NFE and color (L, a*, b*) of flour/AKF supplemented cookies

Treatments		T_0	T_1	T_2	T_3	T_4	T_5
Physico-chemical Parameters	**Moisture%**	2.78±0.09^{a}	3.65±0.12^{b}	4.52±0.15^{c}	5.45±0.19^{d}	6.26±0.21^{e}	7.13±0.25^{f}
	Ash%	0.57±0.06^{a}	0.72±0.25^{ab}	0.86±0.26^{abc}	1.01±0.01^{ab}c	1.16±0.25^{bc}	1.30±0.26^{c}
	Fat %	23.53±0.23^{a}	19.23±0.19^{b}	13.66±0.13^{c}	9.28±0.09^{d}	5.74±0.05^{e}	3.82±0.03^{f}
	Fiber%	0.13±0.01a	0.74±0.07^{b}	1.36±0.13^{c}	1.88±0.18^{d}	2.41±0.24^{e}	2.75±0.27^{e}
	Protein %	6.78±0.07^{a}	9.47±0.09^{b}	13.65±0.14^{c}	16.83±0.17^{d}	20.31±0.20^{e}	24.69±0.25^{f}
	NFE %	44.04±0.91^{a}	66.21±0.62^{b}	66.11±0.82^{b}	65.98±0.98^{b}	65.90±1.61^{b}	65.72±2.98^{b}
	Color						
	L-value	71.39±4.28^{a}	69.47±4.17^{a}	69.62±4.18^{a}	70.46±4.23^{a}	71.31±4.28^{a}	72.35±4.34^{a}
	a*-value	9.35±0.93^{ab}	7.45±0.75^{a}	7.67±0.77^{a}	8.49±0.85^{ab}	9.29±0.93^{ab}	10.33±1.03^{b}
	b*-value	37.56±2.25^{a}	35.77±2.15^{a}	36.15±2.17^{a}	37.09±2.23^{a}	37.61±2.26^{a}	38.11±2.29^{a}

Mean values of replication (n = 3) within rows are significantly ($p \leq 0.05$) different; NFE, nitrogen free extract; AKF, apricot kernel flour; T_0, 100% shortening : 0% AKF; T_1, 80% shortening : 20% AKF; T_2, 60% shortening : 40% AKF, T_3, 40% shortening : 60% AKF; T_4, 20% shortening : 80% AKF; T_5, 0% shortening : 100% AKF.

The current findings are in harmony with the conclusions of Pasha et al. (2002), who studied the impact of sweeteners on cookies and reported a non-significant variation in protein contents among treated biscuits. Similar trend was found by Mushtaq et al. (2010), who reported negligible changes in protein contents during study of xylitol replacement effect on cookies.

3.9. Nitrogen free extracts (NFE)

Nitrogen free extracts of apricot kernel flour supplemented cookies are expressed in Table 1. Within treatments, significant increase in nitrogen free extract (NFE) was recorded by increasing AKF supplementation. Significant increase in nitrogen-free extract (NFE) by increasing apricot kernel flour concentration for cookies preparation might be due to increase in carbohydrates in form of fiber. Amongst the treatments, the increase in T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies) was examined respectively. Minimum value of trait was recorded in T_0 (control). Qaisrani et al. (2014) reported same trend of increase in nitrogen free extracts (69.91±0.01 to 70.61±0.17%) by increasing the concentration of psyllium husk from 5% to 25% for the preparation of dietetic cookies.

3.10. Color

CIELAB color system is used to perform color measurement and its attributes are L*, a* and b* values, where L* is the indicator of lightness to darkness, a* indicates greenish to reddish tonality and b* represents bluish to yellowish tonality.

3.10.1. L, a and b* value*

Means regarding L, a* and b* values of AKF supplemented cookies are illustrated in Table 1. It is obvious from mean vales that treatments owned non-significant effect on L, b* attributes, however treatments significantly affect a* values of AKF supplemented cookies. Treatments vary non-significantly with regards to L value of the cookies, whereas significant increase in a* value and steady increase in b* value took place in all treatments. The maximum value for the traits (L, a* and b*) was observed in T_5 (100% AKF supplemented cookies). However, least value for the trait was observed in T_1 (20% AKF supplemented cookies). L, a* and b* values were significantly ($p<0.01$) correlated with ash content of cookies as demonstrated in Table 3.

Sudha et al. (2007) reported that color of biscuit becomes darker with the increase bran concentration. Similar trend in color change was observed by Ashoush & Gadallah (2011), who supplemented the biscuits with mango peel powder and mango kernel powder. Moreover, it was reported that color of biscuit becomes darker with the increase bran concentration (Fradinho & Nunes, 2015). The findings of the instant research work are quite in harmony with the earlier investigation on properties of kernel flour supplemented cookies (Seker et al. 2010).

3.11. Energy value

Energy values of AKF supplemented cookie are elaborated in Table 2. Treatments had significant effect on energy value of cookies. Highest value were recorded in T_0 (control) followed by T_1 (20% AKF supplemented cookies) and T_2 (40% AKF supplemented cookies) respectively. However, minimum value were observed in T_5 (100% AKF supplemented cookies) as fat content reduced by increasing fiber percentage.

The results of the present investigation closely related with the previous findings. Filipcev et al. (2016) reported that calorie of biscuits reduced from 14.51-16.23% by replacing the fat with fine ground wheat bran at the level of 30, 40 and 50%.

3.12. Total dietary fiber

Total dietary fiber in AKF supplemented cookie is elaborated in Table 2. Treatments had significant effect on total dietary fiber of cookies. Maximum value were recorded in T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies). However, minimum value was observed in T_0 (control).

The results of the present investigation closely related with the findings of Seker et al. (2010), who reported that increasing the level of apricot kernel flour in cookies progressively increased the total dietary fiber content of cookies ranging from 03.24-12.86%.

3.13. Spread factor

The spread factor values of AKF supplemented cookies are presented in Table 2. Results indicate that treatments exhibit highly significant effect on spread factor AKF supplemented cookies. Lowest spread factor was observed in T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies). Whereas, maximum value was found in T_0 (control) followed by T_1 (20% AKF supplemented cookies) and T_2 (40% AKF supplemented cookies) respectively. It was observed that the spread factor decrease gradually as the supplementation level was increased. Spread value reflected significant ($p<0.01$) positive correlation with fat content and significant ($p<0.01$) negative correlation with all other parameters like ash, fiber, and protein, total dietary fiber, nitrogen free extract and water activity of AKF supplemented cookies as shown in Table 3.

The current results are in harmony with the past research findings. Hallen et al. (2004) reported that decrease in spread factor was due to the competing effect among the hydrophilic. Similar results were evaluated by Abu-Salem & Abou-Arab (2011), who recorded that substitution of bambara flour decreased the spread factor.

Table 2. Energy value, total dietary fiber, texture, spread value and water activity of AKF supplemented cookies.

Treatments	T_0	T_1	T_2	T_3	T_4	T_5
Energy value (cal/g)	503.72±0.71^{a}	474.55±0.11^{b}	438.98±0.55^{c}	409.52±1.13^{d}	384.82±1.65^{e}	369.26±2.01^{f}
Total dietary fiber (%)	2.63±0.09^{a}	9.34±0.33^{s}	15.86±0.55^{c}	20.69±0.72^{d}	26.69±0.93^{e}	31.82±1.11^{f}
Spread value (mm)	23.21±0.81^{d}	20.96±0.73^{c}	19.67±0.68^{bc}	19.32±0.68^{bc}	18.06±0.63^{ab}	16.87±0.59^{a}
Texture (N/kg)	3.27±0.11^{a}	3.70±0.13^{b}	3.83±0.13^{b}	4.27±0.15^{c}	5.13±0.18^{d}	6.02±0.21^{e}
Water activity (ERH)	0.48±0.09^{a}	0.58±0.08^{ab}	0.69±0.04^{b}	0.77±0.09^{bc}	0.9±0.04^{c}	0.93±0.05^{c}

Mean values of replication (n = 3) within rows are significantly ($p \leq 0.05$) different; AKF, apricot kernel flour; T_0, 100% shortening : 0% AKF; T_1, 80% shortening : 20% AKF; T_2, 60% shortening : 40% AKF, T_3, 40% shortening : 60% AKF; T_4, 20% shortening : 80% AKF; T_5, 0% shortening : 100% AKF.

3.14. Texture

Texture is an important factor for the consumer acceptance of cookies. Textural measurements were also conducted to study the effect of fat reduction at selected supplementation levels. Texture of AKF supplemented cookies is expressed in Table 2. Treatments owned significant effect on AKF supplemented cookies and behaved differently with regards to texture. Minimum value was observed in T_0 (control) followed by T_1 (20% AKF supplemented cookies) and T_2 (40% AKF supplemented cookies) respectively. However, maximum value for the trait was observed in T_5 (100% AKF supplemented cookies). Texture demonstrated significant ($p<0.01$) negative correlation with fat content and significant ($p<0.01$) positive correlation with all other parameters like ash, fiber, protein, total dietary fiber, nitrogen free extract and water activity of AKF supplemented cookies as shown in Table 3.

Present findings are according to earlier research presented by Srivastava et al. (2012), who reported that hardness and brittleness of the cookies generally increased with fat mimetic such as polydextrose or maltodextrin. Tian et al. (2011) demonstrated that hygroscopic ingredients contribute to decrease in hardness as the moisture contents increased by hygroscopic nature of flour.

Table 3. Correlation coefficient (r) between sensory characterstics, textural attributes and physicochemical properties of flour/AKF supplemented cookies.

	Color			SV (mm)	Tex (N/Kg)	Sensory Properties				
	L-value	a*-value	b*-value			Color	Flavor	Taste	Text	OA
MC (%)	0.24	0.49*	0.31	-0.91**	0.96**	-0.59*	-0.79**	-0.59**	-0.57*	-0.61**
Ash (%)	0.59**	0.65**	0.63**	-0.65**	0.86**	-0.48*	-0.64**	-0.48*	-0.46	-0.49*
Fat (%)	-0.12	-0.39	-0.19	0.95**	-0.92**	0.54*	0.76**	0.54*	0.51*	0.55*
Fib (%)	0.26	0.47*	0.33	-0.90**	0.94**	-0.55*	-0.76**	-0.54*	-0.52*	-0.56*
PC (%)	0.18	0.48*	0.26	-0.93**	0.97**	-0.63**	-0.81**	-0.63**	-0.61**	-0.65**
NFE (%)	0.05	-0.15	-0.01	-0.69**	0.52*	-0.17	-0.29	-0.11	-0.09	-0.13
EV (cal/g)	-0.12	-0.38	-0.19	0.95**	-0.92**	0.53*	0.76**	0.53*	0.50*	0.55*
TDF (%)	0.19	0.44	0.26	-0.94**	0.95**	-0.59**	-0.79**	-0.59*	-0.56*	-0.61**
WA (ERH)	0.43	0.56*	0.48*	-0.80**	0.94**	-0.53*	-0.74**	-0.53*	-0.50*	-0.54*

*, $p<0.05$; **, $p<0.01$; MC, moisture content; Fib, fiber; PC, protein content; NFE, nitrogen free extract; EV, energy value; TDF, total dietary fiber; WA, water activity; L*, lightness; a*, red to green; b*, from yellow to blue; OA, overall acceptability.

3.15. Water activity

Water activity of AKF supplemented cookies is presented in Table 2. Treatments had significant effect on water activity of AKF supplemented cookies. Maximum value of water activity was recorded in T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies) and T_3 (60% AKF supplemented cookies) respectively. The minimum value was evaluated in T_0 (control) followed by T_1 (20% AKF supplemented cookies) and T_2 (40% AKF supplemented cookies) correspondingly.

Results of this research are justified from findings of Zoulias et al. (2002), who evaluated that cookies prepared with the fat mimetic at 35% fat replacement demonstrated significantly higher water activity than the control cookies ranging from 0.08 to 0.21.

3.16. Sensory response of AKF supplemented cookies

Color is the basic criteria which gives the perception about food acceptance. AKF supplemented cookies were evaluated for color, flavor, taste, texture and overall acceptability. T_0 (control) was used as reference sample. Significant difference was recorded in sensory attributes of treatments. Treatment, T_3 (60% AKF supplemented cookies) was rated higher scores for color, flavor, taste, texture and overall acceptability followed by T_2 (40% AKF supplemented cookies). Maximum score was received by T_5 (100% AKF supplemented cookies) followed by T_4 (80% AKF supplemented cookies). T_3 (60% AKF supplemented cookies) was recommended best treatment as compared to all other treatments and control sample due to improved sensory properties as shown in Fig. 1. Color, taste, flavor, texture and overall acceptability reflected significant ($p<0.01$; $p<0.05$) positive correlation with fat content of AKF supplemented cookies as shown in Table 3.

Similar results were recorded by Zoulias et al. (2000), who studied improvement in sensory characteristics of biscuits supplemented with citrus peel and pulp at various levels (0, 5, 15

and 25%). Aggarwal et al. (2016) evaluated that flavor, taste and texture were score high by addition of mango peel powder at different levels for biscuits making. Manohar & Rao (2002) reported that maximum texture score is related to improved eating quality of biscuits. The results for overall acceptability are in line with the research findings of Fradinho & Nunes, (2015), who reported that cookies containing 20% wheat bran and 30% barley bran were highly acceptable.

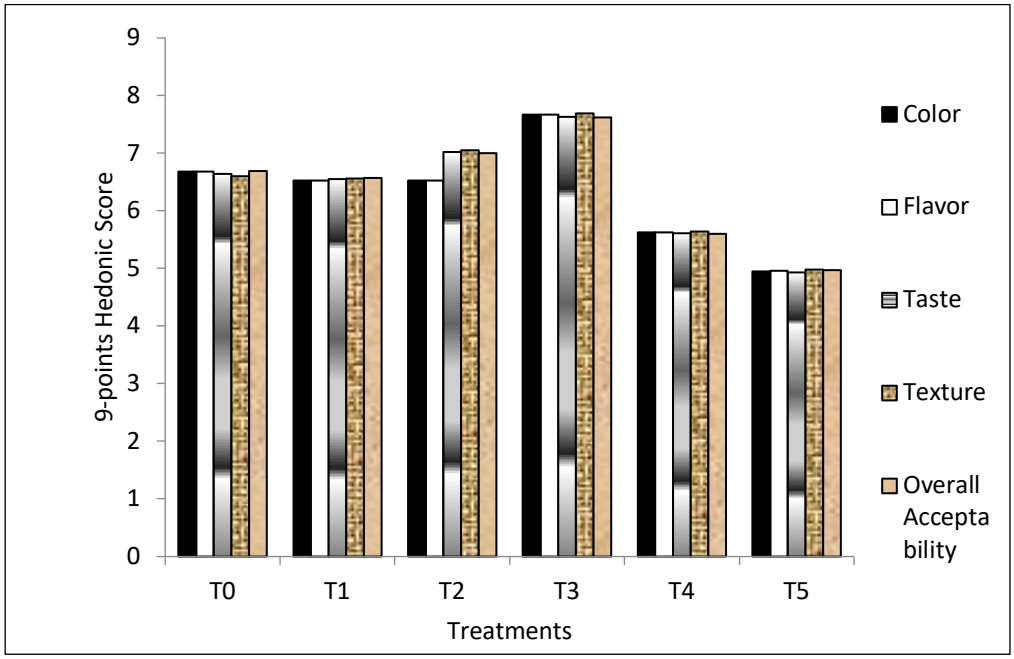

Figure 1. Sensory properties **(**color, flavor, taste, texture and overall acceptability) of AKF supplemented cookies.

AKF, apricot kernel flour; T_0, 100% shortening : 0% AKF; T_1, 80% shortening : 20% AKF; T_2, 60% shortening : 40% AKF, T_3, 40% shortening : 60% AKF; T_4, 20% shortening : 80% AKF; T_5, 0% shortening : 100% AKF.

4. Conclusions

This study revealed that AKF supplemented cookies are good source of fiber, partially reduced fat and protein. Sensory properties of the AKF supplemented cookies were affected in positive way like color, flavor, taste and texture, leading to softer eating quality which is required in cookies. Cookies containing 40% and 60% AKF were recommended on the basis of different parameters including energy value, texture and organoleptic properties.

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BUCKWHEAT STARCH (*Fagopyrum esculentum*): AQUEOUS EXTRACTION, MODIFICATION BY HMT AND CHARACTERIZATION

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ABSTRACT

Buckwheat (*Fagopyrum esculentum*) is a pseudoceral. It grains are nutritionally rich and boast great technological potential for use in the food industry in infant and backed food as well as ingredient of functional products. Flour is considered as an alternative to celiacs because it does not contain gluten. It is an option for the growing appeal for a healthy diet, as well as containing starch as a major component. Starches in native form have restricted use in the industry. So modifications are made to increase their application. These modifications can be chemical or physical. Physical modification is preferable and accepted by consumers. Heat-moisture treatment (HMT) consists of heating starch above gelatinization point with insufficient moisture (<35%). The main objective was extract buckwheat starch, modify by HMT and assess its physico-chemical, thermal and morphological properties. The starch granules from buckwheat have spherical or polygonal shape, with an average size around 1 – 7.5 µm. Morphology of buckwheat starch granules was not altered. The XRD technique showed no signifficative differences between main peaks in diffractograms however the relative crystallinity decrease. DSC analysis allowed to observe that according HMT the enthalpy decrease and gelatinization occurs in higher temperatures.

1.Introduction

Starches are the most abundant carbohydrate reserve in plants. They are found in fruits, seeds, leaves, steems and roots as water-insoluble granules with several shape (oval, spherical, lenticular, etc.) and size (1 up to more than 100 µm). Starches are made up to biopolymers called amylose and amylopectin. There are formed by α-D-glucose units. Amylose is a linear polymer with α-1,4 linked glucose units and amylopectin is an highly branched polymer with α-1,4 linked glucose and with α-1,6 linkages at the branched points (Smith, 2001; Andrade et al. 2014; Alcázar-Alay and Meireles, 2015).

The buckwheat (*Fagopyrum esculentum* Moench) plant is a crop that belongs to the *Polygonaceae* family. It is a pseudo-cereal usually grouped with cereals due the similarity in cultivation and utilization. It is called the common buckwheat starch and is the main species of buckwheat which has been widely consumed and used around the world (Hung et al., 2009; Liu et al., 2015).

The hydrothermal treatment or heat-moisture treatment (HMT) is a physical and safe method of modification of starch. It involves incubation of starch granules at low levels (less than 35% water, w/w) by a certain period of time (15 min to 16 hours) in temperatures higher than the gelatinization temperature (84 to 120 °C). The HMT is important due to alter some physicochemical properties of starch without

destroying its granular structure. It is considered a technique with low cost and used in several food products (Vieira and Sarmento, 2008; Zavareze and Dias, 2011; Sun et al., 2013; Bet et al, 2018). The changes in properties of starches treated by HMT vary according to the conditions employed as starch-to-moisture ratio, temperature and heating time. Regardless of the starch origin, HMT promotes an increase in the gelatinization temperature with widening the gelatinization temperature range (Chung et al, 2009; Zavareze and Dias, 2011; Moraes, Branzani and Franco, 2014).

The main objectives of this study were: the extraction of buckwheat starch by aqueous method and investigation of thermal and pasting properties as well as the morphology and structure of buckwheat starch granules.

2. Materials and methods

2.1. Materials

2.2.1.Samples

The buckwheat seeds of (*Fagopyrum esculentum*, Moench) were supplied by Protecta Co., Ltd., Ponta Grossa-PR-Brazil.

The extraction and treatment of buckwheat starch was performed in the Food Engineering Department Laboratory and analyses in the Multiusuary Laboratories of the State University of Ponta Grossa.

2.2.2.Starch Extraction by Aqueous Method

It was performed in agreement procedure described (Andrade et al, 2014; Barros et al., 2020). The buckwheat seeds were milled vigorously and so obtained the buckwheat flour. An aliquot of this flour was suspended in distilled water (ratio 3:1, water:flour, v/m). A consistent dough was formed and maintained in stirring by 30 minutes. After, this suspension was passed through sieves 200 and 325 mesh, respectively (0.075 mm and 0.043 mm). The suspension was centrifuged (Hettish routine 420R Zentrifugen, Germany) at 8500 rpm and 4ºC by 10 minutes. The precipitate was recovered and dried in an oven with forced air circulation at 40ºC by 24 hours. So, the starch obtained was kept in a desiccator containing anhydrous calcium chloride until analysis and/or modification.

2.2.3.Proximal Composition

This analysis was performed in triplicate according AOAC (2000), and were determined moisture, carbohydrate, protein, lipid and fiber.

2.2.4.Physical Modification of Starch (HMT)

After constant mass, the obtained starch was divided in four portions: the first, called (NAT) was the native or untreated sample. The other parts were treated after the moisture content determined in the proximal composition and TG/DTG. It was added distilled water with micropipette in such a way that samples reached the levels 15, 20 and 25 % of water and called samples (H15), (H20) and (H25). Each sample was homogenized with pistil and mortar to avoid points with higher concentration of water. After homogenized each sample was transferred to hermetically flasks and sealed. So, all samples were maintained by 24 hours. The modification by HMT was performed in an autoclave at 120 ºC for 1 hour (Bet et al., 2018; Barros et al., 2020).

2.2.5.Instrumental Analysis

2.2.5.1. Scanning Electron Microscopy-Field Emission Gun (SEM-FEG)

The morphology of starch granules was observed using a scanning electron microscope with field emission gun, model MIRA 3 (Czech Rep.). The voltage of the electron beam was 15 kV, generated by a tungsten lamp (Bet et al., 2018).

2.2.5.2. Color Analysis

It was used the colorimeter (Miniscan EZ 4500L, Reston, |USA) calibrated previously. The colour parameters, where L* gives us lightness L*=100 (white) and L*=0 (black); the a* value characterizes the redness region +a*= (red) and –a*= (green); the b* value indicates the color range from +b* (yellow) and –b* (blue) (Martins et al., 2020).

2.2.5.3. Pasting Properties (RVA)

The RVA instrument (Newport Scient., Australia) was used to evaluate the pasting properties. A dispersion of 8% of starch (dry basis) in 28 g of total mass. Each sample was heated to 95 ºC and cooled to 50 ºC cycle under

constant stirring (160 rpm) at a heating rate of 6 ^{o}C min^{-1} (Bet et al., 2018).

2.2.5.4. Differential Scanning Calorimetry (DSC)

The instrument DSC model Q-200 (TA Instr., USA) was previously calibrated and checked with standard Indium, purity 99.99%, m.p.= 156.6 ^{o}C, ΔH_m = 28.56 J g^{-1} was used to obtain DSC curves. A mass around 2.5 mg of each starch sample was weighed and mixed to proportional distilled water (≅ 10 μL). Each mixture was added to aluminum crucible and sealed with aluminum lid and so maintained during 60 min. The DSC analysis were performed as follows: air flow of 50 mL min^{-1}, heating rate of 10 ^{o}C min^{-1}, from 30 ^{o}C to 100 ^{o}C (Bet et al., 2018; Barros et al., 2020).

2.2.5.5. Thermogravimetry/Derivative Thermogravimetry (TG/DTG)

The TG/DTG curves were obtained using the TGA-50 microthermobalance (Shimadzu, Japan). Each mass sample was around 10 mg. It was used open α-alumina crucible and each sample was heated from room temperature to 650 ^{o}C. It was performed in air atmosphere with flow of 150 mL min^{-1}. The TG/DTG curves were obtained using TA-60-WS software and mass loss calculated (Bet et al., 2018; Martins et al., 2020).

2.2.5.6. X-Ray Diffractometry (XRD)

The X-ray diffractograms of each sample follow the methodology according Colman, Demiate, Schnitzler, 2014; Chen et al., 2015. The instrument model was Ultima 4 (Rigaku, Japan) under CuKα radiation, λ = 1.5418 Å, configured at 40 kV and 20 mA. The relative crystallinity (RC) was estimated. The diffraction pattern of each sample was determined by occurrence of peaks in angular range from 2 to 50 ^{o} in 2θ.

3.Results and discussions

3.1. Proximal Composition

The results of proximal composition of the native buckwheat starch are depicted in Table 1.

Table 1. Proximal composition of native buckwheat starch.

Native buckwheat starch	Content (%)
Carbohydrate	84.36
Moisture	9.97
Protein	2.09
Lipid	0.24
Fiber	0.61
Ash	2.73

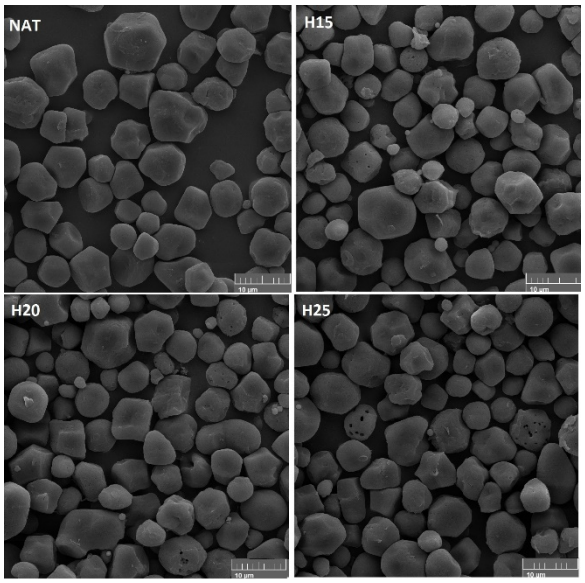
(*) The results are the triplicate average.

These results were determined by the AOAC (2000) methodology. Authors found values from water and ash content (calculated by TG/DTG) that were 10.6 and 2.4%, respectively.

Liu et al., found values from moisture 8,9%, and 2.1% of ash.

3.2. Scanning Electron Microscopy-Field Emission Gun (SEM-FEG)

The microimages of buckwheat starch granules obtained by SEM-FEG are shown in Figure 1. It can be observed that buckwheat starch granules has a bimodal shape (spheric and polygonal) which was not altered after applied modifications. The average diameter of granules was from 1.19 to 7.54 μm. Some cavities appeared on the surfaces, mainly in those that were treated with major moisture content. In the same way, fissures and holes can be observed. In ageement with literature (Liu et al., 2015), the results showed that the effect of HMT on the morphologic structure was moisture dependent. According Watcharatewinkul et al., 2009 and Liu et al, 2015., the cavities and holes were due to recombination of amylose and amylopectin chains. This recombination is result from the termal force given by HMT and contribute to more compact amorphous regions.



F**igure 1.** MEV-FEG microimages of buckwheat starch granules. (magnification 2kx)

3.3. Color Analysis Pasting Properties (RVA)

The color is a criterion for assess starch quality. The obtained values of L*, a* and b* are shown in Table 2.

Table 2. Color Analysis

Sample	L*	a*	b*
NAT	91.61±0.15	0.49±0.03	4.62±0.09
H 15	86.45±0.27	2.75±0.02	8.69±0.14
H 20	85.52±0.08	3.34±0.05	9.3±0.06
H 25	84.24±0.07	3.63±0.02	9.99±0.05

Results presented as mean±standard deviation

According to Sira and Amaiz, 2004, the pure starch present the L* value higher than 90. In this work the found value was 91.61, which decrease according the treatment performed. The a* and b* values increase with increasing amount of water.

In Figure 2, are depicted the pasting obtained after gelatinization of samples.

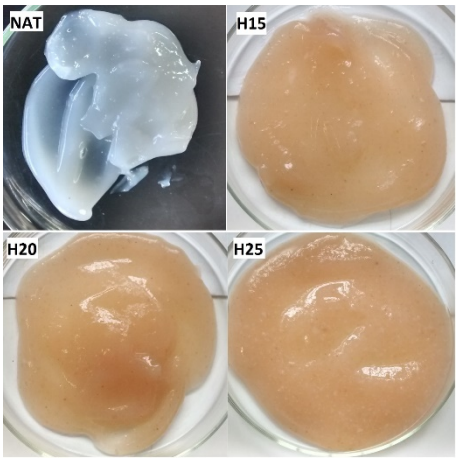


Figure 2. Gelatinized starches: (NAT) native and (H15, H20 and H25) modified starches

It can be observed alterations in color after gelatinization process, which values are in Table 2. The L* value show a decrease in brightness after HMT. The results show that the cromaticity a* and b* were significantly altered.

3.4.Pasting Properties (RVA)

A viscous paste of untreated and modified starches is obtained during heating of granules under excess of water, due to hydration and swelling. An important property, the viscosity, is the main factor for applicability in food processing. The pasting parameters obtained for native and HMT starches are summarized in Table 3.

Higher pasting temperature was presented by starch with 25% water (H25).

The breakdown inicially increase when compared with native starch and show considerably decrease according HMT.

The retrogradation show considerable decrease according were treated the samples.

As reported by Collado and Corke, 1999, hydrothermally treated starches could be utilized in infant and backed foods.

3.5.Thermal Analysis

3.5.1.Thermogravimetry/Derivative Thermogravimetry (TG/DTG)

The obtained TG/DTG curves are shown in Figure 3. There were performed in air atmosphere and heated from room temperature to 650ºC. All curves display mass loss in three steps: the first due to dehydration followed by a stability. After, the second and third mass losses are due to oxidation and decomposition of organic matter with formation of ash as final product (Colman et al., 2014; Lacerda et al., 2015).

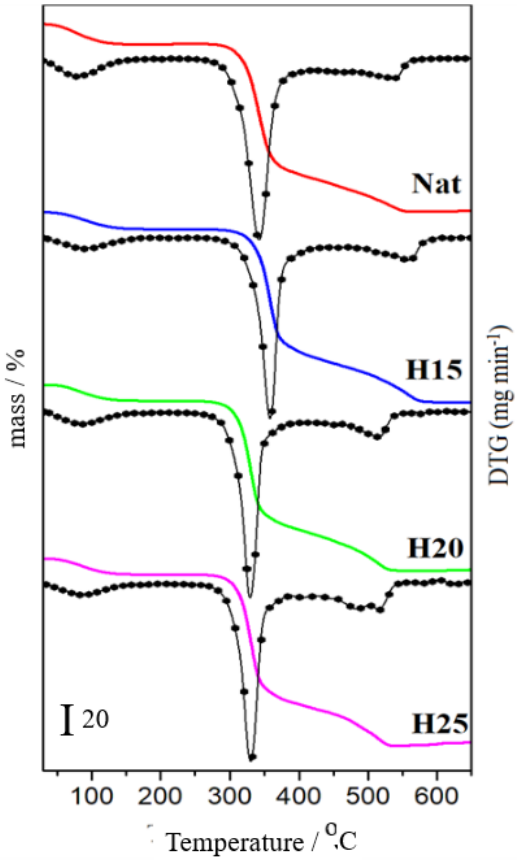


Figure 3. TG/DTG curves

The obtained results of thermal decomposition of native and modified starches are summarized in Table 4.

The ash content after thermal decomposition of untreated and modified starches were 2.4; 1.6; 3.6 and 3.0 %, respectively.

3.5.2.Differential Scanning Calorimetry (DSC)

The DSC technique was used to determine the gelatinization parameters of buckwheat starches. The curves are depicted in Figure 4 and values of T_o, T_p, T_c and enthalpy in Table 5.

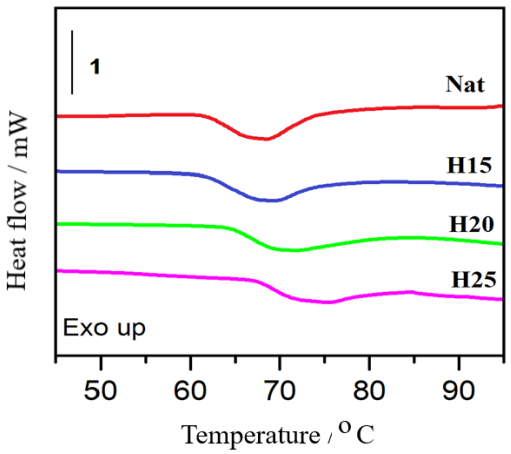


Figure 4. DSC curves of native and modified buckwheat starch

According to literature (Gunaratne and Hoover, 2002; Zavareze and Dias, 2011), these effects are dependent on the moisture level of the treatment, the starch source and the amylose content.

In this work, we observe a displacement of peak temperatures (T_p) to higher values as well as an enlargement in peaks between T_o and T_c.

3.5.3. X-ray difractometry (XRD)

According to literature (Andrade et al., 2014), the main differences in crystallinity between starches can be attributed to some factors: the crystal size, the number of crystalline regions that are influenced by amylopectin content and chain length, the orientation of double helices within the crystalline area and the extent of interactions between the double helices. Other factors that can be considered are the HMT conditions and the starch source.

In Figure 5 are the diffractograms of each sample from 2-50º in 2θ.

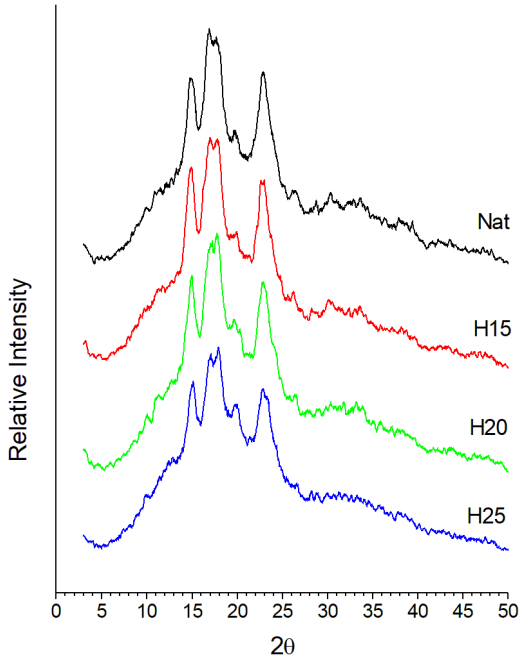


Figure 5. X-ray Diffractograms of of native and modified buckwheat starch

No significative displacements occurs between the main peaks. The relative crystallinity was calculated according Colman et al., 2014.

Table 6. Main results obtained by XRD

Sample	Relative Crystallinity (%)
Nat	21.04 ± 0.45[a]
H15	21.90 ± 0.55[a]
H20	19.56 ± 0.30[b]
H25	16.20 ± 0.21[c]

Averages followed by the same letters in the same column do not differ statistically by Tukey's test (P<0.05).

Table 3. Viscoamylographic (RVA) values of native and modified buckwheat starches

Sample	Pasting Temperature/ °C	Peak Viscosity/ mPa s^{-1}	Retrogradation/ mPa s^{-1}	Breakdown/ mPa s^{-1}	Final Viscosity/ mPa s^{-1}	Peak Time/ s
NAT	83.7	1899	1366	286	2969	600
H15	81.5	2657	1080	703	3034	544
H20	84.4	2384	628	530	2482	552
H25	90.0	1060	63	17	1106	643

Table 4. Results of TG/DTG curves.

Sample	Step	1st mass loss	Stability	2nd mass loss	3rd mass loss
NAT	Ti – Tf/°C	30-160	160-272	272-421	421-573
	Tp/°C	83		340	497
	Δ m/%	10.6		69.7	17.3
H15	Ti – Tf/°C	30-156	156-254	254-403	403-561
	Tp/°C	87		330	488
	Δ m/%	8.7		67.9	21.8
H20	Ti – Tf/°C	30-166	166-256	256-405	405-553
	Tp/°C	89		328	486
	Δ m/%	8.1		67.1	21.2
H25	Ti – Tf/°C	30-151	151-262	262-394	394-550
	Tp/°C	87		329	485
	Δ m/%	8.2		66.6	22.2

Table 5. DSC results of gelatinization of starches

Sample	T_o (°C)	T_p (°C)	T_c (°C)	ΔT (°C)	ΔH_{gel} (J g^{-1})
NAT	61.8±0.1^d	68.6±0.0^c	74.0±0.3^d	12.2±0.2^d	6.2±0.2^b
H15	62.0±0.0^c	68.5±0.0^c	75.5±0.1^c	13.5±0.0^c	5.5±0.0^c
H20	63.6±0.0^b	70.8±0.2^b	81.3±0.4^b	17.7±0.2^a	7.1±0.2^a
H25	65.4±0.0^a	74.4±0.0^a	83.8±0.1^a	18.4±0.1^b	5.5±0.1^c

Averages followed by the same letters in the same column do not differ statistically by Tukey's test ($P<0.05$).

T_o = onset temperature; T_p = peak temperature; T_c = conclusion temperature; ΔT = difference between T_c and T_o; ΔH_{gel} = gelatinization enthalpy.

4. Conclusions

The buckwheat starch was extracted by aqueous process in a satisfactory manner. The heat-moisture treatment (HMT) of starches is an effective technology for change their pasting properties. The TG/DTG curves show similar profile, however it was possible to stablish the main steps of mass loss as well as the stability. Color analysis show proportional darkening according increase the moisture content. SEM-FEG images allowed to observe the spheric and polygonal shape of buckwheat starch with average diameter between 1.19 to 7.54 μm. The DSC analysis show displacement of gelatinization to higher temperatures with decrease of enthalpy. The viscosity of starch is an important fator for applicability to food processing. The HMT of buckwheat starch suggest application in infant and baked foods.

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THE INHIBITION POTENTIAL OF THAI-COLORED RICE EXTRACT AGAINST DIABETES RELATED-ENZYMES AND MELANIN BIOSYNTHESIS-RELATED ENZYME

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ABSTRACT

Thailand has both colored and non-colored rice. Thai people use rice both for consumption and health treatment. This study investigates the phytochemical profile, α-glucosidase and tyrosinase inhibitory activity of water extract, ethanol extract, and methanol extract of 7 cultivars of Thai-colored rice. The results reveal that 7 cultivars of Thai-colored rice had composed of flavonoids, reducing sugar, saponins, and terpenoids. The highest potency against a-glucosidase activity was obtained from rice cultivar 6 of methanol extract with the IC_{50} value of 1.06 ± 0.13 $\mu g.ml^{-1}$, approximately 175-times more efficient than commercial drug acarbose. Moreover, methanol extract of all rice cultivars seems to have higher α-glucosidase inhibitory activity than other solvent extract and acarbose. In comparison, water extract of all rice cultivars had higher tyrosinase inhibitory activity than other solvents. The highest tyrosinase inhibitory efficiency was also obtained from rice cultivar 6 of water extract with the IC_{50} values of 10.93 ± 3.41 $\mu g.ml^{-1}$. Our results indicated that the high-polarity solvent can extract the bioactive compounds from plants with a high potency of α-glucosidase and tyrosinase inhibitory activity. The results also suggested that rice especially colored rice had contained the bio-active compounds which related with α-glucosidase and tyrosinase inhibitory activity.

1.Introduction

Alternative medicine has been used in many countries, especially in Asia (Ekor, 2014). People can use medical plants both for healthcare treatment and for ingredients in some cosmetics (Aburjai and Natsheh, 2003). Diabetes mellitus is a chronic metabolic disorder, and it can be insulin-dependent (type 1) and non-insulin-dependent (type 2), and more than 90% are diabetes mellitus type 2. Insulin is the glucose-level regulated hormone secreted from the Langerhans β cell of the pancreas (Hameed et al., 2015; Slattery et al., 2018; Zangeneh et al., 2003). The therapeutic methods for diabetes mellitus type 2 focused on inhibiting key enzymes from producing high blood sugar levels (Jeremiah et al., 2019; Singh et al., 2018; Slattery et al., 2018; Thilagam et al., 2013;). α-Glucosidase and α-amylase are carbohydrate hydrolyzing enzymes that release glucose from carbohydrates in the intestinal (Chakrabarti and Rajagopalan, 2002). Some standard inhibitors such as acarbose, miglitol, and voglibose have been used to inhibit the glucose releasing enzymes. However, these inhibitors cause many harmful side effects (Dabhi et al., 2013; Khan et al., 2014; Sugihara et al., 2014). Thus, the souse of the new inhibitor from the medicinal plant is to be importantly discovered and developed new

drugs against α-glucosidase and α-amylase enzymes.

The human melanin had synthesized via L-tyrosine oxidation and related with the tyrosinase enzyme (Sari et al., 2019). The tyrosinase activity is also related to Parkinson's diseases (Asanuma et al., 2003). The commercials inhibitor is kojic acid has been used for tyrosinase inhibitory activity. However, kojic acid has been reported to cause cytotoxicity and allergy of skin (Kahn et al., 1997; Nakagawa et al., 1995). Thus the tyrosinase inhibitor with lower side effects from plant extracts is an alternative for melanin disorder and Parkinson's diseases.

Rice is a global staple food in many countries (Fairhurst and Dobermann, 2002). People use rice for consumption and treat their health, and some countries use rice as the ingredient of cosmetics (Hu et al., 2012; Marto et al., 2018). In Thailand, people like to consume brown rice, non-colored rice, and colored rice. Non-colored rice has also been reported to a related risk of cardiovascular diseases (Mohan et al., 2014). Moreover, non-colored rice has also been reported to a related risk of cardiovascular diseases (Krittanawong et al., 2017). The present study investigates the potential inhibition for a-glucosidase and tyrosinase of Thai-colored rice extracted in a different solvent, i.e., water extract, ethanol extract, and methanol extract.

2. Materials and methods

2.1. Materials

2.1.1. Plant materials and reagent

Seven cultivars of Thai colored-rice seed were obtained from Rio-Et province, Thailand. The characteristics of seven different rice cultivars were showed in Figure 1. All rice seeds were sterilized with 0.1% NaClO and washed with deionized water, then baked with hot air in an oven at 60 °C for six h. Before homogenization, the seed-coat was taken out, and the rice-seed samples were homogenized with CryoMill (Retch, Germany) by using liquid nitrogen. The homogenized samples were kept at -20 °C until extraction. The reagent comprising acabose, kojic acid, *p*-nitrophenyl-α-glucopyranoside (4-*p*NPG), L-DOPA, α-glucosidase from *Saccharomyces cerevisiae*, and tyrosinase from mushroom were obtained from Sigma-Aldrich (St. Louis, MO).

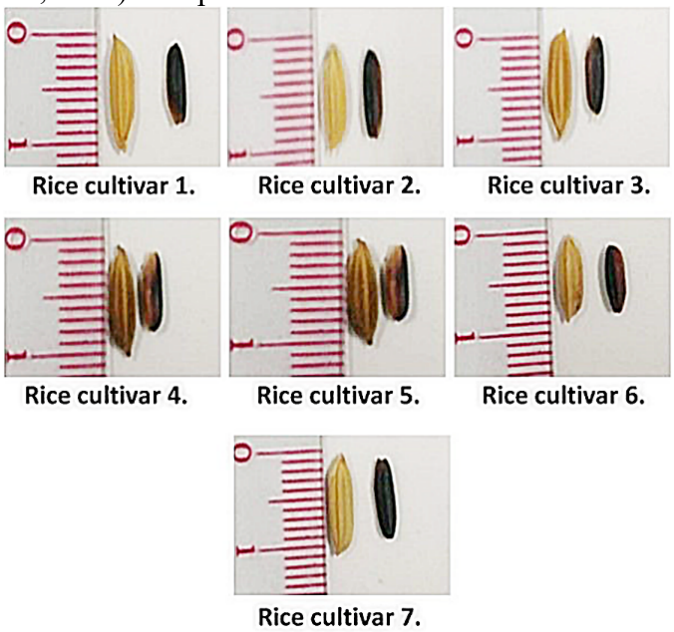

Figure 1. The 7 cultivars of Thai colored-rice harvested from Roi-Et province, Thailand. The seed-coat color of 7 cultivars is different by cultivar 3, 4, 5, and 6 are in brown, whereas cultivar 1, 2, and 7 are in yellow. The seed of 7 cultivars also shown differently by cultivar 1, 2, 3, and 7are in black, whereas cultivar 4, 5, and 6 are in red.

2.2. Methods

2.2.1. Samples extraction

The 500 g of homogenized rice samples were extracted with three solvents, i.e., water (HPLC water), ethanol (95%), and methanol (95%). The extracts were stirred for three days at room temperature. Then, the extracted rice samples were centrifuged, and the solvent was evaporated by hot air in an oven (ThermoStable ON-32, Daihan, Korea) at 60°C. The dry extraction yielding approximately 25% w/w to 40%w/w were kept at -20°C until enzyme activity occurred.

2.2.2. Phytochemical screening

The phytochemical composition of the samples was conducted by modified methods of Sansenya and Nanok, (2020). Flavonoids, reducing sugar, saponins, and terpenoids were evaluated by using the rice samples concentration of 0.10 $mg.mL^{-1}$ for each method.

For the flavonoids test, the rice samples were dissolved with ethyl acetate and then boiled for 5 min. Next, the reaction was filtered with a syringe filter 0.45 µm. Finally, the filtered solution was mixed with ammonia. The yellow color was occurred indicated that the samples were composed of flavonoids.

For the reducing sugar test, the rice samples were dissolved with HPLC water and then the solution was filtered with a syringe filter 0.45 µm for discarded the rice residues. The rice samples solution was mixed with benedict solution under base condition for determine the reducing sugar. Then, the mixture solution was boiled until brick-red precipitate appeared, which indicated that the samples was reducing sugar.

For the saponin test, the rice samples were dissolved with HPLC water and then the solution was filtered with a syringe filter 0.45 µm for discarded the rice residues. The three drops olive oil were added to the rice samples solution and shaken immediately. The mixture solution was incubated for 30 min at room temperature .The formation of emulsion and permanent bubbles indicated that the samples had saponin.

For the terpenoids test, the rice samples were mixed with 2 mL of chloroform and then shaken immediately. The rice samples solution was filtered with a syringe filter 0.45 µm for discarded the rice residues. Three milliliters of concentrated H_2SO_4 was added to the filtrate solution; a reddish-brown color indicated a positive result.

2.2.3. α-Glucosidase inhibitory activity assay

The α-glucosidase inhibitory activity of rice samples was performed in the previous study but slightly modified (Şöhretoğlu et al., 2018). In 100 mL of reaction was mixed with 5 mL of rice extracts (in 50mM sodium phosphate buffer pH 6.9) to give final concentration of 2 to 1,500 $µg.mL^{-1}$ and 5 mL of 5mM 4-*p*NPG and 5 mL of 0.05 $mg.ml^{-1}$ α-glucosidase. The reaction was incubated at 37°C, 10 min. Finally, 100 mL of 0.5 M Na_2CO_3 was added to stop the reaction. The absorbance was carried out at 405 nm using UV/Vis-spectrophotometer (Mapada UV-1200, Shanghai, China). Acarbose is the standard inhibitor used as the positive control. The inhibition percentage was calculated by the reaction below. The IC_{50} values of acarbose and all rice cultivars were calculated by Grafit 5.0 computer program (Erithacus Software, Horley, UK).

% Inhibition = $[(A_b - A_s)/A_b]*100$ (A_b = absorbance without sample; A_s = absorbance with sample). (1)

2.2.4. Tyrosinase inhibitory activity assay

The tyrosinase inhibitory activity assay of kojic acid and all rice samples was performed according to the method of Uchida et al. (2014) with slight modification. In brief, five mM of L-DOPA and 1 $mg.ml^{-1}$ of rice samples were prepared in 5% DMSO. The 0.1 $mg.ml^{-1}$ of tyrosinase from the mushroom was prepared in 20mM sodium phosphate buffer pH 6.8. First, 5mL of rice extracts (final concentration range of 2 $µg.mL^{-1}$ to 1,500 $µg.mL^{-1}$) was mixed with 5mL of tyrosinase (5$µg.mL^{-1}$ in final concentration), then the reaction mixture was incubated at 30°C for 10 min. After that, 5mLof L-DOPA (0.25mM in final concentration) was added to the reaction mixture. Then, the reaction

was continuously incubated at 30°C for 15 min. The dopachrome production was analyzed by the spectrometer at 492 nm using UV/Vis-spectrophotometer (Mapada UV-1200, Shanghai, China). Kojic acid was used as the standard inhibitor, working as the positive control. The inhibition percentage was calculated by the reaction above. The IC_{50} values of kojic acid and all rice cultivars were calculated with the same a-glucosidase inhibitory activity.

2.2.5. Statistical analysis

The experiments including α-glucosidase and tyrosinase inhibitory activity of rice samples were performed in triplicate and these results showed as standard deviation of mean (SD). Statistical differences were determined by one-way analysis of variance (ANOVA), and the differences were considered significant at $P < 0.05$.

3.Results and discussions

3.1. Phytochemical compounds

The phytochemical compounds of 7 colored-rice cultivars in the different solvent extracts were evaluated and shown in Table 1. The compounds including flavonoids and reducing sugar were obtained from all rice cultivars and all solvent extract. In contrast, saponin and terpenoids were identified in some rice cultivars and some solvent extracts. However, saponin seems to have abundantly been found than terpenoids. Flavonoids from the plant have been reported for their biological activity. Flavonoids also have multiple positive health effects, primarily on metabolic disorders, such as cancer, diabetes mellitus and heart disease (Middleton et al., 2000; Al-Ishaq et al., 2019).

Table 1. Phytochemical profile of 7 colored-rice cultivars extracts

Rice cultivars	Samples	Phytochemical compounds			
		Flavonoids	Reducing sugar	Saponin	Terpenoids
1	Water extract	+	+	+	-
	Ethanol extract	+	+	+	+
	Methanol extract	+	+	+	+
2	Water extract	+	+	+	+
	Ethanol extract	+	+	+	+
	Methanol extract	+	+	+	+
3	Water extract	+	+	+	-
	Ethanol extract	+	+	+	+
	Methanol extract	+	+	+	+
4	Water extract	+	+	+	-
	Ethanol extract	+	+	-	-
	Methanol extract	+	+	+	-
5	Water extract	+	+	+	-
	Ethanol extract	+	+	-	-
	Methanol extract	+	+	+	-
6	Water extract	+	+	+	-
	Ethanol extract	+	+	+	-
	Methanol extract	+	+	+	-
7	Water extract	+	+	+	-
	Ethanol extract	+	+	+	+
	Methanol extract	+	+	+	+

+; indicated detected of the compounds. -; indicated not detected of the compounds

Saponin and terpenoids from the plant also have been reported for their biological activity. El Barky et al. (2017) reported that saponin decreased blood glucose levels in diabetes mellitus. The terpenoids have also been reported for metabolic disorders, especially for Type II diabetes (Panigrahy et al., 2021). Our results showed that the biological compounds,

including flavonoids, saponin, terpenoids, had been identified from all 7 colored-rice cultivars. These rice cultivars had also demonstrated the α-glucosidase inhibitory activity. Previous reports and our results indicated that biological activity such as diabetes had related to biological plant compounds such as flavonoids, saponin, and terpenoids.

3.2. Thai colored-rice extracts against α-glucosidase activity

The results show that methanol extract of 7 colored-rice cultivars seems to have effective α-glucosidase inhibition than other solvent extraction and the positive control inhibitor acarbose Table 2.

Table 2. α-Glucosidase inhibitory activity of rice extracts.

Rice cultivars	IC_{50} value (μg.ml^{-1})		
	Water extract	**Ethanol extract**	**Methanol extract**
1	229.31 ± 23.31^{b}	168.21 ± 7.72^{b}	44.04 ± 4.13^{c}
2	286.38 ± 33.17^{a}	115.61 ± 6.94^{e}	37.42 ± 3.13^{d}
3	128.63 ± 12.69^{de}	171.55 ± 7.22^{b}	38.58 ± 6.06^{d}
4	141.24 ± 5.17^{d}	186.26 ± 12.13^{a}	20.44 ± 2.85^{e}
5	179.43 ± 14.31^{c}	153.41 ± 6.84^{c}	171.44 ± 13.29^{a}
6	118.22 ± 10.07^{e}	134.57 ± 11.88^{d}	1.06 ± 0.13^{f}
7	172.01 ± 14.30^{c}	125.76 ± 5.75^{de}	135.42 ± 9.46^{b}
Acarbose	185.92 ± 15.12		

The IC_{50} values were presented as mean ± standard error of the mean of triplicate measurements. The differences superscript letters in the column indicate significant differences between the inhibitory activity of rice extracts ($P < 0.05$).

The IC_{50} values of methanol extract was in the range of 1.06 ± 0.13 μg.ml^{-1} to 171.44 ± 13.29 μg.ml^{-1}. The highest effective α-glucosidase inhibition of methanol extract of rice cultivar 6 (IC_{50} values of 1.06 ± 0.13μg.ml^{-1}) was approximately 175 times higher than acarbose (IC_{50} values of 185.92 ± 15.12 μg.ml^{-1}). While methanol extract of rice cultivar 5 (IC_{50} values of 179.43 ± 14.31 μg.ml^{-1}) was less effective against α-glucosidase activity than other rice cultivars but still higher effective than acarbose. The α-glucosidase inhibitory activity of methanol extract of all colored-rice cultivar was also higher than other solvent extraction except for rice cultivar 5 and cultivar 7. More observation, the α-glucosidase inhibitory potential of ethanol extract also had higher potent than acarbose except for rice cultivar 4. The lower a-glucosidase inhibitory activity of rice extract was obtained from water extract, especially rice cultivar 2 with the IC_{50} values of 286.38 ± 33.17 μg.ml^{-1}. The highest IC_{50} value of rice cultivar 2 had approximately 270.17-times and 1.54-times compared with the lowest IC_{50} value of rice cultivar 6 of methanol extract (highest α-glucosidase inhibitory activity) and IC_{50} value of acarbose, respectively. The α-glucosidase inhibitory activity of rice bran had been reported previously by Sivamaruthi et al. (2018). The highest one against the α-glucosidase activity of rice bran was obtained from ethanol and methanol, similar to our results. The ethanol extract of black and red rice can reduce hyperglycemia, which induces diabetes in the rat by streptozotocin (Tantipaiboonwong et al., 2017). Our results and previous report indicate that the high efficiency of inhibitor against α-glucosidase activity should be extracted under methanol and ethanol conditions and the results also indicate that the inhibitor might have slight polarity.

3.3. Thai colored-rice extracts against tyrosinase activity

The tyrosinase inhibitory activity of 7 colored-rice cultivars was investigated, and their IC_{50} values were shown in Table 3. Kojic acid is the commercial inhibitor that acts as the positive control. All the IC_{50} values of rice extract in

different cultivars and different solvent extracted were shown in higher values than kojic acid. The high tyrosinase inhibitory activity was obtained from water extract with the IC_{50} values were found to be 10.93 ± 3.41 μg.ml^{-1} to 301.39 ± 26.01 μg.ml^{-1}. Rice cultivar 6 of water extract had the highest tyrosinase inhibitory activity efficiency but still lower than kojic acid. The most increased tyrosinase inhibitory activity of rice cultivar 6 (IC_{50} values of 10.93 ± 3.41 μg.ml^{-1}) had approximately 27-times higher than the lowest one of rice cultivar 1 (IC_{50} values of 301.39 ± 26.01 μg.ml^{-1}). Still, its inhibitory activity efficacy had approximately 2.4-times lower than kojic acid (IC_{50} values of 4.64 ± 0.69 μg.ml^{-1}). Furthermore, water extract of rice cultivar 2 to rice cultivar 7 had higher tyrosinase inhibitory activity potent than ethanol and methanol extract. The IC_{50} values of ethanol extract and methanol extract were of similar potential levels. The results indicated that ethanol extract had lower tyrosinase inhibitory activity than methanol extract. The highest IC_{50} values of all extraction were obtained from ethanol extract of rice cultivar 4 and its IC_{50} values were determined to be 488.90 ± 44.92 μg.ml^{-1}. Rice cultivar 4 of ethanol extract and its IC_{50} values were approximately 105-times and 45-times higher than those of kojic acid and rice cultivar 6 (most increased tyrosinase inhibitory activity) water extract, respectively. The results suggest that tyrosinase inhibitor compounds from rice might have strong polarity. Many tyrosinase inhibitor compounds have been isolated from various plants by polarity solvent. The isolated 6 from *Quercus coccifera* (L.) has strong potent on tyrosinase inhibitory activity, and this compound was extracted with methanol. In contrast, the increase of polarity by increase of water ratio was shown in the purification step (Şöhretoğlu et al., 2014). Di Petrillo et al. (2016) also reported the extraction of *Asphodelus microcarpus* with three solvents i.e., water, ethanol, and methanol. The results showed that the high tyrosinase inhibitory activity was investigated from ethanol extract. Fu et al. (2014) also reported the high antioxidant and tyrosinase inhibition activity of *Sapium sebiferum* (L.) Roxb. extracted by water at high temperatures. The previous report may support our results, indicating that the compounds from rice with high tyrosinase inhibition activity might have strong polarity.

Table 3. Tyrosinase inhibitory activity of rice extracts.

Rice cultivars	IC_{50} value (μg.ml^{-1})		
	Water extract	**Ethanol extract**	**Methanol extract**
1	301.39 ± 26.01^{a}	305.90 ± 26.74^{c}	214.48 ± 21.28^{d}
2	37.44 ± 4.27^{cd}	196.10 ± 8.50^{e}	330.88 ± 29.44^{b}
3	31.83 ± 3.50^{d}	322.44 ± 30.50^{b}	180.09 ± 19.13^{e}
4	40.27 ± 4.13^{c}	488.90 ± 44.92^{a}	154.06 ± 9.90^{f}
5	40.31 ± 4.08^{c}	181.20 ± 18.95^{f}	341.61 ± 30.25^{a}
6	10.93 ± 3.41^{e}	195.94 ± 15.99^{e}	299.72 ± 21.19^{c}
7	73.15 ± 6.63^{b}	290.67 ± 20.19^{d}	210.71 ± 13.44^{d}
Kojic acid	4.64 ± 0.69		

The IC_{50} values were presented as mean ± standard error of the mean of triplicate measurements. The differences superscript letters in the column indicate significant differences between the inhibitory activity of rice extracts ($P < 0.05$).

3.4. The efficacy of α-glucosidase and tyrosinase inhibitory activity of colored rice extracts from different solvent extract

The inhibition potential on α-glucosidase and tyrosinase activity of rice extract of different solvent extractions were shown in Figure 2. The higher polarity of solvent seems to give the inhibition potential on tyrosinase activity than the lower polarity solvent. Tyrosinase inhibitory activity of water extract has high efficacy than

other solvents except for rice cultivar 1. Other solvent extracts, ethanol, and methanol get high α-glucosidase inhibitory activity than tyrosinase of all rice cultivars. Three solvent-extracts, water, ethanol, and methanol are the polarity solvents, where water has the higher polarity than ethanol and methanol. The extraction has reported many inhibitors of α-glucosidase and tyrosinase with polarity solvent. Masuda et al. (2007) identifies tyrosinase inhibitor as 4-beta-D-glucopyranosyloxybenzoic acid from leaves of *Nandina domestica*, and this compound is extracted by ethanol, and it is water-soluble. Tyrosinase inhibitor has also been reported from the methanol extract. Identified from *Vitex negundo* Linn, 8 ligands have high potency on tyrosinase inhibitory activity (Malik et al., 2006). Our results agree with the report of Ohta et al. (2002) in that the methanol extract from *Pelvetia babingtonii* (Harvey) De Toni has a high effect against a-glucosidase, sucrase, and maltase activity. The new ester of fatty acid, also extracted by methanol, has potency on α-glucosidase inhibitory activity reported by Mondal et al. (2015). The other polarity solvent, such as ethyl acetate, has been used to extract the a-glucosidase inhibitor. Mauldina et al. (2017) reported that the ethyl acetate extract of *Antidesma bunius* (L.) containing triterpenoid had a-glucosidase inhibitory activity. Thus the previous reports and our results indicate that the α-glucosidase and tyrosinase inhibitor could be extracted from plants by polarity solvent. The rice extracts containing the a-glucosidase and tyrosinase inhibitor may be applied in cosmetic products such as skinning products. Moreover, these extracts may be applied to the drug for diabetes treatment.

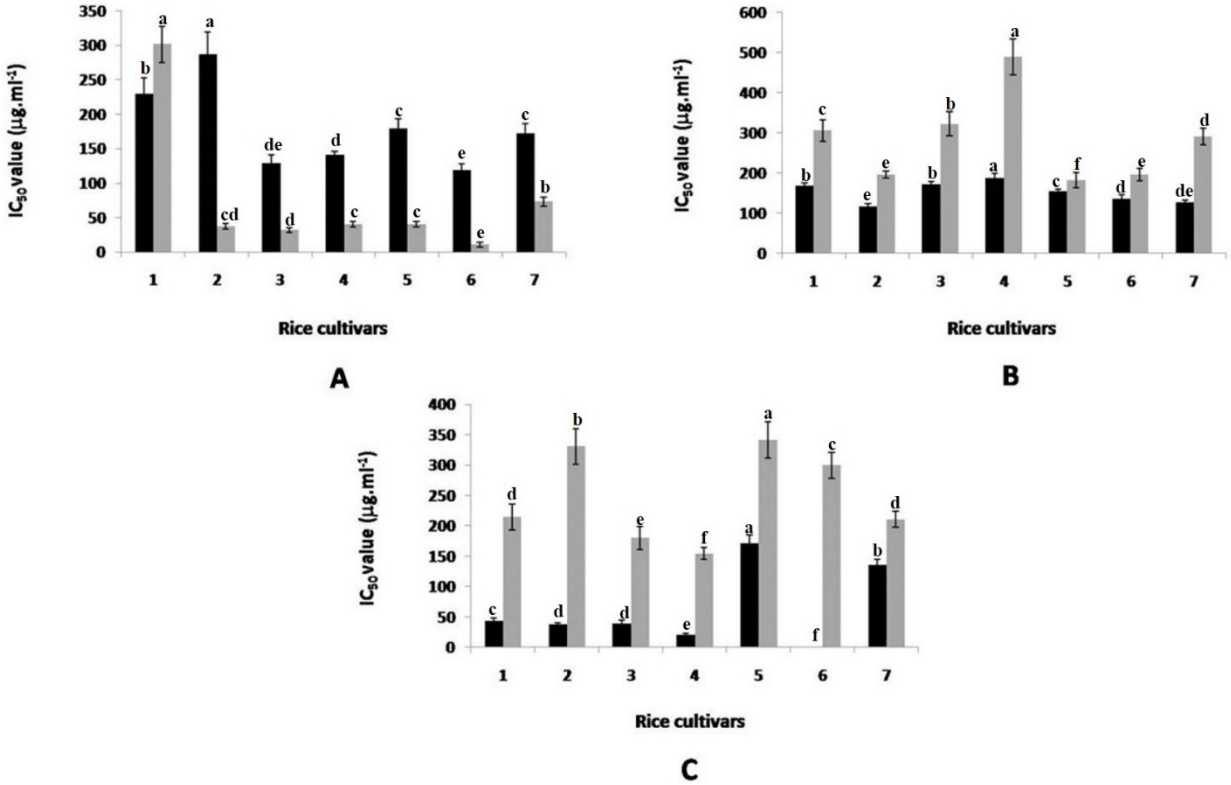

Figure 2. The comparison between α-glucosidase and tyrosinase inhibitory activity from three solvent extracts (water, ethanol, and methanol) of 7 colored-rice cultivars. A; water extract, B; ethanol extract, and C; methanol extract. The black bars represent a-glucosidase inhibitory activity, and the gray bars represent tyrosinase inhibitory activity. The error bar is also shown at the top of each bar graph. The differences letters above the error bar indicate significant differences between the inhibitory activity of rice extracts ($P < 0.05$).

4. Conclusions

We investigated the phytochemical profiled, α-glucosidase and tyrosinase inhibitory activity from water extract, ethanol extract, and methanol extract of 7 colored-rice cultivars. The flavonoids, reducing sugar, saponins, and terpenoids were identified from all colored-rice cultivars. The methanol extract had higher α-glucosidase inhibitory activity than those of other solvent extracts (water and ethanol) and acarbose. In contrast, the water extract seemed to have the lowest α-glucosidase inhibitory activity than those of ethanol extract and methanol extract. On the other hand, the water extract had higher tyrosinase inhibitory activity than other solvents but lower than kojic acid. At the same time, the ethanol extract showed the lowest tyrosinase inhibitory activity than those of methanol and water. The results indicate that rice was contained bio-active compounds that rich in α-glucosidase and tyrosinase inhibitory compounds.

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PREVALENCE OF *SALMONELLA* STRAINS ISOLATED FROM INDUSTRIAL QUAIL EGGS AND LOCAL DUCK EGGS, IRAN

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ABSTRACT

Salmonella is a worldwide public health issue as one of the reasons for foodborne illness for humans and animals. Eggs can be a significant source of this bacterium and the prevalence of salmonellosis. Thus, the control of contamination by *Salmonella* has become essential for the consumer. This study investigates the prevalence, and serotype distribution of *Salmonella* isolates recovered from industrial quail eggs and local duck eggs collected from Qazvin city, Iran, in 2020. In this cross-sectional study, 130 eggs were collected randomly (including 100 industrial quail eggs and 30 local duck eggs) from the retail and stores in Qazvin city, Iran. *Salmonella* was isolated from eggshells and egg contents using conventional culture methods for selective isolation of *Salmonella* and biochemical identification, suspect colonies confirmed by Real-Time PCR assay for the amplification and detection of *Salmonella* using specific primers. A 16.67% prevalence of *Salmonella* was observed from duck eggs; however, no *Salmonella* recovered from quail eggs. *Salmonella* was isolated from 0% (0 groups of 6 groups) and 16.67% (1 group of 6 groups) of eggshells and contents of duck eggs, respectively. Isolates from positive egg samples characterized as *S. Typhimurium*. Although *Salmonella* infection was low in this study, Continuous monitoring is required to prevent health hazards associated with poultry products in this area, and the presence of duck eggs can be a public health problem. The results of this study are essential for the government, consumers, regulators of poultry products, producers like poultry farmers.

1.Introduction

Salmonella is one of the significant foodborne enteric pathogens globally and causing enormous economic losses in the poultry industry. Non-typhoidal *Salmonella* causes 4.07 million Disability Adjusted Life Years (Huang et al., 2016; Kirk et al., 2015). The serotype is a phenotypic trait according to which *Salmonella* is divided into groups A, B, C, D and *Salmonella* with over 2600 serotypes is a widespread zoonotic pathogen (Abdel-Maksoud et al., 2015; Hai et al., 2020).

Salmonella enterica serovar *Typhimurium* and *Salmonella enterica* serovar *Enteritidis* are the most current causes of non-typhoidal salmonellosis throughout the world (Lee et al., 2015); *S. Typhimurium* and *S. Enteritidis* are

causing gastroenteritis and, severe systemic infections may occur in infants, the elderly, and immunocompromised individuals for an instant the HIV-positive, diabetics or rheumatoid arthritis cases (Bonny et al.; Ceyssens et al., 2015; Lee, Runyon, Herrman, PhillipsHsieh, 2015). *Salmonella* is more dangerous in people under the age of 20 (children) and over 70 (elderly) than in other ages (Danesh Ghohar et al., 2017; Nadi et al., 2020).

Salmonella serovars cause of foodborne has a different prevalence in the various times and regions. For example, *S. Typhimurium* caused an outbreak in Australia, while *S. Enteritidis* caused the spread in Europe and United States (J. R. Andrews et al., 2015; Chousalkar et al., 2017; El-Tayeb et al., 2017).

Contamination of egg contents by *Salmonella* can occur in two ways, including 1) contamination of the egg content or vertical transmission before shell formation laid by invasion to ovaries and oviducts, and 2) contamination of the shell surface or horizontal transmission after laid with *Salmonella* infiltration into eggshell membranes after infection oviposition (De Vylder et al., 2013; Gantois et al., 2009).

Salmonellosis is mainly related to the consumption of meat, poultry, eggs and milk, and therefore this organism is a pathogen transmitted through food (Khodadadipour et al., 2016).Eggs and egg products are a significant part of the human diet. Eggs such as Quail eggs are tasty and have much nutritional value, a little fat content (Mir et al., 2015), and they can be a source of foodborne diseases, such as salmonellosis outbreaks worldwide (Moffatt et al., 2016). Non-typhoidal *Salmonella* strains (NTS) separated from eggshell and egg contents of quail and duck in different countries like India, Nigeria, Egypt and other countries (Ashraf et al., 2013; Harsha et al., 2011; Nwaobi et al., 2016; Routhu, 2019; TURGAY, 2004). Duck eggs and duck products were associated with *S. enterica* serovar *Typhimurium* outbreaks in Germany, England (UK) and Northern Ireland at different times (Noble et al., 2012; Owen et al., 2016).

Efficient, rapid and specific methods like DNA methods for detecting and identifying various pathogens such as *Salmonella* species with 95 bp products in a different kind of food are significant for clinical and reporting aims (Adzitey et al., 2012). Polymerase chain reaction (PCR) is a molecular biology technique with high sensitivity. The invA gene of *Salmonella* in the mammalian epithelial cells contains sequences unique to this genus, and it is an appropriate PCR target by a potential diagnostic application (Ashraf, Ahmed, Aisha, FatmaMohammed, 2013).

Due to the low economic conditions of society, high egg consumption, the possibility of egg contamination risk of *Salmonella* for the community, a little information about the distribution of strains in eggs. In this regard, this study aimed to evaluate the prevalence of *Salmonella* contamination and characterization of serotypes in industrial quail eggs and local duck eggs supplied from retail markets of Qazvin, Iran.

2. Materials and methods

2.1. Materials

We collected 130 eggs, including 100 industrial quail eggs and 30 local duck eggs. Samples were collected from retail and stores of Qazvin, Iran, in 2020 (every 100 eggs represented by 20 samples, five eggs constitute one sample). Samples were placed in a separate sterile bag and immediately transferred to the laboratory in cool boxes. The eggs were stored under sterile conditions at four °C until being analyzed.

2.2. Methods

2.2.1. Isolation and detection of Salmonella

The eggs were prepared as described by Bacteriological Analytical Manual (W. H. Andrews et al., 2011). Briefly, a swab (Sterile cotton) technique was used to sample the shell surface of the intact eggs. Swabs dipped in 50 ml of trypticase soy broth (TSB) ((LIOFILCHEM, DIAGNOSTIC, ITALY) pre-enrichment) supplemented with ferrous sulfate (35 mg ferrous sulfate added to 1,000 mL TSB) and incubated at 37 °C for 24 h. The egg was

disinfected with a disinfectant solution to investigate the *Salmonella* contamination under eggshell and egg contents. The disinfection solution Prepared by adding 250 ml iodine/potassium iodide solution to 750 ml 70% alcohol solution and mixed well. Submerge eggs in disinfection solution for 10 seconds (ensured not less than 10 seconds). The eggs were removed from the solution and air-dried in a sterile chamber, then cracked with a sterile knife. Each egg contents were thoroughly mixed. Then, the sample was added to 500 ml of TSB and incubated at 37 °C for 24 h. For selective enrichment broth, 1 ml of the pre-enrichment solution was put in a tube containing 20 ml of Rappaport Vassiliadis (Scharlau, Spain) (incubated at 37°C for 24 h). The RV cultures were streaked onto xylose lysine deoxycholate (LIOFILCHEM, DIAGNOSTIC, ITALY) plates (Selective media) and incubated at 37 °C for 24 h. In the XLD cultures, Salmonella has colonies with black centres. Presumptive colonies were processed and identified by biochemical tests like triple sugar iron agar slant (Scharlau, Spain) and urea agar (LIOFILCHEM, DIAGNOSTIC, ITALY) then incubated at 37 °C for 24 h. (Figure 1). A selective medium of TSIA slant was used to detect the lactose, saccharose and dextrose fermenters and determine the organisms to produce H2S. Black slant and yellow butt or pinkish slant and yellow butt were recorded as the positive reaction for *Salmonella*. The colour of urea agar was yellow (Negative result).

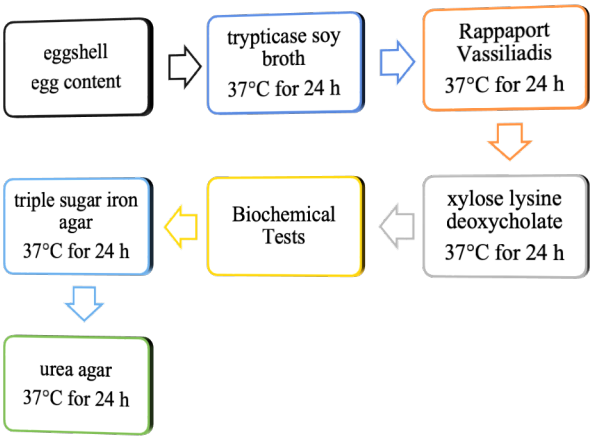

Figure 1. Isolation and identification of *Salmonella*.

2.2.2. Preliminary identification of Salmonella by PCR

The specific primer InvA was used to detect *Salmonella* (Heymans et al., 2018). Primers were used based on the InvA gene sequence designed using Primer-BLAST software and NCBI gene bank. SINACLON, Iran, synthesized the primers. Primers listed in Table 1.

Table 1. Primers designed to detect *Salmonella*.

Gene	Sequence of nucleotides	Primer's size (bp)	Gene size (bp)
InvA	F-GCTGCTTTCTCTACTTAAC	19	
	R-GTAATGGAATGACGAACAT	19	95
SEN1392	F= GGATATGAGGTGCGTTTA	18	
	R= CAGTGCCGGAATTATCTC	18	77
STM4200	F-CACCTGATATAGAGTCCAA	19	
	R- TATAGATGTTGTCGCCAA	18	101

2.2.3. Real Time PCR

Total genomic DNA was extracted by using a pathway boiling. PCR method was used to verify and the identification of isolates. *Salmonella* isolates examined and identified for invA genes in DNA extracted from isolate by multiplex quantitative PCR method was described by Raymond Heymans et al. with some modification (Heymans, Vila, van Heerwaarden, Jansen, Castelijn, van der VoortBiesta-Peters, 2018). A Real-time PCR (Rotor-Gene Q) device was used by a temperature cycle according to Table 2 in 46 cycles with the desired PCR mixture.

Table 2. PCR mixture, and cycling conditions for detection *Salmonella* isolates based on InvA genes.

Composition	**Stock**	**Content in final volume (20 µL)**
Master mix	2 X	10 µL
Forward primer	10µM	0.5µl
Reverse primer	10µM	0.5µl
DDW	-	6µl
DNA	-	3µl (ng)
Stage	**Temperature (°C)**	**Time (s)**
Denaturation	95	15
Annealing	51.5	19
Extension	72	37

2.2.4. Data analysis

All data analyses were performed using SPSS Statistical Software version 25. The dependent variable used in the study included the incidence of *Salmonella*. Independent variables included testing eggshells and egg content. The Chi-square test was used to compare the incidence of *Salmonella* to different variables. The results were considered significant by a P-value < 0.05.

3.Results and discussions

3.1. Prevalence and serotypes

A 16.67% prevalence of *Salmonella* was observed from duck eggs, and no *Salmonella* was recovered from quail eggs of two different groups collected randomly. *Salmonella* was isolated from 0% (0 groups of 6 groups) and 16.67% (1 group of 6 groups) of eggshells and contents of duck eggs, respectively. The isolate identified from positive egg samples were *Salmonella typhimurium* serotype (Figures 2). Shell contamination was significantly less than

content contamination (Table 3). There was a significant relationship between contamination in duck and quail samples and there was a significant relationship between contamination in shell and contamination in contents of duck eggs.

Table 3. Distribution and sources of *Salmonella* isolated.

Sample			Quail egg shell	Quail egg content	Duck egg shell	duck egg content	Total
positive	**pos**	Count	0	0	0	5	5
		% Within egg	0.0%	0.0%	0.0%	16.7%	1.9%
	not	Count	100	100	30	25	255
		% Within egg	100.0%	100.0%	100.0%	83.3%	98.1%
Total		Count	100	100	30	30	260
		% Within egg	100.0%	100.0%	100.0%	100.0%	100.0%
Mean			1.9808				
Std. Deviation			.1376				

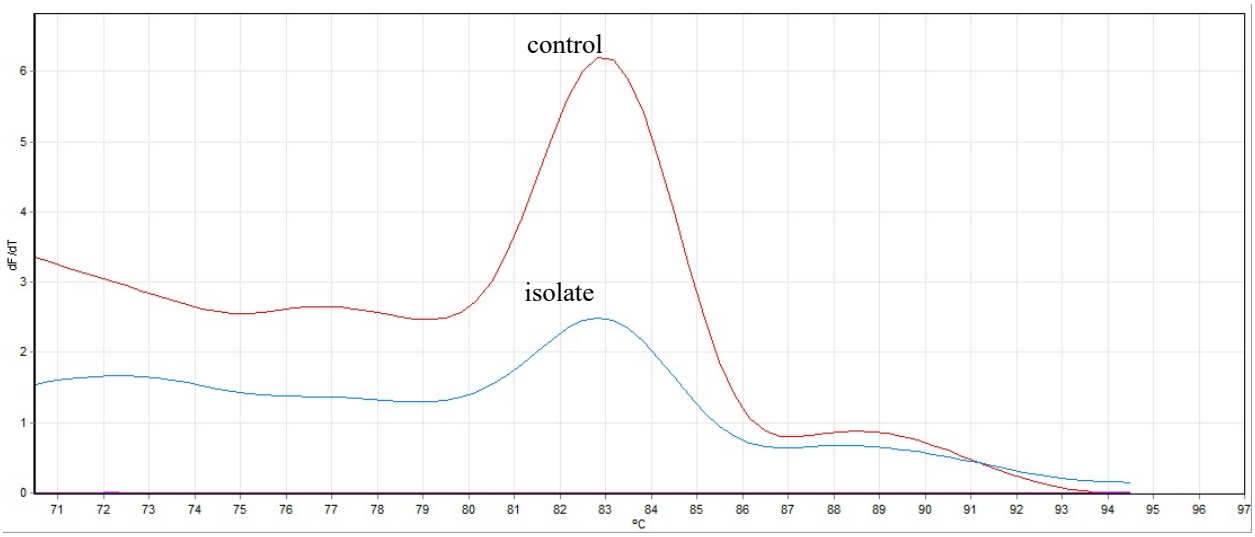

Figure 2. Melting curve analysis of SYBR Green real-time PCR product of *Salmonella Typhimurium*.

3.2. Egg contamination with different *Salmonella* strains and serotypes

Salmonella is one of the reasons foodborne diseases in humans, and salmonellosis is caused by eggs infected with this pathogen and is a significant public health issue worldwide (Rahman et al., 2019). Liquid egg products are implicated in an extensive range of foods that may not receive enough heat treatment' like pasteurization, thus causing contamination of the latest production (TURGAY, 2004).

Salmonella has been isolated from quail eggshells from Indonesia and India (Erina et al., 2019; Harsha, Reshmi, Varghese, Divya, Rahiman Hatha, 2011). also, it has been isolated from quail egg content from different regions, including Nigeria, Iran, and Turkey (Atere et al., 2015; Nwaobi, Kwaga Okolocha, 2016; Hamid Staji et al., 2012; TURGAY, 2004). The prevalence in these studies was higher than in our study. This contamination could be due to environmental contamination and poor storage conditions within the study areas. *Salmonella* has not been isolated from quail eggs from Nigeria, India and Iran which these results agree with our studies (Badouei et al., 2012; Bose et al., 2020; Sangeetha et al., 2019). Quail are reported to be more resistant to infectious diseases than chicken, so it effectively reduces the prevalence of infection and even the absence of infection in eggs (Routhu, 2019). No contamination observed in this study may be due to increased knowledge on the prevention and control of poultry illness.

Some studies have reported infection with *Salmonella* serotypes both in duck eggshells and contents for instance, in a study in India, *Salmonella* contamination was detected in duck egg content (16.66%) and duck eggshell (6%) in which contamination of duck eggshell was higher than the prevalence rates of our study (Harsha, Reshmi, Varghese, Divya, Rahiman Hatha, 2011). These results show that *Salmonella* contamination in retail duck eggs in India is a severe public health concern at that time. Some studies have not reported infection with *Salmonella* serotypes in duck eggshells and contents like in Malaysia and Iran (Adzitey, RusulHuda, 2012; Badouei, GhalejooghiMadadgar, 2012; Sarif et al., 2012).

At the same time, the prevalence of *Salmonella* contamination in duck eggs identified in this study is higher than that reported in duck liver and Fecal swabs in Egypt by a prevalence of 8.3% and 4%, respectively also, the different prevalence of *Salmonella* contamination in local and industrial eggs reported in Asia (Hai, Yin, Lu, Lv, Zhao Bie, 2020; Rahman, Ahmad, Mahmud, Barman, Haque, Uddin Ahmed, 2019; Xie et al., 2019). The differences in the prevalence of *Salmonella* contamination in these studies is due to the different geographical areas, differences in the environmental and breeding conditions, C&D (Cleaning and disinfection), nonmetal duck houses, regardless of the production cycle, rodent control, a relationship of birds with large animals, health and safety of stores and retail, methods of isolation, humidity and temperature associated with the climate although flock size (Small, Medium, Large), dog presence, avian influenza history, and distance to the nearest poultry farm did not significantly affect Salmonella prevalence (Kim et al., 2021). Strategies that are effective for food safety and ultimately the protection of public health are monitoring and good hygiene practices like the development of the management system of breeding places and shops and retail (places, tools and methods of regular cleaning and disinfection) and the use of environmental health standards (dry and clean place of breeding and sales (Chen et al., 2020).

The prevalent serotypes detected in our study was *S. Typhimurium*, which is similar to research that has been done on eggs, duck, duckling and duck eggs around the world (Adzitey, RusulHuda, 2012; Ashraf, Ahmed, Aisha, Fatma Mohammed, 2013; Chen, Bai, Wang, Zhang, Zhan, Shen, Zhang, Wen, Gao Liao, 2020; Lenchenko et al., 2020; Sodagari et al., 2019; H Staji et al., 2017; Wang et al., 2020). Due to geographical location, climatic conditions and type of test method, the distribution of serotypes in the world is different (Han et al., 2020).

The real-time PCR assay is being used as a rapid and reliable tool for controlling and detecting contaminated *Salmonella* samples along the food production chain. The invA invasive gene has been offered as an international standard for detecting *Salmonella* in egg and egg products or food chains by PCR. Thus, invA can act as a reliable and accurate gene for diagnosing *Salmonella* by PCR, and PCR showed that the isolates were *Salmonella typhimurium* (Cheng et al., 2008; Gole et al., 2014; Malorny et al., 2003; Malorny et al., 2004). The study from Bangkok detected *Salmonella* from the eggshell using the invA gene (Loongyai et al., 2010). In this study, *Salmonella* invA invasive gene was also used to detect *Salmonella.*

Like other bird species, healthy ducks can be a source of *Salmonella*, and their gastrointestinal tract and faeces are infected with this pathogen (Adzitey, RusulHuda, 2012), show no signs of infection, and transmit the pathogen to other humans and animals. Also, the consumption of raw or almost cooked eggs can lead to salmonellosis, and this disease causes economic losses through disease and death (Muhammad et al., 2010).

Contamination of egg contents by *Salmonella* can occur in two ways, including 1) direct contamination before shell formation laid, and 2) indirect contamination after laid along the storage time like lack of hygiene at the layer farms, improper washing, grading and packing operations (Pärn et al., 2017). In this study, surface contamination of eggshells was not seen in two sample groups, but internal contamination of egg contents was seen in local duck egg samples, which shows direct contamination of eggs during formation in the reproductive tract.

4. Conclusions

In summary, our findings showed that *Salmonella* was identified in a group of duck eggs. A positive sample infected with *Salmonella* was detected to *Salmonella typhimurium*, and *Salmonella* was not identified in quail eggs. This study can be helpful in risk management options, risk assessment, regulators about food safety.

Our study has a limitation. One of the reasons for the lack of duck eggs was the beginning of autumn, so sampling in early summer and the tropical months of the year is recommended that the more samples, the more accurate the conclusion will be.

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MICROELEMENT COMPOSITION OF BASIC CONSUMPTION PRODUCTS IN THE TRANSCARPATHIAN REGION, UKRAINE

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Traditional food products.

ABSTRACT

Traditional food products (milk and vegetables) form the diet basis and are the main source of essential trace elements (microelements). The contents of trace elements in food products obtained from various landscape areas significantly differ. Transcarpathia has a pronounced tectonic and geological diversity. The young Carpathian Mountains face high tectonic and geological activity, which can affect the microelement composition of food products. A significant difference in the microelement composition of milk and vegetable mix was found for different landscape zones, for instance, food in lowland areas is richer in Fe, Cu, Zn, Mo, Co, P, Se, I, Br, F, Ca, and Mg while food in mountainous areas contains large amounts of As and Mn. Consistent patterns of microelement distribution in the food products from different landscape zones (lowland > foothill > mountainous) were estimated using the Spearman correlation coefficient. For milk samples: Fe: 0.91; Cu: 0.89; Mn: -0.94; Zn: 0.88; Mo: 0.89; Co: 0.94; Ca: 0.91; Mg: 0.91; P: 0.92; As: -0.94; Se: 0.82; I: 0.84; Br: 0.73; F: 0.82, for vegetable mix samples: Fe: 0.91; Cu: 0.85; Mn: -0.94; Zn: 0.79; Mo: 0.90; Co: 0.92; Ca: 0.94; Mg: 0.94; P: 0.80; As: -0.91; Se: 0.84; I: 0.91; Br: 0.73; F: 0.88. Pronounced inter-element correlation of microelements in food products is observed (the value of the Pearson correlation coefficient for all pairs of chemical elements is $r > 0.60$).

1. Introduction

Nutrition is a prerequisite for a healthy and full quality life. Each food item affects human health (Niva, 2007). Therefore, the quality and safety of food products requires special attention (Khan *et al.,* 2017; Sohail *et al.,* 2018; Barrett, 2010; Lam *et al.*, 2013; Röhr, 2005). Similarly, traditional food products should be carefully considered, since they are the basis of any diet (Guerrero *at el.,* 2010).

In most cases, nutritional value of food products is measured by the content of the main components (proteins, carbohydrates, fats, etc.) or biologically active substances (vitamins, polyphenols, etc.) (Chen *at el.,* 2017; Abuajah *at el.,* 2014; Rahaiee *at el.,* 2014; Kadnikova *et al*., 2015; Sharma *et al.*, 2012). However, the

micro- and macronutrient composition of food products is also an important quality criterion (Harmankaya *et al.*, 2012; Kizil and Turk, 2010; Özcan *et al.*, 2013; Rudawska and Leski, 2005; Simsek and Aykut, 2007; Škrbić and Onjia, 2007; Słupski *et al.*, 2005; Xiao *et al.*, 2016). New approaches, which include personalizing the choice of a diet, are an important component of rational and therapeutic nutrition and affect immunity and other crucial health components (Luo *at el.,* 2018; Kovalskys *at el.,* 2015; Shahidi, 2006). An important aspect of assessing food products' quality is their microelement composition, which can be used to measure the degree of ingestion of vital chemical elements into the human body when eating food, which affects human health (Bilandžić *at el.,* 2015; Marles, 2017; Martínez-Ballesta *et al.*, 2010). The content of microelements (essential trace minerals) in food products depends on their content in soils and rocks. Therefore, the geology and geochemistry of the studied areas can significantly affect the contents of essential trace elements in foods.

Based on the reference diet of Ukrainian citizens, milk and dairy products (equivalent to milk) account for about 42% (1,022 kg per day) of the diet, vegetables – about 26% (potatoes – 0.359 kg per day, other vegetables – 0.279 kg per day) of the diet (MHPU, 1997). These products, which are also traditional, form the basis of a person's diet, including residents of the Transcarpathian region. Since most residents of the Transcarpathian region consume household-produced dairy products and vegetables, the latter's microelement composition depends on how they were cultivated and obtained. The Transcarpathian region is characterized by significant landscape diversity (height difference varies from 100 m to 2100 m) including geochemical zones and terrain features. Therefore, traditional food products (milk and vegetables) obtained in different landscape zones can differ much in terms of microelement composition. This affects the entry of vital trace elements into the human body and, therefore, health. No such studies for traditional food products of the Transcarpathian region have been conducted before.

This paper presents data on the microelement composition (based on 16 elements, including Hg and Pb safety indicators) of traditional food products (whole cow's milk and vegetable mix) of the Transcarpathian region taking into account the area's landscape and geochemical diversity. The contents of microelements: Fe, Cu, Mn, Zn, Mo, Co, As, Se, I, Br and F, macroelements: P, Ca and Mg, and safety indicators: Hg and Pb were studied. The choice of Hg and Pb as a safety indicator is based on their high toxicity to humans, even in small amounts (Boudebbouz *at el.,* 2021). Based on the generalization of the results of analyses, major features of the microelement composition of traditional food products have been identified, which may have a decisive effect on the health of the region's population. A similar study was carried out for raw cow milk in Slovakia (Pšenková *at el.,* 2020).

2. Materials and methods

2.1. Study area

The research study was conducted for all landscape zones of the Transcarpathian region in 2018. It covers a mountainous area (3 districts: Rakhiv, Mizhhirya, and Volovets) located on the outer flysch areas (the Krosno Zone) and inner covers and rock zones; a foothill area (6 districts: Svaliava, Irshava, Khust, Tiachiv, Perechyn, and Velykyi Bereznyi) located on different territories and covering the Volcanic Carpathians, the Marmara massif, outer flysch areas, and partially inner covers and rock zones; and a lowland area (4 regions: Berehove, Vynohradiv, Mukachevo, and Uzhhorod) located within the Transcarpathian deflection. Tectonic and geochemical peculiarities of the studied area are considered in the study (Sukharev *et al.*, 2020).

On Figure 1, shows a fragment of tectonic map of the Ukrainian Carpathians (Transcarpathian region) with research areas.

On Figure 2, shows a fragment of a geologic map of the Ukrainian Carpathians (Transcarpathian region) (Sukharev *et al.*, 2020).

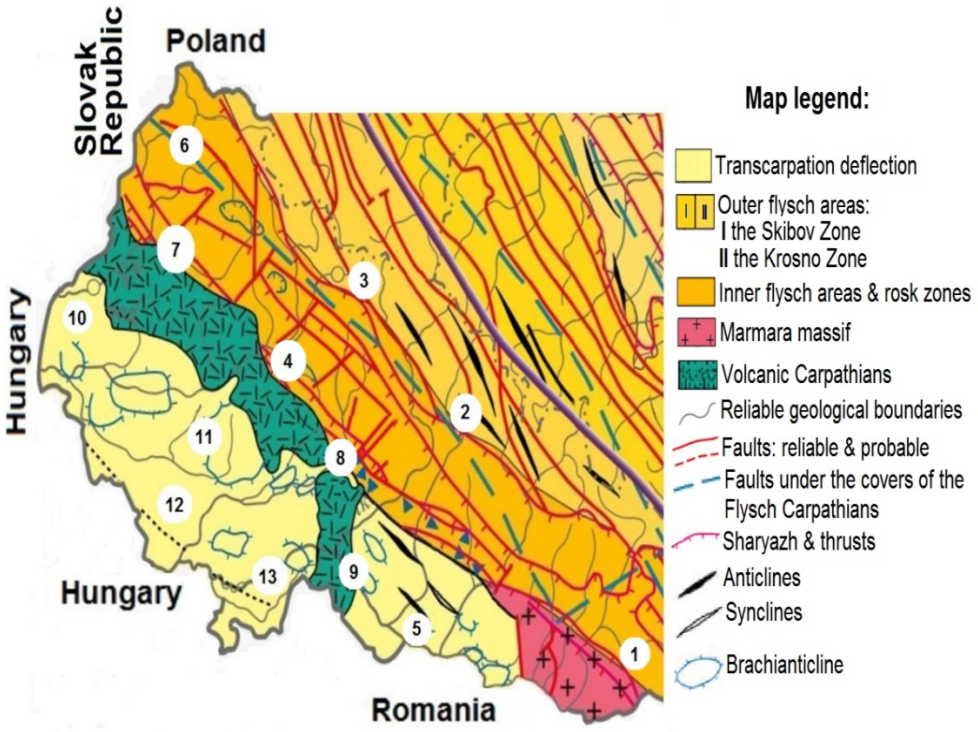

Figure 1. Fragment of tectonic map of Ukrainian Carpathians (Transcarpathian region). Research areas (district):
1 – Rakhiv; 2 – Mizhhirya; 3 – Volovets; 4 – Svaliava; 5 – Tiachiv; 6 – Velykyi Bereznyi; 7 – Perechyn; 8 – Irshava; 9 – Khust; 10 – Uzhhorod; 11 – Mukachevo; 12 – Berehove; 13 – Vynogradiv

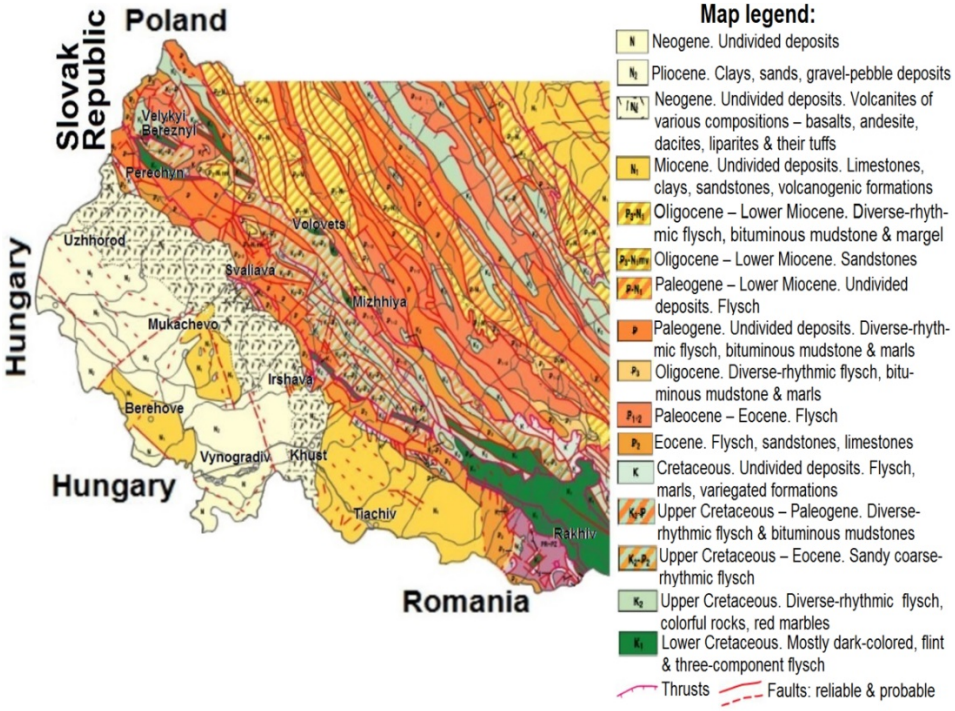

Figure 2. Fragment of geologic map of Ukrainian Carpathians (Transcarpathian region)

2.2. Sampling and sample preparation

Samples of the whole cow's milk were obtained by mixing 10 different milk samples from different households in respective districts taking into account the areas' landscape and terrain features.

Vegetable mix samples (potato : white cabbage : beet : onion = 3:1:1:0.5) were prepared for each district respectively, from different households, averaging 10 samples after grinding. Samples were homogenized. The ratio of vegetable mix components was selected based on consumer needs and considering the reference diet of Ukrainian citizens. Vegetables were grinded (homogenized) using a blender with polymer blades.

When sampling milk and vegetables, we took into account the number of villages in each study district and the ratio of the area of different landscapes in them. The samples were collected in the way that they would represent the features of the territories.

Dry ashing (determination of Fe, Cu, Zn, Mn, Mo, Co, Pb, Ca, and Mg) and wet ashing (determination of P, As, Se, I, Br, F, and Hg, for iodine with KOH) techniques were used for sample preparation. These sample preparation methods are traditional (Lars, 2000; Lars and Joakim, 2000; Nielsen, 2017; Sahrawat *et al.*, 2006). For dry ashing, a muffle furnace was used (Protech, PT-1400M, Italia). The acids (and KOH) used for wet ashing were and preparation of solutions (after dry ashing) of analytical grade.

For example, the preparation of milk samples with dry ashing was carried out as follows. Twenty-five mL of each sample was put in ceramic crucibles and dried in 450°C by heater. After that, crucibles containing the samples were put in the oven at 450°C for 4 hours until the sample turned to ash. In the next step, 5.0 mL of nitric acid (1.0 mol·L^{-1}) was added to the vessel containing the sample and heated to dissolve of ash. After cooling the solution, the volume was increased to 50 mL with nitric acid 0.1 mol·L^{-1}.

2.3. Analytical methods and instruments

All the reagents used in this research were of analytical grade. In the research study, double-distilled water was used. Standard procedures were used to determine microelements in food products (Nielsen, 2017). Standard solutions for making calibration curves (determination of As, Ca, Co, Cu, Fe, Pb, Mg, Mn, Mo, Se, Zn) were obtained by diluting commercial multi-element standard stock solution (SPEX QC-21, USA). Thus, the total content of trace elements in food products was determined.

Atomic absorption spectroscopy was used to determine Cu, Zn, Fe, Pb, Mn, Mo, Co (electrothermal technique: graphite furnace, chemical modifier $Pd(NO_3)_2$), Hg (cold vapor technique), and As (hydride generation technique). Experimental conditions (wavelength, nm): Cu – 324.8, Zn – 213.9, Fe – 248.3, Mn – 279.5, Mo – 313.3, Co – 240.7; Pb – 283.3; Hg – 253.7; As – 193.7. AAS vario® 6 (Analytik Jena AG, Germany), a hydrogen generator (Varian VGA-76, USA), and ultrapure $Pd(NO_3)_2$ (Suprapur®, Merck, Germany) were involved in the study.

Flame atomic emission spectroscopy was used to determine Ca and Mg (wavelength, nm: Ca – 434, Mg – 385; FPA-2-01, LLC LabTime Ltd., Russia). The following methods were used to determine specific elements: F^- and Br^- – potentiometry (SevenCompact S220, Mettler Toledo, USA); iodine – inverse voltammetry (Ecotest-VA-iodine, Russia); Se – spectrofluorimetry (λ_{abs} = 378 nm, λ_{em} = 520 nm, Hitachi F-7000, Hitachi Ltd., Japan); and P – spectrophotometry (Shimadzu UV-1800, Shimadzu Co., Japan).

2.4. Statistics and Mapping

Standard statistical methods were used; in particular, the Pearson and Spearman correlation coefficients were calculated using the SPSS Statistics (IBM) and OriginPro (OriginLab Corporation) programs. The ArcGIS 10.2.1 program was used to map the territory of the Transcarpathian region based on

the results of microelements' determination in the food products.

The results of trace elements' determination in milk samples are presented in Tables 1-3.

3. Results and discussions

Table 1. The results of determination of some trace elements (metals) in the milk from the Transcarpathian region (n = 6; P = 0.95)

Milk samples‡ (district)	Trace element content, mg·L^{-1}					
	Fe	**Cu**	**Zn**	**Mn**	**Mo**	**Co**
Mountain area						
Rakhiv	2.17±0.11	0.13±0.01	2.34±0.12	0.21±0.02	0.031±0.002	0.021±0.002
Mizhhirya	1.96±0.10	0.18±0.01	2.61±0.13	0.19±0.02	0.029±0.002	0.027±0.003
Volovets	2.34±0.12	0.22±0.02	2.53±0.13	0.22±0.02	0.036±0.002	0.025±0.003
Average content	*2.16±0.20*	*0.18±0.05*	*2.49±0.15*	*0.21±0.02*	*0.032±0.004*	*0.024±0.003*
Foothill area						
Svaliava	2.51±0.12	0.31±0.02	3.19±0.19	0.15±0.01	0.052±0.004	0.052±0.005
Tiachiv	2.72±0.13	0.29±0.02	3.08±0.20	0.18±0.01	0.042±0.003	0.056±0.005
Velykyi Bereznyi	2.66±0.12	0.25±0.02	2.88±0.18	0.14±0.01	0.047±0.004	0.044±0.004
Perechyn	2.63±0.13	0.27±0.02	3.27±0.21	0.17±0.01	0.044±0.003	0.048±0.005
Irshava	2.84±0.13	0.33±0.02	3.92±0.22	0.13±0.01	0.051±0.004	0.051±0.005
Khust	2.81±0.14	0.34±0.02	3.60±0.19	0.11±0.01	0.051±0.005	0.066±0.006
Average content	*2.70±0.19*	*0.30±0.05*	*3.32±0.60*	*0.15±0.04*	*0.048±0.006*	*0.053±0.013*
Lowland area						
Uzhhorod	2.89±0.14	0.31±0.02	3.79±0.20	0.09±0.01	0.061±0.005	0.073±0.007
Mukachevo	2.97±0.15	0.38±0.03	4.02±0.22	0.12±0.01	0.054±0.004	0.089±0.007
Berehove	3.09±0.15	0.40±0.03	4.29±0.23	0.10±0.01	0.055±0.004	0.091±0.007
Vynohradiv	3.02±0.14	0.36±0.02	4.11±0.19	0.08±0.01	0.058±0.005	0.087±0.007
Average content	*3.00±0.11*	*0.36±0.05*	*4.05±0.26*	*0.10±0.02*	*0.057±0.004*	*0.085±0.012*

Note: ‡ – milk density is 1.027 kg·L^{-1}; Safety indicators: Hg content from < 0.5 to 1.03 μg·L^{-1}; Pb – 5.7-13.1 μg·L^{-1}. The lowest Hg and Pb content in milk samples from mountainous areas. Permissible content of Hg and Pb in milk ≤ 10 μg·kg^{-1} and ≤ 20 μg·kg^{-1} respectively (EC, 2015; FAO/WHO, 2011).

Table 2. The results of determination of some trace elements (non-metals) in the milk from the Transcarpathian region (n = 6; P = 0.95)

Milk samples (district)	Trace element content, μg·L^{-1}				
	As	**Se**	**I**	**Br**	**F**
Mountain area					
Rakhiv	32.2±3.8	21.4±2.1	50.9±4.8	344±47	48.1±6.6
Mizhhirya	28.3±3.4	19.3±2.0	47.7±4.7	406±55	56.9±6.8
Volovets	27.1±3.3	24.8±2.9	63.0±5.5	368±46	38.5±56
Average content	*29.2±3.0*	*21.8±3.0*	*53.9±9.1*	*373±33*	*47.8±9.3*
Foothill area					
Svaliava	19.9±2.7	52.5±5.7	115±11	492±61	59.4±6.7
Tiachiv	21.3±2.9	43.9±5.1	127±11	890±89	83.9±9.6
Velykyi Bereznyi	25.8±2.9	52.1±5.7	96±9	423±52	63.1±7.7
Perechyn	22.1±2.5	49.3±5.5	112±10	549±63	74.8±8.8
Irshava	23.6±2.6	50.5±5.5	118±10	641±68	73.2±8.7

Khust	17.4±2.2	54.8±5.6	122±11	677±70	68.7±7.5
Average content	*21.7±4.3*	*50.5±6.6*	*115±19*	*612±278*	*70.5±13.3*
Lowland area					
Uzhhorod	12.8±1.7	51.4±5.7	115±10	624±67	77.5±8.8
Mukachevo	14.1±1.8	52.3±5.7	137±12	589±65	91.2±9.6
Berehove	18.7±2.0	61.7±6.6	153±12	753±78	118.7±12.9
Vynohradiv	15.2±1.9	58.8±6.5	144±12	805±82	104.3±11.1
Average content	*15.2±3.5*	*56.1±5.6*	*137±22*	*693±112*	*97.9±20.8*

Table 3. The results of macroelements determination in the milk from the Transcarpathian region (n = 6; P = 0.95)

Milk samples (district)	**Macroelement content, mg·L^{-1}**			**Ca/Mg**
	P	**Ca**	**Mg**	
Mountain area				
Rakhiv	97±5	1985±109	173±10	11.5
Mizhhirya	111±5	2077±108	189±11	11.0
Volovets	103±5	2104±110	182±11	11.6
Average content	*104±7*	*2055±70*	*181±8*	*11.4±4*
Foothill area				
Svaliava	154±7	2197±116	221±12	9.9
Tiachiv	151±7	2136±103	213±12	10.0
Velykyi Bereznyi	129±6	2098±109	218±12	9.6
Perechyn	136±7	2174±115	202±11	10.8
Irshava	163±7	2263±106	243±13	9.3
Khust	168±8	2389±107	267±14	9.0
Average content	*150±21*	*2210±179*	*227±40*	*9.8±1.0*
Lowland area				
Uzhhorod	177±8	2410±113	279±15	8.6
Mukachevo	179±8	2373±111	231±13	10.3
Berehove	163±8	2488±114	253±14	9.8
Vynohradiv	182±8	2421±116	298±16	8.1
Average content	*175±12*	*2423±65*	*265±34*	*9.2±1.1*

The results of trace elements' determination in milk samples are presented in Tables 1-3.

The studied milk samples are characterized by rich microelement composition while safety indicators (content of Hg and Pb) confirm their safety. Our data are consistent with the results of determining the content of heavy metals in cow's milk from the mountainous area of Romania (Cadar *at el.,* 2015). The level of essential trace elements in milk samples significantly varies (mg·L^{-1}): Fe: 1.96-3.09; Cu: 0.13-0.40; Zn: 2.34-4.29; Mn: 0.08-0.22; Mo: 0.029-0.061; Co: 0.021-0.091; P: 97-182; As: 0.013-0.032; Se: 0.019-0.062; I: 0.048-0.153; Br: 0.34-0.81; F: 0.039-0.119. The content of the majority of trace elements (Fe, Cu, Zn, Mo, Co, P, Se, I, Br, F) in milk samples from the mountainous area is significantly lower than in milk samples from the foothill and lowland areas. The exception is As and Mn, the content of which is higher in milk from the mountainous area than in milk from the foothill and lowland areas. For example, Figure 3 shows a map of the Transcarpathian region according to the distribution of arsenic content in milk, with the highest content being

observed in the milk sample from Rakhiv district (it has the highest average height above sea level). This is obviously associated with the relatively high As content in this region's soils as evidenced by the presence of arsenide mineral waters in the Rakhiv district.

Microelement distribution in milk samples from different landscape zones (lowland area > foothill area > mountainous area) was estimated using the Spearman coefficient: Fe: 0.91; Cu: 0.89; Mn: -0.94; Zn: 0.88; Mo: 0.89; Co: 0.94; Ca: 0.91; Mg: 0.91; P: 0.92; As: -0.94; Se: 0.82; I: 0.84; Br: 0.73; F: 0.82. Thus, consumption of milk from the lowland landscape zone of the Transcarpathian region provides a more complete supply of microelements to the human body. The authors (Falandysz *et al.*, 2008; Falandysz *et al.,* 2012) also established similar patterns of content of some trace elements in mushrooms of the mountains and lowland areas.

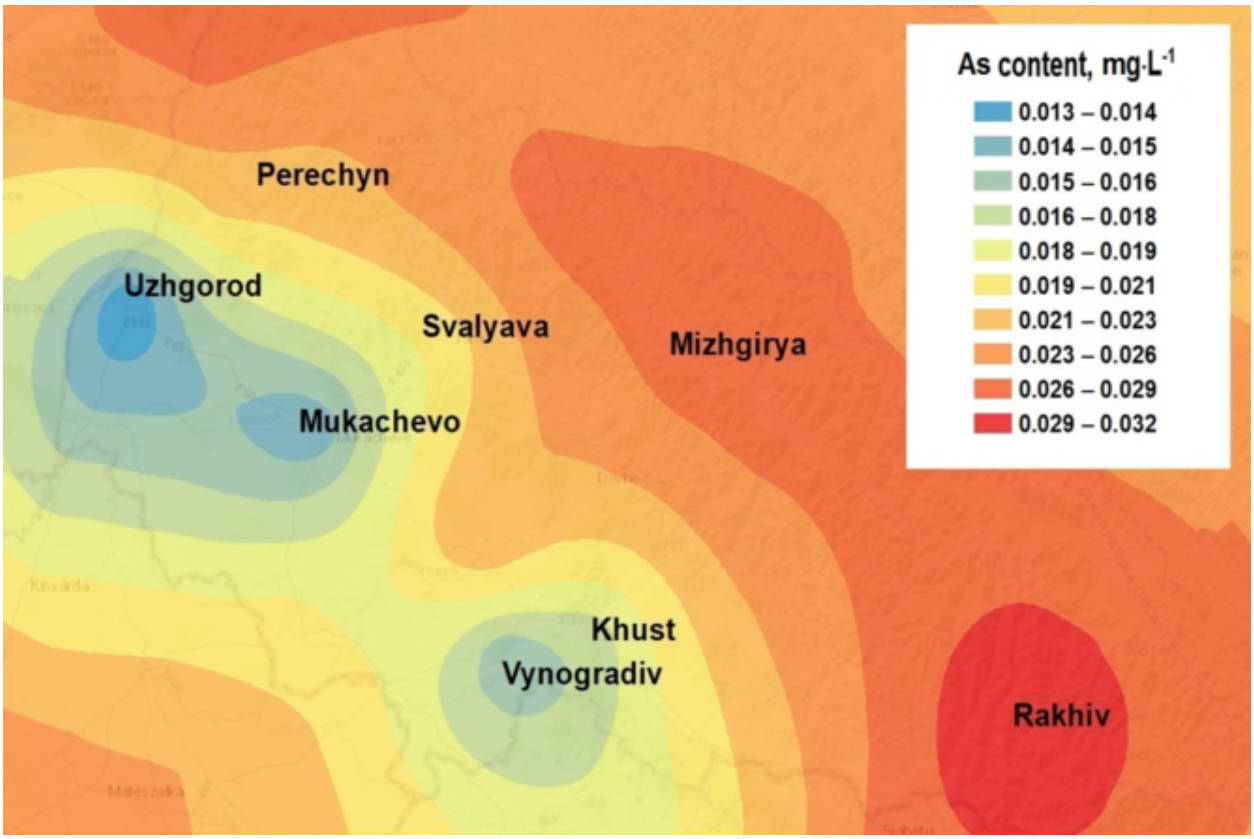

Figure 3. Map of arsenic distribution in milk samples from the Transcarpathian region

A similar consistent pattern is observed for the macroelement content in milk samples from different landscape zones. For example, Ca content in milk samples varies at the rate of 1985-2488 mg·L^{-1}, Mg – 173-298 mg·L^{-1}, and their ratio (Ca/Mg) varies between 8.1 and 11.5. Though Ca and Mg content in milk samples from the mountainous area is lower than in foothill and lowland areas, the ratio of Ca/Mg content in milk samples from the mountainous area is higher (Fig. 4).

Importantly, the content of different trace elements in milk samples correlates with each other, and the values of the Pearson coefficient of inter-element correlation are high ($r > 0.60$) as can be seen in Table. 4. A particularly pronounced correlation is observed for such pairs of microelements as (values of the Pearson coefficient): Fe:Cu – 0.92; Fe:Zn – 0.92; Fe:Mo – 0.90; Fe:Co – 0.91; Fe:Se – 0.91; Fe:I – 0.95; Cu:Zn – 0.95; Cu:Co – 0.93; Cu:P – 0.92; Cu:Se – 0.90; Cu:I – 0.95; Zn:Co – 0.94; Zn:P – 0.93; Zn:I – 0.91; Mo:P – 0.92;

Mo:Se – 0.91; Co:P – 0.92; Co:I – 0.92; Co:F – 0.90; P:I – 0.90; Se:I – 0.94. An inverse pattern regarding other trace elements is observed for Mn and As (Table 4). This may indicate a relative stability of the chemical composition of milk even from different landscape zones. In order to illustrate the revealed inter-element patterns of microelement content in milk samples, Figure 5 presents the respective correlation diagram. The authors (Pilarczyk *at el.,* 2013) also showed significant very high or high positive inter-element correlations in milk.

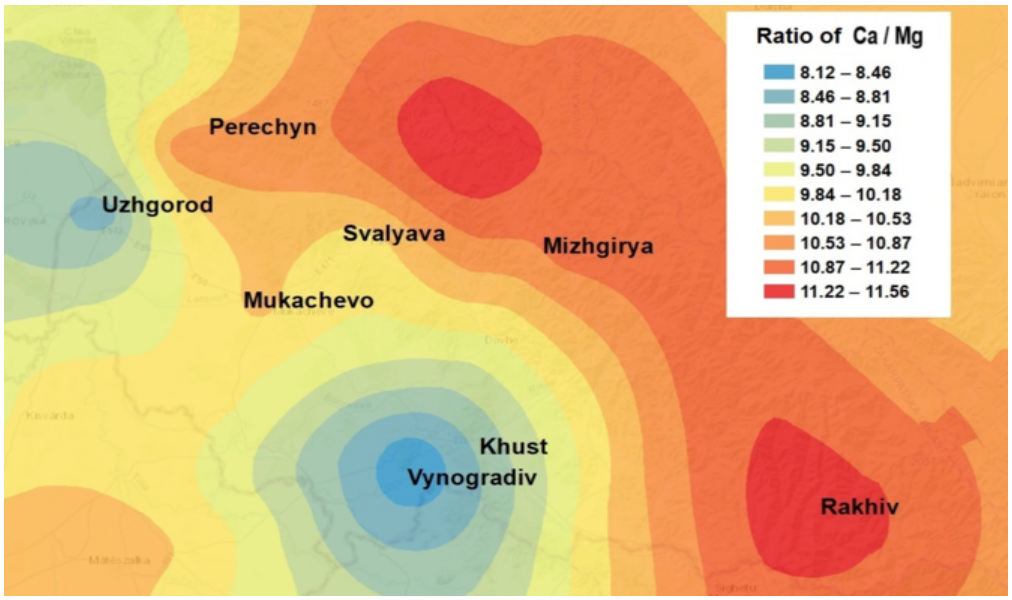

Figure 4. The map of the Transcarpathian region by the ratio of Ca/Mg in milk

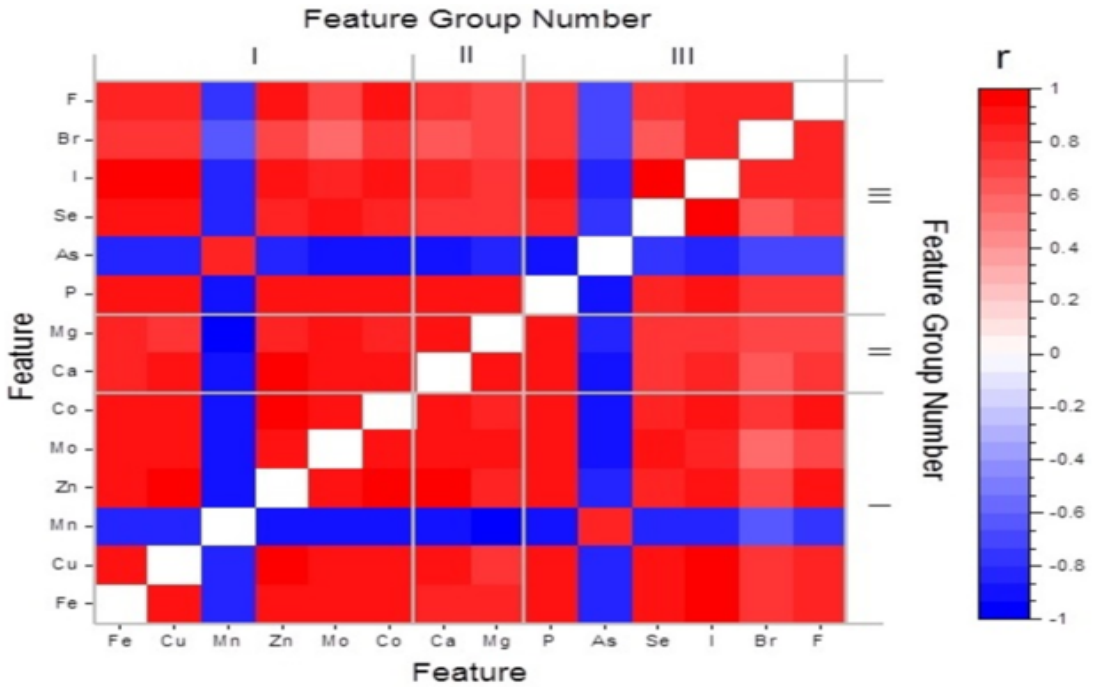

Figure 5. Correlation diagram of inter-element correlation of microelement content in milk samples (Pearson's correlation coefficient): I – trace elements metals; II – macro elements; III – trace elements non-metals

Table 4. The values of the Pearson coefficient for consistent patterns of inter-element composition of milk from the Transcarpathian region ($\sigma < 0.05$)

-	Fe	Cu	Mn	Zn	Mo	Co	Ca	Mg	P	As	Se	I	Br	F
Fe	X	0.92	0.85	0.92	0.90	0.91	0.86	0.82	0.88	-0.81	0.91	0.95	0.76	0.82
Cu	0.92	X	-0.83	0.95	0.88	0.93	0.91	0.79	0.92	-0.85	0.90	0.95	0.74	0.81
Mn	-0.85	-0.83	X	-0.90	-0.92	-0.89	-0.91	-0.96	-0.91	0.83	-0.86	-0.80	-0.61	-0.75
Zn	0.92	0.95	-0.90	X	0.89	0.94	0.94	0.85	0.93	-0.83	0.86	0.91	0.73	0.87
Mo	0.90	0.88	-0.92	0.89	X	0.87	0.87	0.89	0.92	-0.88	0.91	0.86	0.60	0.68
Co	0.91	0.93	-0.89	0.94	0.87	X	0.93	0.83	0.92	-0.90	0.84	0.92	0.74	0.90
Ca	0.86	0.91	-0.91	0.94	0.87	0.93	X	0.89	0.89	-0.88	0.79	0.83	0.67	0.79
Mg	0.82	0.79	-0.96	0.85	0.89	0.83	0.89	X	0.89	-0.83	0.79	0.77	0.69	0.69
P	0.88	0.92	-0.91	0.93	0.92	0.92	0.89	0.89	X	-0.93	0.86	0.90	0.76	0.75
As	-0.81	-0.85	0.83	-0.83	-0.88	-0.90	-0.88	-0.83	-0.93	X	-0.77	-0.82	-0.67	-0.68
Se	0.91	0.90	-0.86	0.86	0.91	0.84	0.79	0.79	0.86	-0.77	X	0.94	0.67	0.74
I	0.95	0.95	-0.80	0.91	0.86	0.92	0.83	0.77	0.90	-0.82	0.94	X	0.83	0.87
Br	0.76	0.74	-0.61	0.73	0.60	0.74	0.67	0.69	0.76	-0.67	0.67	0.83	X	0.80
F	0.82	0.81	-0.75	0.87	0.68	0.90	0.79	0.69	0.75	-0.68	0.74	0.87	0.80	X

Table 5. The results of determination of trace elements (metals) in vegetable mix from the Transcarpathian region ($n = 6$; $P = 0.95$)

Samples of vegetable mix (district)	**Trace element content, mg·kg^{-1}**					
	Fe	**Cu**	**Zn**	**Mn**	**Mo**	**Co**
Mountain area						
Rakhiv	3.92±0.29	0.62±0.05	2.55±0.17	3.18±0.29	0.053±0.004	0.018±0.002
Mizhhirya	4.14±0.28	0.73±0.06	2.74±0.18	2.93±0.27	0.062±0.005	0.025±0.002
Volovets	4.39±0.31	0.71±0.06	2.61±0.17	3.32±0.29	0.069±0.005	0.021±0.002
Average content	*4.15±0.24*	*0.69±0.07*	*2.63±0.11*	*3.14±0.21*	*0.061±0.008*	*0.021±0.004*
Foothill area						
Svaliava	4.77±0.33	0.86±0.07	3.66±0.23	2.18±0.21	0.082±0.006	0.032±0.003
Tiachiv	5.45±0.35	0.71±0.06	3.18±0.20	2.74±0.25	0.069±0.005	0.035±0.003
Velykyi Bereznyi	5.22±0.34	0.77±0.06	2.93±0.18	2.07±0.20	0.066±0.005	0.028±0.003
Perechyn	5.09±0.34	0.81±0.07	3.47±0.22	2.54±0.24	0.071±0.006	0.029±0.003
Irshava	6.61±0.42	0.92±0.07	3.69±0.22	1.91±0.19	0.077±0.006	0.037±0.004
Khust	5.88±0.36	0.94±0.07	3.28±0.21	1.52±0.15	0.087±0.006	0.042±0.004
Average content	*5.50±1.11*	*0.84±0.13*	*3.37±0.44*	*2.16±0.64*	*0.075±0.012*	*0.034±0.008*
Lowland area						
Uzhhorod	6.92±0.41	0.83±0.07	3.57±0.22	1.28±0.13	0.117±0.008	0.037±0.003
Mukachevo	6.69±0.39	1.07±0.08	3.96±0.23	1.67±0.17	0.084±0.006	0.051±0.005
Berehove	7.81±0.44	1.21±0.09	4.69±0.27	1.19±0.13	0.093±0.007	0.053±0.005
Vynohradiv	7.63±0.43	1.32±0.10	4.51±0.26	1.34±0.14	0.108±0.007	0.048±0.005
Average content	*7.26±0.57*	*1.11±0.28*	*4.18±0.61*	*1.37±0.30*	*0.101±0.017*	*0.047±0.010*

Note: Safety indicators: Hg content from < 0.5 to 3.1 μg·kg^{-1}; Pb – 3.4-21.4 μg·kg^{-1}. The lowest Hg and Pb content in vegetable mix samples from mountainous areas. Permissible content of Hg and Pb in vegetable ≤ 10 μg·kg^{-1} and ≤ 100 μg·kg^{-1} respectively (EC, 2015; FAO/WHO, 2011).

Table 6. The results of determination of some trace elements (non-metals) in vegetable mix from the Transcarpathian region (n = 6; P = 0.95)

Samples of vegetable mix (district)	Trace element content, μg·kg^{-1}				
	As	Se	I	Br	F
Mountain area					
Rakhiv	92.9±9.9	3.13±0.47	7.07±0.81	102±16	101±19
Mizhhirya	88.1±9.5	4.19±0.66	6.24±0.70	125±19	121±21
Volovets	78.7±8.7	5.38±0.85	7.85±0.83	113±17	92±18
Average content	*86.6±7.9*	*4.23±1.15*	*7.05±0.81*	*113±12*	*105±16*
Foothill area					
Svaliava	59.3±7.5	7.04±0.95	23.7±2.1	153±26	132±24
Tiachiv	61.7±7.7	7.98±0.99	33.0±2.8	258±36	169±25
Velykyi Bereznyi	77.0±8.6	5.07±0.75	14.4±1.4	140±25	137±24
Perechyn	66.2±8.1	6.32±0.86	18.5±1.8	161±26	153±25
Irshava	70.5±8.5	8.05±0.98	26.9±2.5	192±29	144±24
Khust	51.4±7.5	7.11±0.87	38.3±3.6	204±32	148±24
Average content	*64.4±13.0*	*6.93±1.86*	*25.8±12.5*	*185±73*	*147±22*
Lowland area					
Uzhhorod	33.4±4.6	8.86±0.97	43.1±3.9	181±28	172±26
Mukachevo	60.9±7.6	8.90±1.01	30.9±2.8	165±27	181±27
Berehove	54.2±7.5	10.4±1.03	53.8±4.7	231±33	228±29
Vynohradiv	47.1±6.6	11.1±1.04	51.0±4.5	246±35	217±29
Average content	*48.9±15.5*	*9.82±1.28*	*44.7±13.8*	*206±41*	*200±28*

Table 7. The results of macroelements determination in vegetable mix from the Transcarpathian region (n = 6; P = 0.95)

Samples of vegetable mix (district)	Macroelement content, mg·kg^{-1}			Ca/Mg
	P	Ca	Mg	
Mountain area				
Rakhiv	334±17	118±6	151±8	0.78
Mizhhirya	359±18	144±8	142±8	1.01
Volovets	349±17	157±8	179±9	0.88
Average content	*347±13*	*140±22*	*157±22*	*0.89±0.12*
Foothill area				
Svaliava	401±19	192±10	218±11	0.88
Tiachiv	398±19	176±9	213±11	0.83
Velykyi Bereznyi	355±18	168±9	204±11	0.82
Perechyn	380±19	184±9	185±9	0.99
Irshava	413±19	201±10	231±11	0.87
Khust	393±19	219±11	255±12	0.86
Average content	*390±35*	*190±29*	*218±37*	*0.88±0.11*
Lowland area				
Uzhhorod	409±19	241±12	273±13	0.88
Mukachevo	422±20	203±11	266±13	0.76
Berehove	431±21	253±12	311±14	0.81
Vynohradiv	438±20	232±11	307±14	0.76
Average content	*425±16*	*232±29*	*289±23*	*0.80±0.08*

In general, milk can be considered a microelement-rich food product. Based on the daily microelement intake, it is possible to calculate to what extent consumption of traditional food products satisfies human need for micronutrients (Goldhaber, 2003; Prashanth *et al.*, 2015; Santos *et al.*, 2004). A person's need for essential trace elements varies between 18-50 mg per day (Mertz, 1981). The degree of digestibility of microelements from food should also be considered. For example, when consuming milk in generally accepted amounts, a resident of the Transcarpathian region satisfies the need for As: in mountainous areas – by 116% (calculation according to the maximum recommended amount), in foothill areas – by 100%, in lowland areas – by 100% (calculation according to the minimum recommended amount). The recommended amount for arsenic is 15-25 mg per day (Uthus and Seaborn, 1996). For iodine (the recommended amount is 150 mg per day), a different situation is observed. For instance, a resident of the mountainous area of the Transcarpathian region consuming milk in generally accepted quantities satisfies the need for iodine by only 36%, while a resident of lowlands – by 91.5%. This should be considered when developing diets.

The results of microelements' determination in vegetable mix samples are presented in Tables 5-7. Chemical composition of the vegetable mix (Tables 5-7) significantly differs from the microelement composition of milk (Tables 1-3).

Vegetable mix is richer in Fe, Cu, Mn, Mo, P, As, and F than milk, but milk contains larger amounts of Co, Se, I, Br, and Ca. In particular, the mean contents of iron in vegetable mix is 5.64 $mg \cdot kg^{-1}$ versus 2.62 $mg \cdot L^{-1}$ in milk; copper – 0.88 $mg \cdot kg^{-1}$ versus 0.28 $mg \cdot L^{-1}$ in milk; manganese – 2.22 $mg \cdot kg^{-1}$ versus 0.15 $mg \cdot L^{-1}$ in milk; molybdenum – 0.079 $mg \cdot kg^{-1}$ versus 0.046 $mg \cdot L^{-1}$ in milk; phosphorus – 387 $mg \cdot kg^{-1}$ versus 143 $mg \cdot L^{-1}$ in milk, and fluorine – 151 $\mu g \cdot kg^{-1}$ versus 72.1 $\mu g \cdot L^{-1}$ in milk. Herewith, milk contains significantly larger amounts of cobalt (0.054 $mg \cdot L^{-1}$ versus 0.034 $mg \cdot kg^{-1}$ in vegetable mix); selenium (42.8 $\mu g \cdot L^{-1}$ versus 6.99 $\mu g \cdot kg^{-1}$ in vegetable mix); iodine (102 $\mu g \cdot L^{-1}$ versus 25.9 $\mu g \cdot kg^{-1}$ in vegetable mix); bromine (559 $\mu g \cdot L^{-1}$ versus 168 $\mu g \cdot kg^{-1}$ in vegetable mix), and calcium (2229 $mg \cdot L^{-1}$ versus 187 $mg \cdot kg^{-1}$ in vegetable mix). Given that milk and vegetables constitute the basis of the human diet (about 68%), milk can be considered the main source of Ca, I, Se, and Co while vegetables are the main source of Fe, Cu, P, Mn, Mo, As, and F.

The chemical composition of vegetable mix also demonstrates relative stability. Like in milk, microelement content of vegetable mix samples from different landscape zones is significantly different. A vegetable mix from lowlands tends to be richer in trace elements (except for As and Mn) than a vegetable mix from foothills and mountains, which was estimated using the Spearman coefficient with the following values (lowland area > foothill area > mountainous area): Fe: 0.91; Cu: 0.85; Mn: -0.94; Zn: 0.79; Mo: 0.90; Co: 0.92; Ca: 0.94; Mg: 0.94; P: 0.80; As: -0.91; Se: 0.84; I: 0.91; Br: 0.73; F: 0.88. Thus, vegetables from the lowlands of the Transcarpathian region are a more complete source of trace elements for people.

Unlike milk, for which Ca/Mg ratio is 8.1–11.5, for vegetable mix Ca and Mg content is comparable and Ca/Mg ratio varies from 0.76 to 1.01 (Figure 6). For vegetable mix, as well as for milk samples, pronounced correlations of inter-element composition are also observed, which was estimated using the Pearson coefficient (Table 8). The most pronounced are the correlations for such pairs of chemical elements (values of the Pearson coefficient): Fe:Zn – 0.90; Fe:Co – 0.91; Fe:Ca – 0.92; Fe:Mg – 0.96; Fe:P – 0.90; Fe:Se – 0.93; Fe:I – 0.92; Fe:F – 0.91; Cu:Zn – 0.93; Zn:Co – 0.90; Zn:P – 0.94; Zn:Se – 0.91; Zn:F – 0.91; Mo:Ca – 0.90; Co:Mg – 0.92; Co:P – 0.92; Co:Se – 0.90; Co:F – 0.90; Ca:Mg – 0.94; Ca:Se – 0.90; Ca:I – 0.93; Mg:P – 0.90; Mg:Se – 0.94; Mg:I – 0.96; P:Se – 0.97; P:I – 0.90; Se:I – 0.93; Se:F

– 0.90; I:F – 0.91. For clarity of the revealed correlations of inter-element composition of vegetable mix, Figure 7 presents the respective correlation diagram.

Table 8. The values of Pearson coefficient for consistent patterns of inter-element composition of vegetable mix from the Transcarpathian region ($\sigma < 0.05$)

-	Fe	Cu	Mn	Zn	Mo	Co	Ca	Mg	P	As	Se	I	Br	F
Fe	X	0.87	-0.90	0.90	0.82	0.91	0.92	0.96	0.90	-0.77	0.93	0.92	0.75	0.91
Cu	0.87	X	-0.80	0.93	0.70	0.89	0.80	0.88	0.85	-0.60	0.85	0.81	0.63	0.85
Mn	-0.90	-0.80	X	-0.82	-0.85	-0.87	-0.93	-0.92	-0.83	0.82	-0.81	-0.87	-0.62	-0.81
Zn	0.90	0.93	-0.82	X	0.73	0.90	0.86	0.89	0.94	-0.70	0.91	0.87	0.70	0.91
Mo	0.82	0.70	-0.85	0.73	X	0.71	0.90	0.87	0.79	-0.94	0.84	0.85	0.57	0.71
Co	0.91	0.89	-0.87	0.90	0.71	X	0.87	0.92	0.92	-0.72	0.90	0.89	0.75	0.90
Ca	0.92	0.80	-0.93	0.86	0.90	0.87	X	0.94	0.89	-0.91	0.90	0.93	0.72	0.83
Mg	0.96	0.88	-0.92	0.89	0.87	0.92	0.94	X	0.90	-0.85	0.94	0.96	0.74	0.88
P	0.90	0.85	-0.83	0.94	0.79	0.92	0.89	0.90	X	-0.79	0.97	0.90	0.80	0.88
As	-0.77	-0.60	0.82	-0.70	-0.94	-0.72	-0.91	-0.85	-0.79	X	-0.84	-0.88	-0.69	-0.72
Se	0.93	0.85	-0.81	0.91	0.84	0.90	0.90	0.94	0.97	-0.84	X	0.93	0.83	0.90
I	0.92	0.81	-0.87	0.87	0.85	0.89	0.93	0.96	0.90	-0.88	0.93	X	0.86	0.91
Br	0.75	0.63	-0.62	0.70	0.57	0.75	0.72	0.74	0.80	-0.69	0.83	0.86	X	0.82
F	0.91	0.85	-0.81	0.91	0.71	0.90	0.83	0.88	0.88	-0.72	0.90	0.91	0.82	X

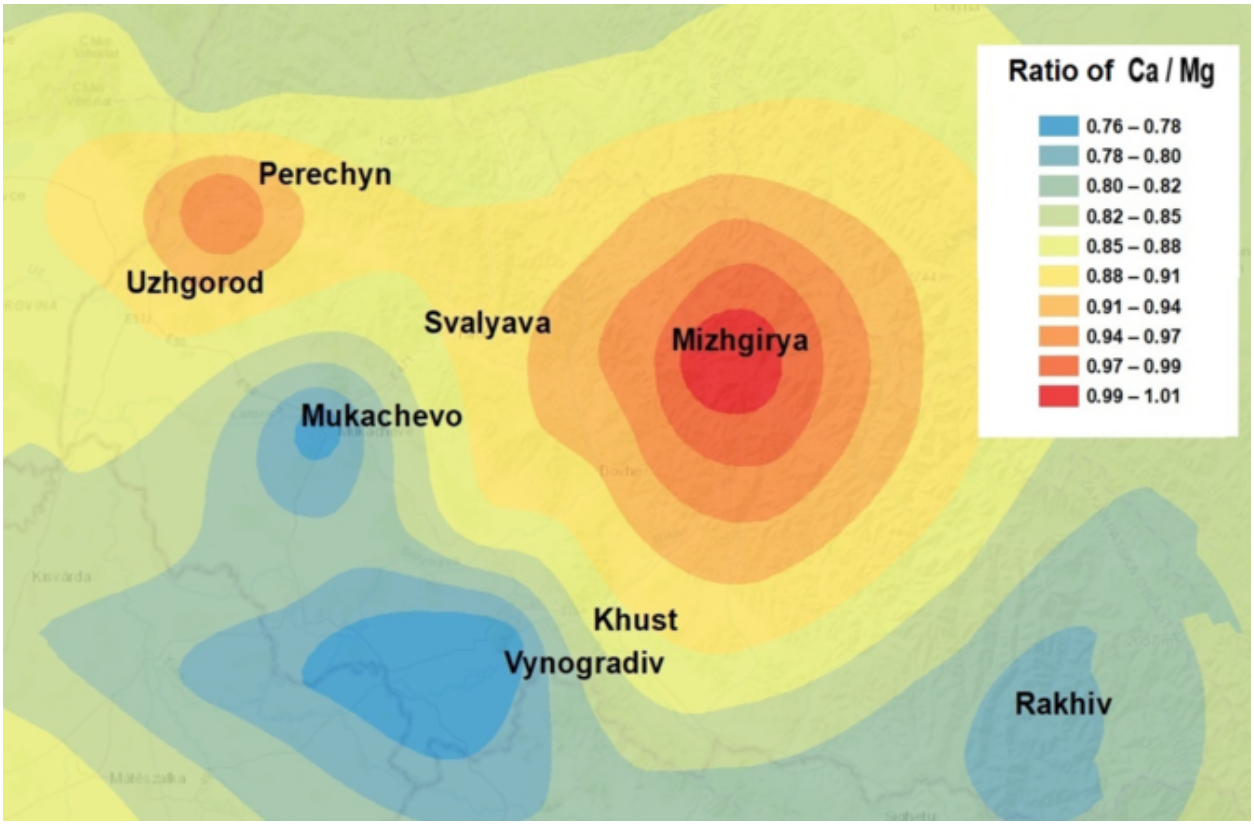

Figure 6. The map of the Transcarpathian region by the ratio of Ca/Mg in vegetable mix

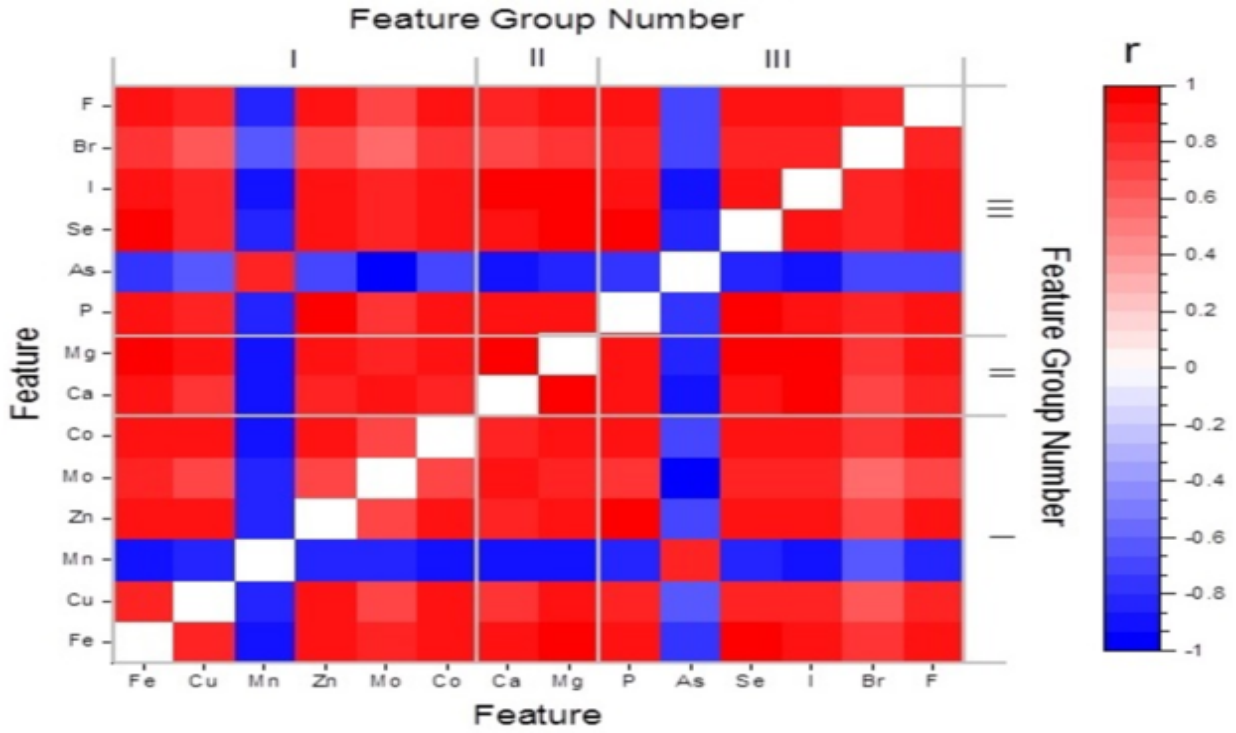

Figure 7. Correlation diagram of inter-element correlation of microelement content in vegetable mix samples (Pearson's correlation coefficient): I – trace elements metals; II – macro elements; III – trace elements non-metals

The content of toxic elements in vegetable mix is low (Hg to 0.0031 mg·kg^{-1}; Pb to 0.0214 mg·kg^{-1}) but is higher than in milk samples.

In general, milk and vegetables from the Transcarpathian region are rich in microelements (with the exception of I, F, and Se) and some macroelements (Ca and Mg), and they contain a low amount of toxic components (Hg and Pb). Therefore, traditional food products play an important role in providing the human body with trace elements and maintaining population health.

4. Conclusions

The microelement composition (based on 16 chemical elements) of traditional food products (milk and vegetable mix) from the Transcarpathian region was compared and it was proven that milk can be considered the main source of Ca, I, Se, and Co for human body while vegetables are the main source of Fe, Cu, P, Mn, Mo, As, and F. A significant difference was found in the microelement composition of milk and vegetables from different landscape zones; food products from the lowland area are richer in Fe, Cu, Zn, Mo, Co, P, Se, I, Br, F, Ca, and Mg while food products from the mountainous area have large amounts of As and Mn. This is obviously connected with the geochemical features of these areas. Consistent patterns of trace element distribution in food products from different landscape zones: lowland area > foothill area > mountainous area. It was found that the food products from the Transcarpathian region demonstrate relative stability of chemical composition. There is pronounced inter-element correlation of trace element content in food products, the value of Pearson coefficient for all pairs of chemical elements is $r > 0.60$. Results generalization suggests that traditional food products from the Transcarpathian region, taking into account their share in the human diet, almost completely satisfy daily human need for essential microelements (except for I, Se, and F).

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PRODUCTION OF CHOCOLATE PROBIOTIC DESSERT BASED ON CAMEL MILK USING *LACTOCASEIBACILLUS CASEI*

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ABSTRACT

A functional food is a food that contains nutrients, which has a positive effect on one or more functions in the human body. Probiotic foods are also a functional food that by consuming them can be benefited the nutritional properties of probiotic bacteria. Inoculation of lactic acid Proteolytic bacteria to milk leads to production of fermented dairy products rich in bioactive peptides. This research was conducted in two stages. At first, *Lactocaseibacillus* (*L.*) *casei* was inoculated into milk (cow and camel) and incubated for 12 h. The incubation time had a significant effect on the growth of *L. casei* ($p \leq 0.05$). The growth of *L. casei* in camel milk was significantly different from cow milk. ($p \leq 0.05$). Changes in pH and acidity during incubation were significant ($p \leq 0.05$) and there was a significant difference between cow and camel samples. During incubation of both milk increased proteolytic activity, antioxidant activity was observed while antioxidant activity in camel milk was more than cow milk. In sensory evaluation, no significant difference was observed between the two types of milk ($p > 0.05$). Four samples of chocolate dessert were prepared by inoculation of *L. casei* to cow and camel milk containing two types of sweeteners of sucrose and sucralose. During 28 days of storage, the survival of *L. casei* in all samples was more than 8 Log cfu/g. Two sweeteners and milk type showed significant impact on the survival of *L. casei* ($p \leq 0.05$). Survival of *L. casei* in desserts prepared with camel milk and sucrose sweetener was higher. Changes in acidity and pH in the samples were significant during 28 days of storage ($p \leq 0.05$) Desserts prepared with camel milk and sucrose sweetener had higher acidity and lower pH. The desserts prepared with cow's milk and sucrose sweetener have higher elasticity. The sensory evaluation test does not show any significant difference in odor, taste and overall acceptability with blank ($p > 0.05$).

1.Introduction

Milk and dairy products are an important part of human diet due to their nutritional and biological value. A group of dairy products that are world famous are dairy desserts (cream, puddings, cocktails, whipped cream) and the most important factor for this group of products is their rheological properties (viscosity and jelly). (Peter and Glyn, 2014). Creamy milk chocolate dessert as high accepted dairy products, could be alternative for incorporation by probiotics and prebiotics (Amna et al., 2015; Valencia et al., 2016)

Dairy dessert contains at least 50% of fresh milk or reconstituted milk and food additives (e.g. flavorings, sweeteners, thickeners and stabilizers), after passing thermal processes such as pasteurization, pasteurization with extended

shelf life, sterilization (Ibrahim et al., 2015; Kanmani et al., 2013; Kaur et al., 2015; Khaskheli et al., 2005; Konuspayeva et al., 2009; Kumar et al., 2016). Types of desserts including pudding, custard, mousse, flan, porridge and rice milk, and milk desserts are drinks. There are two common camel species, the Arabian dromedary (*Camelus drumderius*) and the Bactrian camel (*Camelus bactrianus*), the camel found in the mountains ((Bayarri et al., 2010; Beresford et al., 2001; Cardarelli et al., 2008; Ibrahem et al., 2016)). Food and Agriculture Organization (FAO) approximately estimates that more than 5.3 million tons of camel milk is produced worldwide (Ayyash et al., 2018). The dromedary camel is known for producing camel milk as a nutritious source in raw and fermented form. More than 60% of the dromedary population (totaling 23 million worldwide) is found in the arid and desert regions of Northeast Africa (Jilo, 2016). Camel milk which has been considered as an important ingredient in the diet in different continents, can been produced up to 3500 L for 18 months lactation of camel (Elagamy, 2000).

In the development of new probiotic products, the main goal is bacterial survival during the production and storage. A wide spectrum of variables has been reported as influencing factors on microbial survival including temperature, pH, acidity, the presence of other microorganisms, and probiotic strain (Valencia et al., 2016).

Chocolate milk desserts are one of suitable carriers for probiotic pH > 6 and humidity above 70% and there are no competing microorganisms (Valencia et al., 2016). Due to the lack production of chocolate dairy desserts, and according to the mentioned benefits for camel milk and probiotic bacteria in this study, using the probiotic bacterium *Lactocaseibacillus* (*L.*) *casei*, the possibility of producing chocolate dessert based on camel milk will be investigated.

The main purpose of this study is the simultaneous use of the properties of probiotic bacteria and camel milk for production of a functional food. So, by applying camel milk as carrier for probiotic *L. casei*, survival of microorganism during shelf life was investigated. The antioxidant, nutritional, rheological and sensory properties of produced dairy dessert was also characterized.

2. Material and methods

2.1. Production of probiotic chocolate dessert

Milk (camel and cow) was pasteurized at 80°C for 20 minutes in a water bath and then cooled to 43°C. Under the laminar hood, *L. casei* was weighed and 0.01% was added to the cooled pasteurized milk (camel and cow) mixed well and incubated at 37°C for 2 h. Changes in pH, acidity, bacterial growth, and antioxidant activity were examined during the 12 h incubation time. The final product was evaluated in terms of sensory properties. In order to produce chocolate dessert, milk (camels and cows) was first pasteurized and then gelatin was added to one third of this milk and placed in a water bath until complete solvation of gelatin in the milk.

Cocoa powder, sugar or sucralose and carrageenan were added to the rest of the formulation milk at 45°C and mix the ingredients thoroughly in the milk. Milk and gelatin were added to the rest of the ingredients and pasteurized in a water bath at a temperature of 80 to 85°C for 20 min. The ingredients were cooled in a cold-water bath to 43°C under sterile conditions. Then 0.05% of *L. casei* was added to samples. The chocolate dessert was produced with 4 different formulations according to Table 1.

Two different sweeteners, sucrose and sucralose, were used to supply equal sweetness of the product. Then, 40 g in each of the glass containers were packaged, cooled, and stored at 4°C for 28 days. (Argon Allegro, 2007). All 4 dessert samples were examined on the 1st,7th, 14th, 21st and 28th days for microbial count, pH, and acidity. Rheology and sensory evaluation were carried out on the first day of production.

Table 1. The amount of used raw ingredients to produce chocolate dessert in terms of weight percentage (per 100 grams of dessert).

Treatments	Camel milk	Cow milk	Water	Cocoa powder	Gelatin	Carrageenan	Sucrose	Sucralose+ Maltodextrin	*L. casei*
Dessert 1	80.45	-	-	5	1.3	0.2	13	-	0.05
Dessert 2	80.45	-	3	5	1.3	0.2	-	10	0.05
Dessert 3	-	80.45	-	5	1.3	0.2	13	-	0.05
Dessert 4	-	80.45	3	5	1.3	0.2	-	10	0.05

2.2. Microbial test

A serial dilution was prepared for microbial count, 10^{-5}, 10^{-6} and 10^{-7} dilutions. Triplicated culture of probiotic in MRS agar medium was cultured as a pour plate incubated for 72 h at 37°C and number of colonies per gram was reported (Argon Allegro 2007).

2.3. Physicochemical Analysis

The pH, and acidity of milk samples were determined according to AOAC methods (AOAC, 2005). The pH value of milk samples was evaluated with a pH meter (Metrom, Switzerland) at room temperature. The titratable acidity was determined in milk by titration method and in dessert samples by potentiometric method.

2.4. Antioxidant activity using the 1, 2-diphenyl 1-picrylhydrazyl radical scavenging method (DPPH)

To measure the antioxidant activity of probiotic milk (camel and cow), first aqueous extract solution was prepared, for this purpose, using hydrochloric acid or 1M sodium hydroxide, the pH of milk samples was increased to 4.6 and it was centrifuged at 4 ° C for 15 minutes at 9000 g. The luminaire was smoothed using Whatman paper with a pore size of 0.45 µm; then 3.8 ml of methanol solution containing 0.1 mmol of DPPH radical was added to 0.2 ml of the prepared extract. The mixture was shaken evenly for one minute and placed in a dark place at room temperature for 30 minutes. Then the absorbance of the tested samples was measured by using a spectrophotometer at 517 nm against the control sample. The percentage of free radical scavenging effect was obtained by Eq. 1 (Ayash et al., 2018).

DPPH percentage of inhibition effect (%)

$$= (Ac\text{-}As)/Ac \times 100 \quad (1)$$

where, A_S and A_c are the sample and control absorption.

In order to investigate the rheological behavior of dessert samples, a Physica MRC 301 Rheometer (Anton-par Company of Austria) was used. The oscillation test was performed on the first day. The device was equipped with a water circulator to control the temperature and all tests were performed at a temperature of 10±1 °C. The probe of the device was in the form of a plate and the distance between the plates was 1 mm. Strain oscillation test was performed in the strain range of 0.01-100 and a constant frequency of 1 Hz. Frequency scan oscillation test was performed on a fixed strain of 0.5 in the frequency range of 0.01-100 Hz and the storage modulus (G'), dissipation modulus (G"), drop tangent or tan δ, complex modulus and complex viscosity were measured by Eqs 2 and 3 (Bayarri et al., 2010).

$$\tan \delta = G''/G' \quad (2)$$

$$G^* = \sqrt{(G')^2 + (G'')^2} \quad (3)$$

2.5. Sensory evaluation

Two samples of milk (camel and cow) were coded with random three-digit codes and according to the 8-point hedonic method by 20 male and female evaluators, different properties of the sample including taste, aroma and general

acceptance were scored (A larger number indicated greater utility). This test was performed at the sixth hour of incubation (Abolfazli et al., 2014). Sensory evaluation of dessert samples was performed on the second day. All four dessert samples were coded with random three-digit codes and according to the 8-point hedonic method by 28 male and female evaluators, different properties of the sample such as taste, aroma, texture and general acceptance were scored.

2.6. Statistical analysis

To investigate the effect of two samples of milk (camel and cow) on changes in *L. casei* growth, acidity and pH, antioxidant changes and sensory evaluation, a completely randomized design in the form of factorial test. In order to investigate the effect of two variables of dessert base type (cow milk and camel milk) and two dessert formulations with sugar and sucralose on a total of four samples, a completely randomized plan was used as a factorial test. The experiments were performed in three replications. Analysis of variance and mean comparison were performed using LSD test at 95 percent confidence level. In all stages, statistical analysis of data was performed using SPSS software. Excel software was used to draw the graphs.

3. Results

3.1 Acidity and pH

The initial pH of camel milk before starter inoculation was 6.4 and that of cow milk was 6.5; during 12 h of incubation in both types of probiotic milk (camel and cow), the pH decreased to 3.5 in camel milk and 5.4 in cow milk, respectively. The results showed that camel milk had lower pH and higher acidity than cow milk during incubation. Comparison of the mean of the main effects showed that the type of milk (camel and cow) and incubation time had a significant effect on pH changes ($p \leq 0.05$) Interactions of milk type (camel and cow) and incubation time had no significant impact on pH changes (Figure 1) ($p > 0.05$).

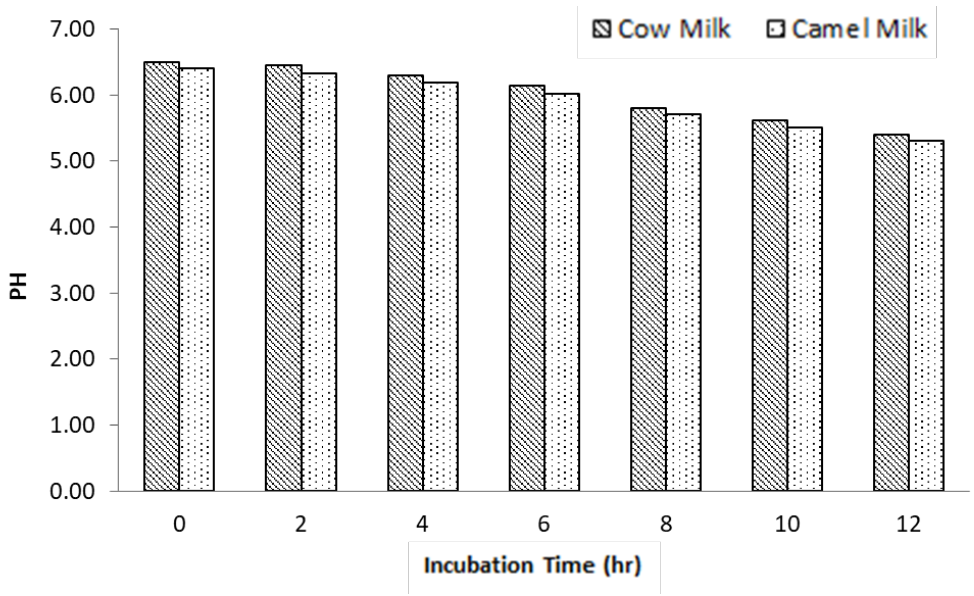

Figure 1. Comparison of the interaction effects of milk factors and incubation time on the pH response variable.

Camel milk acidity before starter inoculation was 0.22% lactic acid and the acidity of cow milk was 0.17 percent lactic acid and the difference between them was significant ($p \leq 0.05$). As the incubation time increased, the acidity of both types of probiotic milk (camel

and cow) increased significantly. After 12 hours of incubation, the acidity of camel milk reached 0.46 % lactic acid and in cow milk it reached 0.43 % lactic acid. Comparison of the mean of main and interaction effects of milk type and incubation time showed that these two levels of camel milk and cow milk and incubation time have a significant effect on acidity changes ($p \leq 0.05$) (Figure 2).

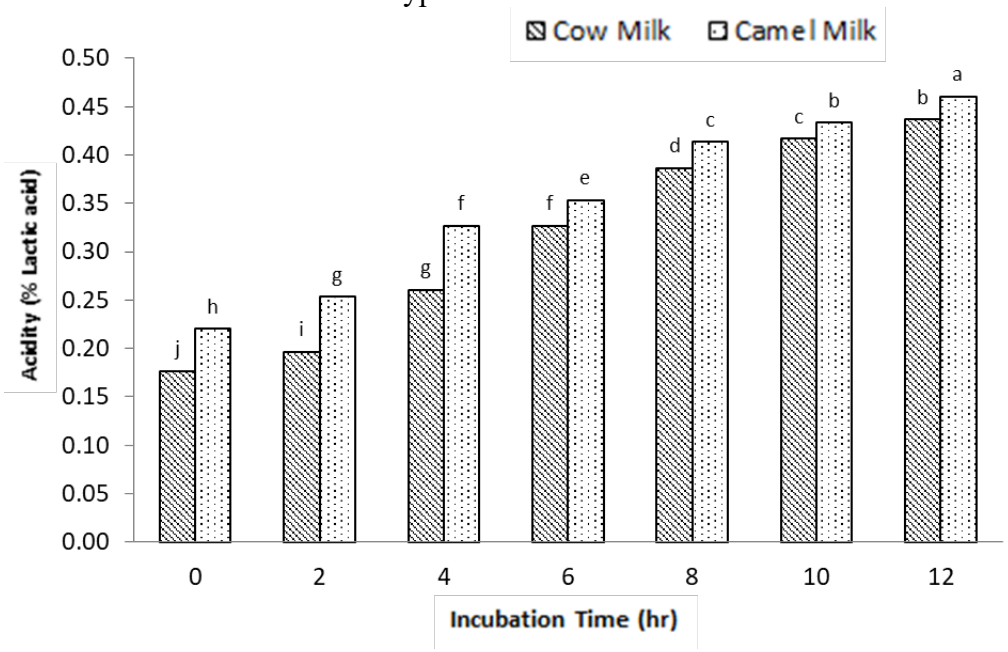

Figure 2. Comparison of the interaction effects of milk factors and incubation time on the acidity response variable.

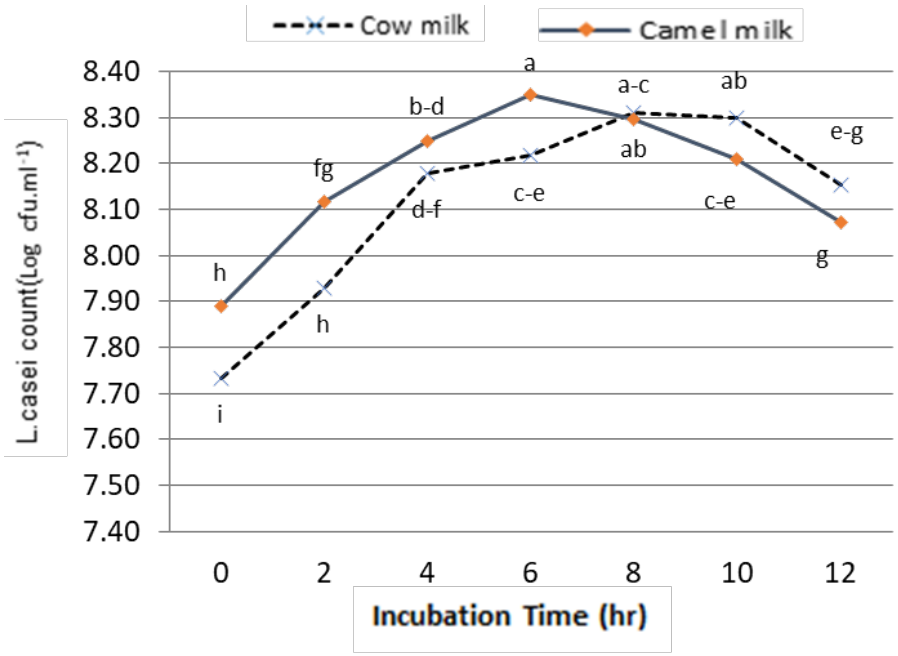

Figure 3. *L. casei* growth curve during incubation time.

3.2. Growth of *L. casei*

Bacterial growth changes of *L. casei* were examined during 12 hours of incubation. During 12 hours of incubation, the growth of *L. casei* in both types of milk (camel and cow) was higher than 7 Log cfu/ml. Microbial growth and count in camel milk was higher than cow milk and the difference between them was significant ($p \leq 0.05$). Microbial count in camel milk at zero moment 7.89 Log cfu/ml after 6 hours of incubation reached its maximum level (8.31 Log cfu/ml) and in cow milk at zero moment 7.73 Log cfu/ml and the maximum microbial count (8.31 Log cfu/ml) was observed after 8 hours of incubation, after which the growth of *L. casei* in both types of milk decreased with a slight slope. Comparison of the mean of the main effects of milk type (camel and cow) and incubation time showed that these two factors have a significant effect on microbial growth and count ($p \leq 0.05$). Comparison of the average interaction effects of milk (camel and cow) and incubation time on the growth variable of *L. case*i showed that their effect is significant ($p < 0.05$) Figure 3.

3.3. Antioxidant activity

During 12 hours of incubation, the antioxidant activity of both types of milk (camel and cow) was examined. At first hour after inoculation of *L. casei*, the level of antioxidant activity of camel milk and cow milk was not different ($p > 0.05$). During incubation, the antioxidant activity of both types of milk (camel and cow) increased with a higher percentage of antioxidant activity in camel milk than cow milk. Before incubation, the percentage of antioxidant activity in camel milk was 1.04± 0.1 and after 6 h of incubation reached 24.84 ± 0.28, in cow milk, the antioxidant activity before incubation was 0.79 ± 0.21% which reached 16.7±0.28% after 6 h incubation; The maximum amount of antioxidant activity was observed in camel milk after 6 hours and in cow in the eighth hour of incubation. The percentage of antioxidant activity in camel milk decreased after 6hours and in cow milk after 8 hours of incubation. Comparison of the mean of the main effects showed that two factors, milk type and incubation time have a significant effect on changes in antioxidant activity ($p \leq 0.05$) (Figure 4)

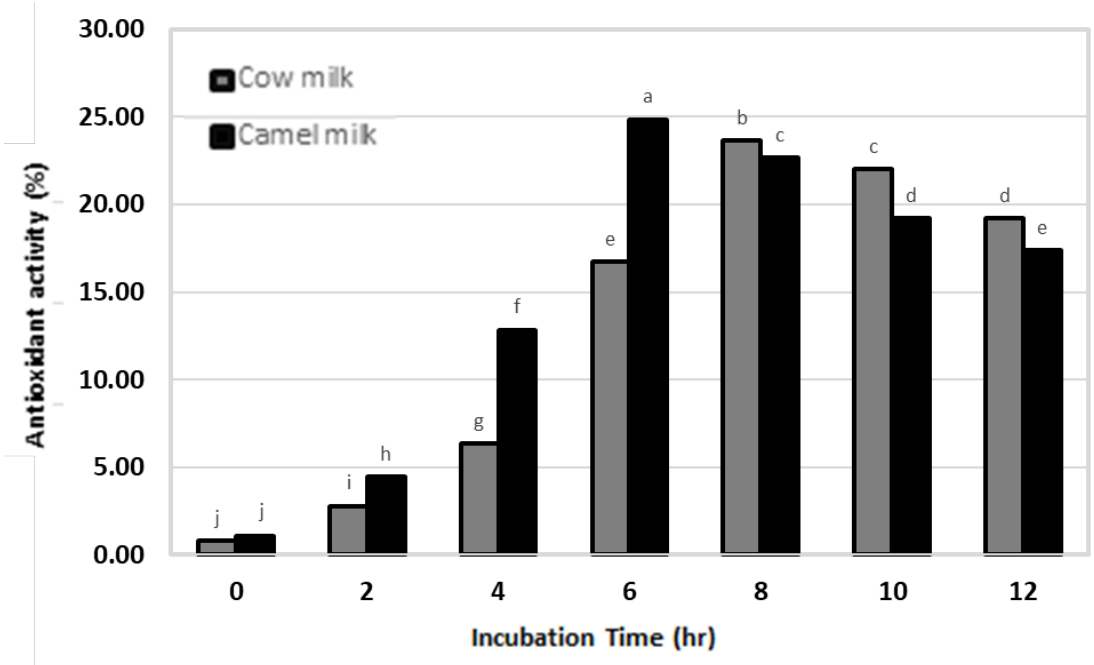

Figure 4. Comparison of the interaction effects of milk factors and incubation time in the response.

3.4. Sensory properties

After 6 hours of incubation, the sensory properties of milk (cow and camel) were examined. Mean comparison showed that the type of milk (cow and camel) has no significant effect on the variables of taste, aroma and overall evaluation (p>0.05), and both types of fermented milk (cow and camel) in terms of taste parameters, aroma and overall rating received low scores. Generally, in the sensory evaluation, fermented cow milk received a higher score than fermented camel milk. (Table 2).

Table 2. Comparison of the average type of milk on the variables of taste, aroma and overall evaluation.

Milk	Variable traits		
	Flavor	Aroma	overall evaluation
camel milk	2.65 ± 0.67 [a]	2.45 ± 0.6 [a]	2.60 ± 0.50 [a]
cow milk	3.00 ± 0.79 [a]	2.35 ± 0.74 [a]	2.65± 0.74 [a]

Means that have common letters don't have significant difference (p>0.05).

3.5. pH and acidity

During 28 days of storage, the PH decreased in all dessert samples from 6.2 on the first day to 5.26 on the 28th day. During 28 days of storage at refrigerator temperature, the acidity of the samples increased from 0.24 percent lactic acid on the first day to 0.46 percent lactic acid on the 28th day. The results showed that time factor had a significant effect on changes in acidity and PH of all dessert samples (P<0.05) (Tables 3).

Table 3. Comparison of the average effect of day on pH and acidity.

Factor	Levels	Variable traits	
		pH	Acidity (% lactic acid)
	First	6.20 ± 0.07 [a]	0.24 ± 0.02[e]
	Seventh	6.08 ± 0.11[b]	0.28 ± 0.03[d]
Day	fourteenth	5.74 ± 0.35[c]	0.36 ± 0.08[c]
	twenty-first	5.49 ± 0.23[d]	0.41 ± 0.05[b]
	twenty-eight	5.26 ± 0.24[e]	0.46 ± 0.05[a]

Means that have common letters don't have significant difference (p>0.05).

Both levels of camel milk and cow milk had a significant effect on increasing acidity and decreasing pH (P<0.05) the rate of increase in acidity in chocolate probiotic dessert based on camel milk was higher than chocolate probiotic dessert based on cow milk and the rate of decrease in PH in camel milk based on probiotic dessert samples was significantly different compared to cow milk- based dessert samples (p<0.05) (Table 4).

Table 4. Comparison of the mean effect of milk type on pH and acidity.

Factor	levels	Variable traits	
		pH	Acidity (% lactic acid)
Milk	cow	5.94±0.30[a]	0.30 ± 0.07 [b]
	camel	5.56±0.43[b]	0.39±0.09 [a]

Means that have common letters don't have significant difference (p>0.05).

The comparison of the mean interactions of day and type of milk (camel and cow) on the pH and acidity variables was significant (P<0.05). The sample of probiotic desserts prepared with camel's milk on the 28th day had higher acidity and lower PH, respectively, compared to the samples of probiotic desserts prepared with cow's milk.

Comparison of the mean of two type of sweeteners, sucrose and sucralose-maltodextrin, showed that these 2 factors have a significant effect on the acidity and pH of the product (p≤0.05). The increase in acidity and decrease in pH in the sample of desserts prepared with sucrose sweetener was more than the sample of desserts prepared with sucralose-maltodextrin.

3.6. Survival of the *L. casei*

The survival of *L. casei* was examined in a sample of probiotic desserts during 28 days off refrigeration. Data showed the survival of *L. casei in* cow and camel milk 8.76±0.02 and 8.83±0.21. Comparison of the mean effect of milk type on the survival of *L. casei* demonstrated that two levels of camel milk and cow milk had a significant effect on the survival of *L. casei* (p≤0.05) and *L. casei* survival in probiotic desserts prepared with camel milk was higher than probiotic desserts prepared with cow milk.

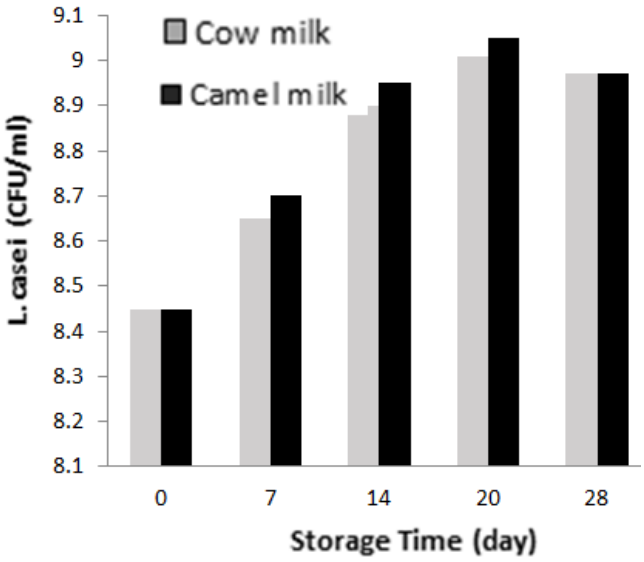

Figure 5. Interactions of milk type and time (day) on the survival of *L. casei*.

Comparison of the mean time factor on the survival of *L. casei* showed that its effect was significant (p<0.05) and survival of *L. casei* increased during 21 days of storage; the survival rate of *L. casei* in chocolate desserts samples was 8.45 Log cfu/g on the first day and reached 9.01 Log cfu/g on the 21st day and then decreased. Survival of *L. casei* during 28 days

of storage at 4C^{o} in the sample of probiotic desserts prepared with camel milk was higher compared to the sample of probiotic desserts prepared with cow's milk. On the 21st day, the survival of *L. casei* in probiotic desserts based on camel milk was 9.05 Log cfu/g and in probiotic desserts based on cow milk in the 21st day was 8.97 Log cfu/g. Comparison of the mean interactions of time (day) and type of milk (cow and camel) on the survival of *L. casei* was not significant ($p>0.05$) (Figure 5).

Survival of *L. casei* in milk containing sucrose and sucralose maltodextrin were 8.82±0.21and 8.76±0.20. These results showed that two sweetener levels (sucrose and sucralose maltodextrin) had a significant effect on survival of *L. casei* which was higher in the sample of probiotic desserts prepared with sucrose sweetener than probiotic desserts prepared with sucralose maltodextrin sweetener.

3.7. Rheological feature

The two most important parameters obtained from the oscillation tests are the storage modulus or elastic modulus (G') and the viscosity modulus or loss modulus (G"). In this test, at a constant frequency of 1 Hz, the effect of strain changes on the storage and loss modulus is investigated. The amount of dissipation and storage modulus at the point of intersection in 4 different dessert samples. The results showed that G' and G" at point of intersection (Pa) of the samples 1, 2, 3, and 4 were 91.57, 65.00, 100.02, and 78.82. According to these data, dessert No. 3, which is actually a probiotic dessert prepared with cow milk and sucrose sweetener, has the highest amount of G' and G". Adding the sucralose maltodextrin sweetener instead of sucrose in both probiotic dessert formulations prepared with camel milk and cow milk, Reduced G 'and G" at the point of intersection (Table 6).

3.8. Frequency scanning oscillation test

In this test, at a constant strain of 0.5%, the frequency is changed from 0.01 to 100 Hz and the amount of storage modulus, loss modulus, complex modulus, complex viscosity and drop tangent are determined. In three selected frequencies of 0.25, 2.5 and 25 Hz, the values of the parameters obtained from this test are compared. The results of comparing the mean showed that the two levels of camel milk and cow milk have no significant effect on the rheological characteristics of probiotic dessert samples ($p>0.05$). The two sweetening levels of sucrose and maltodextrin sucralose had a significant effect on the rheological properties of probiotic dessert samples ($p\leq0.05$) and the measured parameters for the sample of probiotic desserts that used sucrose sweetener in their formulation were higher than the sample of probiotic desserts with maltodextrin sucralose sweetener. Comparison of the mean interactions of milk (camels and cows) and sweeteners (sucrose and sucrose maltodextrin) had no significant effect on the rheological characteristics of probiotic desserts (Table 6).

3.9. Sensory characteristics

4 dessert samples were examined in terms of aroma, texture and overall evaluation. The effect of milk type (camel and cow) and sweetener (sucrose and maltodextrin sucralose) on the variable of aroma response was significant, the samples of desserts prepared with cow milk had a higher score in terms of aroma compared to the samples of desserts prepared with camel milk, Also, desserts that used sucralose sweetener in their formulation scored higher in terms of aroma parameter compared to sucrose sweetener. The mean of the main effects of milk (camels and cows) and sweeteners (sucrose and sucrose maltodextrin) on the variables of texture properties and overall product evaluation did not make a significant difference and all dessert samples received a score above 5 in terms of texture and overall evaluation (Table 7).

Table 6 a. Comparison of mean milk and sweetening factors on the rheological properties of dessert texture.

Factor	levels	mean traits								
		G'(Pa)			G"(Pa)			G*(Pa)		
		0.25	2.5	25	0.25	2.5	25	0.25	2.5	0.25
Milk	cow	337.25±59.41[a]	379±64.38[a]	447.75±82.12[a]	38.02±6.11[a]	41.75±6.51[a]	59.97±8.92[a]	339.33±59.70[a]	381.27 ± 64.77[a]	451.17 ± 82.42[a]
	camel	316.25±67.14[a]	358.50±74.17[a]	419.75±83.59[a]	35.2 ±6.96[a]	38.55±7.76[a]	56.75±11.07[a]	318.17±67.52[a]	360.58 ± 74.58[a]	422.85 ± 84.17[a]
Sweetener	sucrose	381± 14.67[a]	427.75±15.90[a]	501.75±38.35[a]	41.97±2.91[a]	46.17±2.39[a]	66.75±3.17[a]	383.28±14.82[a]	430.26 ± 16.06[a]	505.37 ± 38.48
	sucrose maltod extrin	272.5±16.42[b]	309.75±19.80[b]	365.75±21.31[b]	31.25±2.47[b]	34.12±2.66[b]	49.97±3.42[b]	274.22±16.57[b]	311.59 ± 19.93[b]	368.65 ± 21.7[b]

Table 6 b. Comparison of mean milk and sweetening factors on the rheological properties of dessert texture.

Factor	levels	mean traits					
		Complex Viscosity (Pa.S)			Damping factor		
		0.25	2.5	25	0.25	2.5	25
Milk	cow	1357 ± 245.13 [a]	152.25 ± 27.35 [a]	18.05 ± 3.15 [a]	0.11 ± 0.005 [a]	0.11 ± 0.002[a]	0.13 ± 0.005 [a]
	camel	1307.5 ± 326.94 [a]	136 ± 21.83 [a]	16.62 ± 3.05 [a]	0.11 ± 0.002 [a]	0.1 ± 0.001 [a]	0.13 ± 0.004 [a]
Sweetener	sucrose	1567.5 ± 126.06 [a]	164 ± 17.18 [a]	19.92 ± 1.38 [a]	0.1 ± 0.004 [b]	0.1 ± 0.002 [b]	0.13 ± 0.005 [a]
	sucrose maltod extrin	1097.5 ± 74.1 [b]	124.25 ± 7.22 [b]	14.75 ± 0.96 [a]	0.11 ± 0.000 [aa]	0.1 ± 0.002 [a]	0.13 ± 0.003 [a]

Table 7. Comparison of the mean effect of the main effects of milk type and sweetener on the variables of aroma response, texture and overall evaluation.

			mean traits	
Factor	levels	Aroma	Texture	overall evaluation
milks	cow	5.60 ± 1.03 [b]	6.26 ± 1.31 [a]	5.87 ± 1.36 [a]
	camel	6.57 ± 1.10 [a]	6.28 ± 1.28 [a]	6.17 ± 1.56 [a]
sweetener	sucrose	5.78 ±1.00 [b]	6.17± 1.25 [a]	5.76 ± 1.36 [a]
	sucrose maltodextrin	6.39± 1.26 [a]	6.37 ± 1.34 [a]	6.28± 1.53 [a]

Means that have common letters don't have significant difference ($p>0.05$).

4. Discussions

4.1. pH and acidity

Before fermentation, the pH of camel milk was lower than that of cow milk, which may be due to the high content of vitamin C and organic acids in camel milk (Farah et al., 2007). During incubation, for 12 hours, the pH of both types of milk (camel and cow) decreased and the acidity increased due to the activity of the beta-galactosidase enzyme released by lactic acid bacteria during fermentation, which breaks down lactose and produces lactic acid, acetic acid, citric acid, butyric acid, etc. The mentioned acids lead to an increase in acidity and decrease in pH in the fermented product (Ayash et al., 2018). Camel milk had a lower pH and higher acidity than cow milk during incubation; the results of this study are consistent with the report of Ayash et al. (2018). They stated that camel milk fermented by *Lactoplantibacillus plantarum* extracted from camel milk had higher acidity and lower pH, respectively, than cow fermented milk, because antimicrobial compounds in camel milk are higher than in cow milk and some probiotic species are more compatible with camel milk, which as a result leads to their growth and production of more organic acids. Abu-Tarbush reports (1996) showed that the difference in pH between camel milk and fermented cow milk was significantly different from four species of Bifidobacterium and decrease in pH in camel milk was more than cow milk, which could be due to low buffering capacity of camel milk compared with cow milk and the difference in buffering capacity between camel milk and cow milk is related to the difference in the ratio of specific proteins and salts in each type of milk. Monteagudo-Mera studies (2011) showed that there is a significant difference in the acidity of camel milk and cow milk fermented for 6 hours with the same probiotic. The results of Felfoul et al. (2017) research on fermented milk (camel and cow) by *Enterococcus faecium* and *Streptococcus macedonicus* showed that the acidity of fermented camel milk for 20 hours at 42°C was higher than cow milk. In another study by Ayash et al. (2018) on camel milk fermented by *Lactococcus lactis* extracted from camel milk and compared with cow milk, the results showed that camel milk had higher acidity than cow milk during 21 days of storage. The results of this study are consistent with similar studies.

According to Bresford et al. (2001), the best pH for most bacteria to grow is close to neutral and pH below 5 stops them from growth. During storage of the product for 28 days, the pH of the product decreased significantly. This decrease in pH is consistent with the report of Irkin and Goldaz (2011) who stated that because the

growth of *L. casei* and its ability to produce acid is high, it reduces the pH during the storage period of the crop.

Valencia et al. (2016) also reported an increase in acidity of desserts increased during the storage period of 28 days. This increase in acidity is expected from *L. paracasei* as an arbitrary heterofermentative bacterium and producers of acetic and lactic acid as well as CO_2. Other factors such as 5°C and the addition of sugar, which can be broken down into glucose and fructose. Then glucose can be converted to lactic acid, which affects the metabolism of this strain.

Argon Allegro et al. (2007) stated that the acidity of probiotic desserts decreases during 28 days of storage time which is due to the presence of the probiotic bacterium *L. paracasei*.

The results of Patel et al. (2008) study on probiotic and synbiotic chocolate mousse showed that during 28 days of storage in probiotic samples containing *L. paracasei* and synbiotic samples containing *L. paracasei* and inulin compared to the control sample, the increase in acidity was significantly higher due to the presence of *L. paracasei* and the production of lactic acid by this bacterium.

4.2. Growth changes of *L. casei*

Bacteria need strong proteolytic and glycolytic systems to provide the necessary nutrients for their growth to function properly in milk. While glucose is essential to meet the basic needs of bacterial growth, the supply of amino acids needed to sustain bacterial growth is provided by complex proteolytic systems that lead to bacterial growth in milk (Elfahri et al., 2016). In this research, the growth of *L. casei* during the fermentation process increased with increasing incubation time and the growth rate of this bacterium in camel milk was significantly different from cow milk and its growth in camel milk was higher than cow milk, this is consistent with the results of a study by Varga et al. (2013); they reported that the microbial count of Lactobacillus acidophilus in fermented camel milk was higher than that of fermented cow milk. *L. casei* growth decreased after 6 hours of incubation in camel milk and after 8 hours of fermentation in cow milk; this is consistent with the results of the research by Leclerc et al. (2002). They stated that the growth of *Lactobacillus helveticus* decreased slightly after 10 hours of milk fermentation due to the increase in lactic acid concentration. Type of selected probiotic species, the presence of hydrogen peroxide in the environment due to bacterial metabolism, inoculation temperature, the concentration of organic acids produced by the bacterium, inoculation level, also fermentation time affects the viability of lactic acid bacteria during fermentation (Rybka & Kailasapathy, 1996).

Abu-Tarbush (1996) showed that the growth of different species of Bifidobacterium in two types of camel and cow milk during incubation for 36 hours at 37°C is significantly different. Some of these species have higher growth in camel milk and some in cow milk.

Ayash et al. reported that in camel milk and cow milk fermented by two species of probiotic bacteria, *Lactococcus lactis* K782 extracted from camel milk and *Lactobacillus acidophilus*, *Lactococcus lactis* k782 count during 21 days of storage was higher in camel milk, and *Lactobacillus acidophilus* count was higher in cow milk. They suggested that this may be due to the presence of antimicrobial compounds in camel milk and the greater compatibility of *Lactobacillus lactis* k782 with camel milk compared to *L. acidophilus*.

Research by Ayash et al. (2018) on the growth of four species of probiotic bacteria in camel and cow milk showed that some bacteria grow more in camel milk and some in cow milk and one of the for this is the higher antimicrobial compounds in camel milk compared to cow milk and its effect on the growth of some bacteria. The probiotic food should include 10^6 cfu/g at the time of consumption (Boylston et al., 2004). The viability of *L. casei* in chocolate dessert was evaluated in this study, and the results showed that during 28 days of refrigeration the viability of *L. casei* was higher than Log 8 cfu/g and during 21 days of storage at 4°C. This is consistent with research by Patel et al. (2008)

they reported that the growth of *L. paracasei* in chocolate mousse increased during 28 days of refrigerated storage temperature.

Argon Allegro et al. (2007), in a study on probiotic and synbiotic chocolate mousse stated that lowering the pH during 28 days of refrigerated chocolate mousse was not sufficient to reduce the viability of *L. paracasei*. Its viability in all chocolate mousse was > 7 Log CFU/g after 28 days storage. Viability of *L. paracasei* increased during 21 days of storage at 5°C.

Valencia et al. (2016) in a study on chocolate milk dessert containing *L. paracasei* stated that the viability of this bacterium during 28 days of storage was higher than 8 Log CFU/g, which is more than recommended for probiotic products.

Heenan et al. (2004) reported that with the addition of probiotic bacteria to frozen herbal desserts, the bacterial population remains at about 10^7 CFU/g during six months of storage. The authors stated that dessert is accepted as a suitable food with sensory properties to transmission of probiotic bacteria. Helland et al. (2004) evaluated the growth and metabolism of four probiotic species of *Bifidobacterium animal*, *Lactobacillus acidophilus* La5 and *Lactobacillus rhamnosus* in pudding. They concluded that probiotics survival was between 8Log to 9.1 CFU/g for 21 days. The buffering capacity of food is an important factor for the viability of probiotic bacteria. So, milk is a suitable carrier with a stable pH (Silva et al., 2012).

4.3. Evaluation the changes in antioxidant activity

Bioactive compounds in foods, especially dairy-fermented products, may reduce the effect of superoxidase, hydroxyl, peroxyl, and radicals formed by cell oxidation. These bioactive peptides, especially peptide-derived proteins, neutralize free radicals by donating electrons (Ayash et al. 2018; Gadhiya et al., 2015). Bioactive peptides as antioxidants in fermented milk may inhibit peroxidation of essential fatty acids (El-Salam & El-Shibiny, 2013). According to the results of this test, the level of antioxidant activity of both types of milk (camel and cow) increased during incubation, which is consistent with the results of the study of Elfahri et al. (2016); They reported that the antioxidant activity of milk containing *Lactobacillus helveticus* increased during incubation due to increased bacterial proteolytic activity, which led to the production of bioactive peptides that have antioxidant properties.

The antioxidant activity of camel milk during fermentation was higher than that of cow milk. This is consistent with the results of the research of Ayash et al. (2018); they stated that the reason for the high antioxidant activity of fermented camel milk compared to fermented cow milk is the higher proteolysis rate in camel milk and the nature of the bioactive peptides in camel milk.

Similarly, Moslehi Shad et al. (2013) stated that fermentation of milk (camel and cow) by *L. rhamnosus* increases antioxidant activity, which may be due to the hydrolysis of s1α and β-casein by proteolytic and peptidolytic enzymes of *Lactobacillus rhamnosus*. In fact, peptide fragments extracted from camel milk fermented by *Lactobacillus rhamnosus* showed higher antioxidant activity than cow milk. They stated that these findings indicate that the nature and composition of peptides are not same in camel milk and fermented cow milk, and these peptides play an important role in neutralizing ABTS radicals and antioxidation activity.

Felfoul et al. (2017) by studying the antioxidant activity of fermented camel milk and comparing it with fermented cow milk stated that the free radical scavenging activity of camel milk is higher than cow milk, which may be due to the richness of camel milk with vitamin C comparing to cow milk. Another reason is the high antioxidant activity of peptides derived from camel milk caseins, especially β-casein. The results of Amal and Salinity (2013) research showed that yogurt made from soy and camel milk has higher antioxidant activity than yogurt made from soy and cow milk. The maximum level of antioxidant activity was observed in camel milk at sixth hour of incubation and in

cow milk at the eighth hour after which it decreased in both types of milk (camel and cow). Elfaheri et al. (2016) stated that the antioxidant activity of milk containing *Lactobacillus helveticus* increased from zero time to 12 during incubation and then decreased slightly, which could be due to the hydrolysis of some antioxidant components by *Lactobacillus helveticus* which leads to decreased antioxidant activity. They also reported that antioxidant activity in fermented milk depends on the metabolic activity of lactic acid bacteria, which varies between different bacterial species, in addition to bacterial resistance and growth at low pH conditions.

In sensory evaluation of milk (camel and cow) after 6 hours of incubation, both types of milk received a low average score in terms of smell, taste, and overall evaluation. In terms of taste and overall evaluation, camel milk received a lower score than cow milk. Due to the increased acidic taste in both types of milk (cow and camel) during the incubation period, the product was not accepted. The results of this study are consistent with the results reported by Felfoul et al. (2017). They stated that cow milk fermented by *Enterococcus faecium* received a higher score than camel milk fermented by this bacterium; It may be due to the differences in the structure and composition of both types of milk (camel and cow); such as differences in the amount of lactose in camel milk compared to cow milk, high salt content in camel milk, camel milk is richer in vitamin C compared to cow milk.

Ranadheera et al. (2016), in a study on fermented cow and goat milk, stated that both types of milk received low scores in terms of sensory evaluation due to the development of an unpleasant acidic taste that is produced during fermentation in the product. The results of Gomes et al. (2013) evaluation of fermented dairy beverages made with cow milk and goat milk and a mixture of both types of milk showed that the fermented beverages had a highly acidic taste during 28 days of storage. They stated that taste and aroma are important factors in the acceptance of the product by the consumer and the decision to buy dairy products and the addition of fruit and flavorings and sugar largely obscures the sour taste of the product.

The higher the strain point of the intersection, the greater the tolerance of the sample to mechanical stress and transport, in other words, the more stable it is (Tarrega and Costell, 2006). According to the above mentioned information, the amount of G' and G'' at the intersection point also indicates the structural cohesion and intermolecular connections of the sample; the higher the value of these two parameters, the stronger the intermolecular connections and the more cohesive the structure. The maximum amount of G' and G" at the intersection of the dessert was based on cow milk and sucrose sweetener.

4.4. Frequency scanning oscillation test

The information shows that in all samples of the storage modulus either G' is above the dissipation modulus or G''; therefore, all dessert samples show solid viscoelastic behavior. The maximum amount of storage modulus or elastic and viscosity of the complex was related to a dessert prepared with cow milk and sucrose sweetener. Arcia et al. (2010) reported that desserts with higher inulin concentration have higher viscoelastic properties and storage modulus curve or G' was above the loss modulus or G''. Tarrega and Costell (2006) reported on starch-based low-fat-dairy desserts at frequency of 1 Hz in different dessert with increasing starch concentration (2.5, 3.25, and 4 percent), the storage modulus and complex viscosity increases. The tangent decreases, indicating a relative increase in the elasticity of the sample to viscoelasticity. Bavarri et al. (2010) stated that the binding of casein micelles to kappa carrageenan through electrostatic bonding stabilizes the gel and increases the viscoelastic behavior. Thomas et al. (2008) stated that desserts that use kappa carrageenan in their formulation require more energy to break down their structure than samples that do not use kappa carrageenan in their formulation. These results indicate the suitability of kappa carrageenan as a structural hydrocolloid for

dairy products. Considering that gelatin and kappa carrageenan were used in the formulation of all probiotic dessert samples and according to the properties mentioned for kappa carrageenan, between dessert samples prepared with two types of milk (camel and cow) in terms of rheological features no significant differences were observed.

Adding sucralose instead of sucrose did not have a significant effect on sensory properties, which is consistent with a report by Demorais et al. (2015). They evaluated the sensory properties of dietary probiotic chocolate desserts and found that sucralose is the best sucrose substitute for probiotic chocolate milk desserts compared to other sweeteners such as aspartame, neotam, and stevia, because it makes the least changes in sensory properties.

Irkin and Guldas (2011) stated that chocolate pudding containing *L. casei* received the lowest score in sensory evaluation of texture. They stated this is probably due to the high proteolytic activity of *L. casei*. Also, the pudding prepared with *L. casei* had the lowest taste and smell score compared to the puddings prepared with Lactobacillus acidophilus and *Bifidobacterium lactis*. In sensory evaluation in terms of texture, all four dessert samples had a score above 6, considering that in the formulation of all four dessert samples, texture factors were used, this issue helped to improve the oral texture of the samples. According to Lethuaut et al. (2003), the composition and structure of food are involved in understanding mouth feel and the use of gelling and thickening agents changes the texture of food. However, textural factors may alter taste perception and vice versa. In terms of average scores related to sensory evaluation in all parameters of aroma, texture, and overall evaluation, each four dessert samples had a score above 5and this is consistent with the results of research by Kardley et al. (2008). They showed suitability of chocolate mousse dessert as probiotic carrier with acceptable viability and sensory properties. Their results showed no significant impact on taste and aroma of chocolate mousse containing *L. paracasei* during 7 days of storage. Argon Allegro et al. (2007) investigated on chocolate mousse containing *L. paracasei* and inulin reported that there was no significant difference in the results of sensory evaluation between control, probiotic and synbiotic samples. They stated that the addition of probiotics and prebiotics does not change the sensory evaluation of products by the consumer. Similarly, the results of the study by Patel et al. (2008) showed that there was no significant difference in terms of sensory evaluation between the control sample and the probiotic and synbiotic samples.

5. Conclusions

Investigating of the growth of Lactobacillus casei in milk (camel and cow) during 12 hours of incubation led to increased acidity and decreased the pH of both types of milk (camel and cow). Camel milk had significantly higher acidity and lower pH compared to cow milk ($p<0.05$). The growth of this bacterium during incubation in camel milk was significantly higher than cow milk ($p<0.05$). The fermentation of both types of milk (camel and cow) with *L. casei* increased their antioxidant activity during incubation time and the level of antioxidant activity in camel milk was higher than cow milk. In sensory evaluation, fermented camel milk received a lower score compared to fermented cow milk. The results of examining the properties of probiotic chocolate dessert based on camel milk in this study showed that this product has suitable conditions for the growth of *L. casei*. The growth and viability of this bacterium in dessert after 28 days of storage were higher than recommended for probiotic products. Due to the use of Kappa carrageenan and gelatin in the formulation of probiotic dessert, no significant difference was observed between different samples of probiotic dessert based on camel milk and cow milk ($p>0.05$). In addition, the chocolate dairy dessert evaluated in this study can be an example of functional foods with sensory properties accepted by the consumer.

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EFFECT OF VELVET TAMARIND JUICE-TO-SUGAR RATIO ON THE QUALITY OF HALAL JELLY

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ABSTRACT

The development of Halal velvet tamarind (VT) jelly products is important for Muslim consumer confidence. This study analyzes the proximate composition of VT fruit and develop VT fruit jelly. Based on proximate analysis, the VT pulp contains 11.23 ± 0.86% moisture, 1.53 ± 0.23% ash, 1.67 ± 0.19% protein, 1.12 ± 0.15 fat, 79.48 ± 0.82 carbohydrates, 8.24 ± 0.02% total soluble solids, and has a pH level of 2.89 ± 0.04. The ratio of VT juice and sugar is varied as 4:1, 3.5:1.5, 3:2, 2.5:2.5, and 2:3. There is a significant difference ($p \leq 0.05$) between jellies with varying VT juice-to-sugar ratio in terms of the brightness (L*), red value (a*), and yellow value (b*) of the finished product. The ratio shows that both L* and b* values increase with higher amounts of sugar in the mixture, leading to an overall dark color. Meanwhile, a* increases with higher amounts of sugar, rendering a reddish color on the jelly. The water activity and pH of the jelly are 0.88 ± 0.01 and 3.01 ± 0.01, respectively. Our results show that a ratio of 3:2 has the highest overall likeness score at 7.96 ± 0.92 (like moderately). Therefore, the quality of the halal chewy products was found to be acceptable by the sensory evaluation panel members.

1.Introduction

Velvet tamarind (VT) is an important fruit in the 3 southern border provinces (SBPs) of Thailand (Chedoloh & Chemalee, 2019). Most often found as red fruits, VT fruits are harvested from the trees that grow in southern Thailand. The people in Yala, Patani, and Narathiwat provinces know these trees as Lukyee and Kerayee. They have average height of about 30 m, densely leafy crown, and smooth greyish barks (Chedoloh, 2018). Their leaves are hairy and their flowers are usually whitish. Meanwhile, the fruits are almost circular and flattened. Its pulp is edible and sweet, has a delicate aroma and high levels of ascorbic acid and fiber (Obasi *et al*., 2013), and is a good source of minerals and antioxidants (Afolabi *et al*., 2018). The fruit is offered as a candy-like snack in the 3 SBPs, often dried, sugar-coated and spiced with chili (Chedoloh, 2018). The dried fruit has a powdery texture and is orange in color with a tangy flavor. However, each product has distinctive characteristics and flavors depending on the producers owing to unique processes and ingredients utilized during production. Despite these differences, the end-product is still traditional and entrepreneurs lack development strategies for new products, which limit the choices for consumers. To widen the range of choices and enhance the market competition of households and housewife groups in the 3 SBPs, creating new products

derived from VT is necessary. An alternative product to candied VT fruit, which is now gaining popularity, is jelly. The demand for jelly is driven by consumers ranging from children up to the working age group because jelly is classified either as a dessert or a snack.

The main components of jelly are fruit juices, sugar, acids, and gel-causing agents (Curi *et al*., 2017). In jelly production, sugar contributes to the product's structure, given that gels with high methoxyl pectins are formed only if sucrose is present at a concentration greater than 55% (Acosta *et al*., 2008). Consumers like to eat 14.7% of dried jelly. Of the consumers, 96% are interested if there are jelly products with health benefits available in the market. Apart from the health benefits, producers must study the use of ingredients approved by Islam. The producers also need to address the customers' safety concerns and demand for consistent quality products by raising the quality of production. In addition, VT-derived souvenirs from the 3 SBPs should include Halal products to support the Association of Southeast Asian Nations (ASEAN) market in the future.

The aim of this study is to develop jelly products from VT, with texture and sensory properties similar to the standard products, by replacing sucrose partially or totally with an appropriate substitute combination of VT fruit juice and sugar.

2. Materials and methods

2.1. Procurement of Raw Material and Chemical

VT fruits (*Dialium indum* L.) dried in May 2016 was collected from the Amani Luk Yee Factory, located in the Muang District of Yala province. The glucose, sugar, syrup, salt, copper pan, stainless steel tray, silicone mold, and refrigerator, which were used for jelly production, were obtained from the Yala market, Thailand. The fish gelatin was from the Halamic Company International (Thailand). The strength of the gelatin was 247 g Bloom while its pH, moisture content, and protein were 5.6, 11.1%, and more than 86.5%, respectively. All the chemicals and reagents used in this study were analytical grade and supplied by Sigma Chemical Co.

2.2. Chemical Analysis of Velvet Tamarind Dried Fruit

The method described in AOAC (2000) was used to estimate the moisture content in the dried VT fruits with the temperature maintained at 100-105 °C for 24 hours. It was also used to determine the protein, ash, crude fat, and ascorbic acid content of the samples. The color of each VT samples was evaluated using the three parameters in the color space defined by the International Commission on Illumination (CIE), which are L* (lightness/darkness value), a* (green/red value), and b*(blue/yellow value). These parameters, collectively known as CIE-Lab, were measured using a reflectance calorimeter. The dried fruits' pH was measured by a pH meter. Finally, the total soluble solids (TSS) were determined by a refractometer.

2.3. Effect of Juice-to-Sugar Ratio on Quality of Halal Jelly

Jelly production from the VT fruits started with a mixture of VT pulp and water with a ratio of 1:3. The VT pulp and water mixture was blended for 3 minutes using a blending machine and was filtered with a cheesecloth that is folded 2 times to extract the juice. Sugar was added to the juice with a VT fruit juice-to-sugar ratio (VJS) of 4:1, 3.5:1.5, 3:2, 2.5:2.5 and 2:3 (Table 1). In addition, gelatin (20%), warm water (18%), glucose syrup (31.50%), and salt (0.5%) were also added to the different formulations while keeping the total volume across all formulations constant. The ingredients were placed in a brass pan and the mixture was stirred for 15 minutes until the ingredients were combined well. After stirring, the final mixture was poured into silicone molds with depth that is about 1 cm, and with sides that are approximately 1.5 cm long. The silicone molds were then placed in a large plastic bag to prevent contamination and were refrigerated at a temperature of 4-5 °C until the VT jelly sets, which takes about 8 hours. Finally, the jellies

were taken out of the molds and were placed in PP plastic bags.

Table 1. List of all ingredients in jellies with different VT fruit juice-to-sugar ratio

Ingredients	VT fruit juice-to-sugar ratio				
	4:1	**3.5:1.5**	**3:2**	**2.5:2.5**	**2:3**
VT fruit juice (%)	40.00	35.00	30.00	25.00	20.00
Sugar (%)	10.00	15.00	20.00	25.00	30.00
Gelatin (%)	20.00	20.00	20.00	20.00	20.00
Water (%)	18.00	18.00	18.00	18.00	18.00
Glucose Syrup (%)	31.50	31.50	31.50	31.50	31.50
Salt (%)	0.50	0.50	0.50	0.50	0.50

2.4. Physical and Chemical Properties

The VT jelly was analyzed for its physical and chemical properties. Specifically, the physical property investigated was the color of the jelly. The same parameters as those used for the dried VT fruit (CIE-Lab) were used to describe the color. On the other hand, the chemical properties analyzed were pH, the amount of vitamin C (mg/100 g), and the TSS. All methods were analyzed gravimetrically following the AOAC method (2000).

2.5. Sensory Evaluation

Sensory testing was performed in Yala Rajabhat University Sensory Lab. Fifty (50) non-trained panelists were selected to equally represent genders with ages between 18 to 45 years old. The panelists were asked questions about the color, flavor, taste, adhesion of meat, chewiness, the difficulty of swallowing, sweetness, saltiness, sourness, and overall liking. All questions were ranked using a 9-point hedonic scale, with 1 corresponding to dislike extremely and 9 to like extremely (Meilgaard *et al*., 1990).

2.6. Texture Analysis of Fresh and Dried VT Fruits

Texture analysis of the VT fruit was performed using a Brookfield's CT*3* texture analyzer. Data for the breaking force were collected in Newton (N). A texture analyzer program outputs the hardness and texture curves from three types of fresh and dried VT fruits. Five separate scans were performed for each sample.

2.7. Statistical Analysis

The statistical analysis followed a completely randomized design for physical and chemical properties evaluation (by conducting 3 trials) and a randomized complete block design for the sensory tests. A linear mixed model was implemented using the SPSS software to analyze different treatments (e.g. type and source of VT). Duncan's new multiple range test was used to compare means of treatments for statistical significance ($p \leq 0.05$).

3.Results and discussions

3.1. Initial Raw Material Analysis

Various chemical properties in the raw materials were analyzed first. Table 2 lists the values of pH, water activity (a_w), moisture, TSS, fiber, carbohydrate, fat, protein ash, and vitamin C contents of dried VT. The results suggest that dried VT is a good source of nutrients and antioxidants. The 23 mg/100g vitamin C content is in good agreement with the 33.33 mg/100 g vitamin C previously reported by Niyi *et al*. (2015). The dried VT pulp has high acidity with a pH of 2.89. It also has a moisture content and a_w value of 11.23 ± 0.86 g/100 g and 0.46 ± 0.01, respectively. Osanaiye *et al*. (2013) who did a comparative study of the chemical composition of Africa's *Dialium guineense* samples reported a moisture content of 10.53%. The low moisture and a_w help to extend the shelf life of the raw materials. The VT pulp was low in fat and protein, at 1.67 ± 0.19 and 1.12 ± 0.15 g/100 g, respectively, and contains 4.82 ± 0.75% dietary fiber, which can help in the excretion as well.

Table 2. Analysis of physical and chemical properties of VT fruit

Properties	VT fruit
Color L*	25.12±1.32
a*	12.37±0.25
b*	8.64±0.05
pH	2.89±0.04
a_w	0.46±0.01
Moisture (g/100g)	11.23±0.86
TSS (°Brix)	8.24±0.02
Fiber (g/100g)	4.82±0.75
Carbohydrate	79.48±0.82
Fat (g/100g)	1.12±0.15
Protein (g/100g)	1.67±0.19
Ash (g/100g)	1.53±0.23
Vitamin C (mg/100g)	23.70±0.12

3.2. Effect of VT Juice-to-Sugar Ratio on Physical and Chemical Properties of the Jelly

3.2.1 Physical Properties

VJS in the jelly affects the color attributes (L*, a*, and b*) significantly ($p \leq 0.05$). The parameters L*, a*, and b* were selected as responses to the experimental design and formulation variables (Garrido *et al*., 2015). As summarized in Table 3, L* increases with VJS. Consequently, Fig. 1 shows that the jelly with VJS = 4:1 is darker compared to the jelly with VJS = 2:3. The experimental values of L*, a*, and b* ranges from 21.85 to 29.49, 4.53 to 2.93 (reddish), and 6.94 to 8.57 (yellowish), respectively, as listed in Table 3. These values reflect the yellow-brown color of our jellies.

Table 3. Physical property analysis of VT jelly

Physical properties	VT fruit juice-to-sugar ratio				
	4:1	**3.5:1.5**	**3:2**	**2.5:2.5**	**2:3**
L*	21.85±0.01^{e}	24.46±0.01^{d}	24.48±0.01^{c}	27.80±0.01^{b}	29.49±0.01^{a}
a*	4.53±0.03^{a}	3.93±0.02^{b}	3.44±0.01^{c}	3.26±0.01^{d}	2.93±0.01^{e}
b*	6.94±0.01^{e}	7.37±0.01^{c}	7.05±0.02^{d}	7.61±0.01^{b}	8.57±0.01^{a}

Different characters in the landscape have significant differences ($p \leq 0.05$).

4:1 3.5:1.5 3:2 2.5:2.5 2:3

Figure 1. Representative velvet tamarind jellies with decreasing juice-to-sugar ratio from left to right

3.2.2 Chemical Properties

Table 4 summarizes the proximate composition (%) of the VT jellies. There is a significant change ($p < 0.05$) in the properties as VJS is varied. The moisture content of the jellies ranges from 29.60 g/100g for VJS = 2:3 to 40.83

g/100g for VJS = 4:1. The moisture content decreases as VJS increases, that is, there is a higher amount of sugar in the jelly. Likewise, a_w decreases as the amount of sugar in the jelly increases. Fat, protein fiber, and ash contents in the VT jelly are very low possibly due to the low fat content in VT fruits as previously reported by Adetuyi and Ibrahim (2014). The carbohydrate content of each jelly was significantly different ($p < 0.05$) from each other and ranges from 59.70 g/100g to 66.62 g/100g. The increase in the carbohydrate content could be because VT is rich in glucose and fructose (Obasi *et al*., 2013). Lastly, the pH affected the texture of the jelly. The addition of sugar in jelly processing decreases the pH of the final product. Standard alkalinity of jelly products is between 2.8 and 3.5, while the optimum acidity/alkalinity is between 3.08 and 3.20 according to the Thai Industrial Standards Institute (2004) No. 263-2521 (TIS. 263-2521). The addition of VT juice can contribute to an increased taste, improved gelling, and stabilized natural color of the resulting product. H+ ions that causes the sour taste in the jelly, are derived from an organic acid molecules, which are found mainly in lemon. The ionized molecule releases its proton as H+. Large quantities of H+ ions result to acidic solutions and lower pH (Nazir and Adrian, 2016).

Table 4. Chemical property analysis of VT jelly product

Chemical properties	VT fruit juice-to-sugar ratio				
	4:1	**3.5:1.5**	**3:2**	**2.5:2.5**	**2:3**
Moisture (g/100g)	40.83±0.32^{a}	36.24±2.90^{b}	33.26±1.21^{c}	31.25±1.48^{c}	29.60±1.20^{d}
a_w	0.92±0.01^{a}	0.90±0.01^{b}	0.89±0.01^{c}	0.88±0.01^{c}	0.83±0.01^{d}
Fiber (g/100g)	1.65±0.04^{b}	1.78±0.05^{a}	1.83±0.06^{a}	1.43±0.1^{d}	1.54±0.02^{c}
Ash (g/100g)	0.65±0.04^{b}	0.78±0.05^{a}	0.83±0.06^{a}	0.43±0.01^{d}	0.54±0.02^{c}
Protein (g/100g)	1.10±0.01^{a}	1.08±0.01^{b}	1.02±0.01^{c}	1.09±0.001^{ab}	1.03±0.02^{c}
Fat (g/100g) ^{ns}	0.65±0.01	0.66±0.01	0.67±0.01	0.65±0.01	0.66±0.01
Carbohydrate (g/100g)	59.70±2.98^{b}	54.85±0.34^{c}	59.38±1.16^{b}	65.13±2.49^{a}	66.62±0.59^{a}
pH	3.08±0.01^{c}	3.00±0.01^{d}	3.01±0.01^{d}	3.25±0.02^{a}	3.20±0.02^{b}

Different characters in the landscape have significant differences ($p \leq 0.05$)
^{ns} is Non- significant differences ($p > 0.05$).

3.2.3 Chemical Properties

Table 5 summarizes the sensory evaluation results for different VJS investigated in this study. The results show that VJS has a significant effect on the sensory acceptance in consumers such as the taste, sweetness, sourness, and overall liking ($p \leq 0.05$). On the other hand, there is no significant difference ($p > 0.05$) in the color, flavor, chewiness, and saltiness. The sensory test results reveal that consumers gave their highest overall likeness score of 7.96 (like moderately) to VJS = 3:2 (by weight) among all other VJS investigated in this study.

3.2.4 Effect of Ratio on Texture Analysis

Table 6 depicts the effects of VJS on the jelly texture. The hardness, cohesiveness, gumminess, springiness, and chewiness of the jellies increase significantly with decreasing VJS. As other conditions known to affect the formation of jelly, such as cooking time, temperature, and concentration of sugar (Royer *et al*., (2006)), were fixed in this study, only the amount of sugar added in the jelly influences the textural properties of the gels, except for the adhesiveness (Lee *et al*., 2010).

Table 5. Sensory evaluation results of VT jelly products

Attributes	VT fruit juice-to-sugar ratio				
	4:1	**3.5:1.5**	**3:2**	**2.5:2.5**	**2:3**
Color ^{ns}	7.34±0.89	7.29±1.03	7.74±0.92	7.24±0.68	7.55±0.82
Flavor^{ns}	7.44±0.94	7.51±0.91	7.67±0.90	7.31±0.83	7.27±0.84
Taste	7.00±1.25^{d}	7.38±0.76^{bcd}	7.90±0.78^{a}	7.48±0.76^{abc}	7.80±0.87^{ab}
Chewiness^{ns}	7.03±1.23	7.25±0.85	7.38±0.88	7.03±1.19	6.89±1.22
Sweetness	7.31±0.71^{bc}	7.67±0.65^{ab}	7.38±0.49^{bc}	7.41±0.50^{bc}	7.80±1.01^{a}
Saltiness ^{ns}	7.17±1.07	7.26±1.31	7.48±0.96	7.32±0.90	7.16±1.03
Sourness	7.03±1.09^{b}	7.16±1.10^{b}	7.77±0.66^{a}	7.41±0.67^{ab}	7.16±0.77^{b}
Overall liking	7.30±1.29^{b}	7.51±1.02^{ab}	7.96±0.92^{a}	7.48±0.85^{ab}	7.67±0.70^{ab}

Different characters in the landscape have significant differences ($p \leq 0.05$).
^{ns} is Non- significant differences ($p > 0.05$)

Table 6. The results physical properties of each VT jelly products

Physical properties	VT fruit juice-to-sugar ratio				
	4:1	**3.5:1.5**	**3:2**	**2.5:2.5**	**2:3**
Hardness (N)	37.43±1.30^{c}	49.18±0.74^{b}	38.41±0.94^{c}	98.61±3.83^{a}	95.89±3.37^{a}
Cohesiveness	0.89±0.02^{b}	0.91±0.02^{ab}	0.89±0.03^{ab}	0.93±0.02^{a}	0.93±0.01^{a}
Gumminess (N)	33.40±0.94^{c}	44.95±1.00^{b}	34.59±1.05^{c}	91.25±3.62^{a}	88.95±3.55^{a}
Springiness	0.97±0.02^{ab}	0.94±0.01^{c}	0.96±0.01^{b}	0.98±0.01^{a}	0.94±0.03^{c}
Chewiness (N)	32.69±1.54^{d}	42.47±0.95^{c}	33.22±1.04^{d}	89.42±4.31^{a}	83.87±3.68^{b}

Different characters in the landscape have significant differences ($p \leq 0.05$)

4. Conclusions

VT jelly with VT fruit juice-to-sugar ratio of 3:2 gave the highest overall likeness score of 7.96 as evaluated by a sensory panel in terms of color and smell. The ingredients of the jelly were 30% VT fruit juice, 20% sugar, 20% gelatin, 18% water, 31.5% glucose syrup, and 0.5% salt. Our results show that the jellies could be used as ingredients for Halal production and development of new products to provide alternatives for consumers in the future.

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