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EFFECT OF BAKING TIME AND TEMPERATURE ON THE BAKING QUALITY AND SENSORY ATTRIBUTE OF CAKE PRODUCED FROM WHEAT-TIGERNUT POMACE FLOUR BLENDS BY SURFACE METHODOLOGY

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ABSTRACT

This study was carried out to evaluate the effect of baking temperature and baking time on the baking quality and sensory attribute of cake from wheat-tigernut pomace flour blends. Response surface methodology using Box-Behnken design was employed with three independent variables at three levels of variations resulting into 17 experimental runs. The independent variables are baking temperature (160-180 ºC), baking time (30-40mins) and level of tigernut pomace flour (5-15%) added to the wheat flour. Cake was baked from wheat and tigernut pomace flour and was analyzed for physical properties, colour attributes and sensory qualities. There was significant effect ($p < 0.05$) in the physical properties of wheat-tigernut pomace cake such as the cake weight and oven spring ranged from 50.75g to 69.80g and 2.3cm to 3.5cm. The crumb and crust colour of cake from wheat-tigernut pomace varied significantly ($p < 0.05$) and ranged from 55.84 to 66.77, 0.16 to 2.54, 24.65 to 31.77 and 36.71 to 86.36, 9.76 to 13.32 and 19.96 to 34.51.The result of the sensory evaluation revealed that cakes from wheat flour with low amount of tigernut pomace and those baked at lower temperature and time were rated alike in almost all the quality attributes. The addition of tigernut pomace into wheat flour for cake baking had a significant effect on the physical and sensory qualities. The resultant effect of the baking temperature, baking time and tigernut pomace significantly affected the cake oven spring and led to decrease in cake weight. The lightness, redness and yellowness of the cake samples varied significantly among different levels of the substitution. However, cake can be baked from wheat- tigernut pomace flour between 160-170°C for 30-35 minutes with inclusion of 10% tigernut pomace flour to yield cake of enhanced qualities

1. Introduction

Cakes are a form of food that is usually sweet and often baked (Clerk and Herbert 2000). They are usually prepared from flour, sugar, shortening, baking powder, egg, and flavour as principal ingredients (Clerk and Herbert 2000; Atef *et al.,* 2011). Wheat is the major flour used in cake production. Cakes have enjoyed a relatively constant place in our diet for a long time and its continuous popularity has encouraged the development of newer and more attractive products that are

available in the market today. Wheat flour has also been known to be expensive in Nigeria compared to other cereals mainly because of wheat not been cultivated locally. However, there are so many other crops grown in Nigeria and attempts have been made by many researchers and government to complement wheat flours with non-wheat flour (Oyeyinka *et al.,* 2014). Wheat are grown in few developing countries with the exceptions of where there is a temperate zone caused by high latitude or high altitude or both (examples are Mexico, Northern India, Eastern Africa) (Dendy, 2001).Tigernut is imported from temperate countries most especially where they are cultivated. Due to urbanization and rapid population growth, wheat imports to Nigeria have grown rapidly. According to United States Department of Agriculture, Nigeria imported 4.1 million metric tonnes of wheat in 2011. These imports are paid for with scarce foreign currency and this is depleting Nigeria's external currency earnings and reserve. In the bid to lower or stop out rightly imports of wheat, the Nigerian government & Food and Agriculture Organization (FAO) have encouraged the use of composite flours and blends of non-wheat flours or meals for the production of aerated products such as cake, bread, biscuit, sausage roll, doughnut, etc. Replacing a portion of wheat flour with tigernut pomace flour for the production of cake will therefore decrease the demand for imported wheat, increase the utilization of tigernut and hence the production of fibre-enriched cake.

Consumer attention in dietary fibre has continued to rise as more information about its potential impact on health keeps emanating. In order to tackle the problem of malnutrition in Africa, there is need to explore the underutilized crops; some of which are reported to be rich in dietary fibre. Among the underutilized crops in Nigeria is tigernut which could find useful application in baking industry because of its high level of dietary fibre and other inherent properties (Ade-Omowaye *et al.,* 2008).

Tigernut (*Cyperus esculentus L*) belongs to the division Magnoliophyta, Class-liliopsida, order-cyperales and family-cyperaceae and was found to be a cosmopolitan perennial crop of the same genus as the papyrus plant. Other names of the plant are earth almond as well as yellow nut grass (Odoemelan, 2003; Belewu and Belewu, 2007). In Nigeria, where three varieties (Black, Yellow and Brown) are cultivated,tigernut is kown as `Aya' in Hausa, `Ofio' in Yoruba and Akiausa in Igbo.Tiger nut has been demostrated to be a rich source of good quality oil (Dubois, 2007;Yeboah *et al.,* 2011) and contain a moderate amount of protein (Oladele and Aina, 2007). It has been reported to be high in dietary fibre content (Joy-Toran and Farre-Rovira, 2003), which could be effective in treatment and prevention of many diseases including colon cancer (Adejuyitan *et al.,* 2009), coronary heart disease (Chukwuma *et al.,* 2010), obesity, diabetes, gastrointestinal disorders (Anderson *et al.,* 2009) and losing weight (Borges *et al.,* 2008).

Tigernut pomace is the solid residues which remain after milling and pressing of tigernut for milk and oil production. Tigernut pomace is one of the most common functional ingredients in food products and has been used as fat replacer, fat reducing agent during frying, volume enhancer, binder, bulking agent and stabilizer. Tigernut pomace which is a by-product from tigernut milk production is a suitable fibre source (Sánchez-Zapata *et al.,* 2009). Tigernut pomace has a unique sweet taste, which is ideal for different uses. It is a good alternative to many other flours like wheat flour, as it is gluten free and good for people who cannot take gluten in their diets. It is considered good flour or additive for the bakery industry, as its natural sugar content is high, avoiding the necessity of adding extra sugar (Anderson *et al.,* 2009). The flour is used to make cakes and biscuits and the oil is used for cooking (Wise, 2009).

Tigernut seeds are cheap and readily available but grossly underutilized and need more attention because of its nutritional

qualities such as high fibre. Recent application of tigernut for baked product has been studied extensively. Ade-omowaye *et al.* (2008) reported the use of brown variety of tigernut in bread making using 10-50% dilution of wheat flour with tigernut flour. The report further shows that only bread baked from 10% dilution of wheat with tigernut (brown variety) flour was acceptable. Oke *et al.* (2017a) also reported the use of yellow variety of tigernut in bread making using 2-10% dilution of wheat flour with tigernut flour. The report further shows that incorporation of tigernut flour into wheat flour bread production dramatically improved the parameter investigated as well as bread quality. Oke *et al.* (2016) also reported the proximate, functional, pasting and rheological properties of dilution of wheat flour with tigernut flour. The study showed that addition of tigernut flour has the advantage of improving the mineral and fibre content of flour. However, there is dearth of information on the use of tigernut pomace in cake baking. The nutritional content of wheat is low in fibre due to the various processes the whole wheat might have undergone.

Also, controlling parameters like time and temperature combination during baking has been an engineering problem which is critical to successful implementation of commercial composite flour baking technology. In most developing countries such as Nigeria, baking of cake are mostly done with ovens that lack temperature-time control at cottage level in which fewer small and medium scale bakeries uses automated devices (Shittu *et al.,* 2007). Baking time differs widely and high subjective means developed through long time baking experience are being used by bakers for fueling of ovens and temperature control. Therefore, the inclusion of tigernut pomace flour would serve as a source of fibre supplement for the production of cake and the use of recent and emerging technological ovens for baking of cake will also assist in the design and development of appropriate process for baking cake from the blend. The objective of this study is to investigate effect of baking time and temperature on the baking quality and sensory attribute of cake produced from wheat-tigernut pomace flour blends

2. Materials and methods

2.1. Materials

Wheat flour, tigernut (brown variety) and ingredients such as baking flour, sugar, nutmeg, and margarine were purchased from Osiele market in Abeokuta, Nigeria.

2.2. Methods

2.2.1. Tigernut Pomace Preparation

Tigernut pomace was prepared according to the method described by Oke *et al.* (2017b). Brown tigernut (*Cyperus esculentus*) was sorted to remove unwanted materials like stones, pebbles and other foreign materials before washing with tap water. It was soaked inside the water for eight hours; the soaked nuts was wet milled using laboratory hammer mill. The tigernut co-products were pressed inside the muslin cloth to obtain the extract which is tigernut pomace. The tigernut pomace was dried in the cabinet at 60^{o}C for 24hours. The tigernut pomace was packed and sealed in polyethylene bags until further analysis.

Table 1. Uncoded values for baking variables

Variables	-1	0	1
Baking time (mins)	30	35	40
Baking temperature (^{o}C)	160	170	180
Tigernut pomace (%)	5	10	15

Table 2. Experimental design for the baking runs using response surface methodology

Run	Baking Temperature (°C)	Baking Time (minutes)	Tigernut pomace (%)
1	170.00	35.00	10.00
2	180.00	40.00	10.00
3	160.00	40.00	10.00
4	180.00	35.00	5.00
5	170.00	35.00	10.00
6	180.00	35.00	15.00
7	160.00	35.00	15.00
8	170.00	35.00	10.00
9	170.00	35.00	10.00
10	170.00	40.00	15.00
11	160.00	30.00	10.00
12	180.00	30.00	10.00
13	160.00	35.00	5.00
14	170.00	40.00	5.00
15	170.00	30.00	15.00
16	170.00	35.00	10.00
17	170.00	30.00	5.00

2.2.2. Experimental design

Response surface methodology using Box-Behnken design was employed with three independent variables at three levels of variations resulting into 17 experimental runs. The independent variables are baking temperature, baking time and level of tigernut pomace flour added to the wheat flour. Table 1 shows the range of baking variables while Table 2 shows the baking runs for the production of wheat-tigernut pomace cake.

2.2.3. Preparation of cake produced from wheat-tigernut pomace flour blends

Cake was prepared from the flour blends according to the modified method described by Akubor and Ishiwu (2013) with little modification of recipe. The recipes for the preparation of cake include sugar (50g) baking powder (0.5g), egg (60g), vanilla flavour (1.5g) and margarine (80g). The dry ingredients was weighed and mixed thoroughly in a spiral mixer (for 5minutes). Cream was also made by mixing sugar and margarine thoroughly using an electric hand mixer till a fluffy texture is obtained. The mixed dried ingredient was added and folded in until thoroughly kneaded. The batter was shared out and baked in greased pans in the baking oven.

2.2.4. Determination of physical properties of cake produced from wheat-tigernut pomace flour blends

The cake weight was determined using the method of Shittu *et al.* (2007). The cake weights was determined with the aid of weighing balance after cooling and the weight values was recorded for each sample.

Cake volume was measured using the modified method described by Feili *et al.* (2013). Cake volume was determined using sorghum seed displacement method. The sorghum seeds were poured into a container to measure the volume and were measured in a graduate cylinder and mark as V_1. Thereafter, the sample was placed in the same container and seeds were poured till the test cake is covered. Again, the sorghum seeds were measured in another graduated cylinder and mark as V_2**.** The volume of sample was then calculated based on the following equation.

$$\text{Cake volume (ml)} = V1 - V2 \quad (1)$$

Where: *V_1 represents the volume of the sorghum seeds in the empty container (ml),*

V_2 represent volume of the sorghum seeds in the container containing sample (ml).

The specific volume was also calculated using the method described by Feili *et al.* (2013) as shown in the following equation below:

$$\text{Specific volume (cm}^3\text{/g)} = \frac{Cake\ volume}{Cake\ weight} \quad (2)$$

The cake density was determined using method described by Feili *et al.* (2013). The cake density was calculated by dividing the weight of cake obtained by the volume of cake.

$$\text{Density (g/cm}^3\text{)} = \frac{cakeweight}{cakevolume} \quad (3)$$

Oven spring was determined from the differenced in the height of dough just before and after baking using the method described by Idowu *et al.* (1996).

$$Height\ of\ cake\ before\ baking - Height\ of\ cake\ after\ baking \quad (4)$$

2.2.5. Colour attribute of cake produced from wheat-tigernut pomace flour blends

Crust and crumb colour measurement was measured by the method described by Feili *et al.* (2013). Minolta chroma meter was used based on (CIE) L*a*b* scale. After calibrating the instrument by covering a zero-calibration mask followed by white calibration plate, crust and crumb was analyzed by placing them on the petri dish and the image was captured on the samples. The colour attributed such as lightness (L*) and (0 = black and 100 = white) and chromatically coordinated (a* corresponds to the colour range from red-green coordinates (- is given, while + is red)), (b* corresponds to the colour range from blue-yellow coordinates (- is blue with + indicating yellowness) was recorded.

2.2.6. Sensory evaluation of cake produced from wheat-tigernut pomace flour blends

The method described by Iwe (2002) was used. The sensory panel consisted of 50 members who were familiar with the product and they were asked to score the Cake using a 9-point hedonic scale based on their degree of likeness where 9 = like extremely; 5 = neither like nor dislike; 1 = dislike extremely. Cake quality attributes evaluated are: Crust colour, Crust texture, aroma, taste, and overall acceptability.

2.3. Statistical analysis

All experimental data obtained were done in triplicate, which equal to the sum of fifty one (51) samples. Data obtained were subjected to means, analysis of variance using SPSS version 21.0 and the difference between the mean values were evaluated at $p<0.05$ using Duncan multiple range test. The effect of optimization procedure was investigated using Design expert based on Box Behnken design and significant effects of the independent variables were determined at 5% confidence level.

3. Results and discussion

3.1. Physical properties of cake produced from wheat-tigernut pomace flour blends

The physical properties of cake produced from wheat-tigernut pomace blends are presented in Table 3. Weight basically is determined by the amount of dough baked and the amount of moisture and carbon dioxide diffused out of during baking (Shittu *et al.,* 2007; Oke *et al.,* 2017a). The weight of the cake ranged between 50.75 g and 69.08g. From the regression coefficient table (Table 4), the main effect of baking temperature, baking time and tigernut pomace and the quadratic effect of tigernut pomace had a significant effect ($p<0.05$) on the weight while the quadratic effect of baking time had a negative effect on the weight.

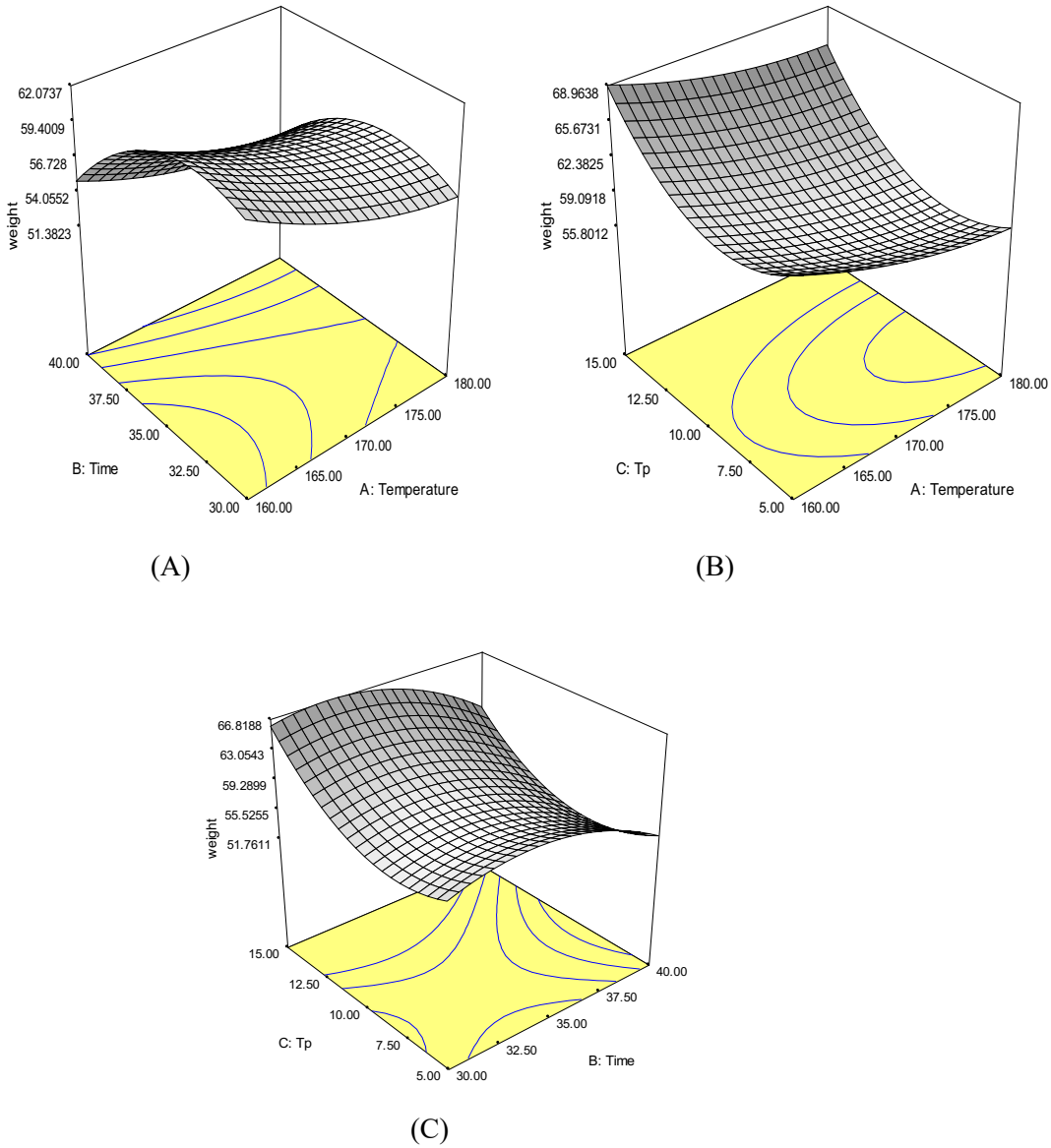


Figure 1. Response surface plot for weight of cake from wheat-tigernut pomace flour blends

The response surface plot for the weight (Figure 1) reveals that increase in baking time and baking temperature when tigernut pomace was held constant led to a decrease in weight. Also, increase in baking temperature and tigernut pomace when baking time was held constant resulted to decrease in weight and while the baking temperature was held constant, increase in baking time and tigernut pomace led to decrease in weight. This can be attributed to lower level of gluten network in the dough and therefore decreasing the ability of the dough to rise (Aluko and Olugbemi, 1989; Oke *et al.,* 2017a).

Table 3. Physical properties of cake produced from wheat- tigernut pomace flour blends

Baking Temperature (°C)	Baking Time (minutes)	TPF (%)	Weight (g)	Oven spring (cm)	Volume (cm^3)	Specific volume (cm^3/g)	Density (g/cm^3)
170	35	10	57.96^{abcd}	2.80^{e}	450^{abc}	7.76^{bc}	0.13^{ab}
180	40	10	50.75^{a}	2.60^{c}	600^{de}	11.82^{g}	0.09^{a}
160	40	10	53.72^{ab}	2.60^{c}	550^{cd}	11.17^{fg}	0.09^{a}
180	35	5	57.33^{abc}	2.85^{e}	450^{abc}	7.85^{bc}	0.13^{ab}
170	35	10	57.96^{abcd}	2.80^{e}	450^{abc}	7.76^{bc}	0.13^{ab}
180	35	15	64.74^{de}	2.70^{d}	350^{a}	5.41^{a}	0.19^{b}
160	35	15	69.08^{e}	2.45^{b}	600^{de}	8.69^{bcd}	0.12^{ab}
170	35	10	57.96^{abcd}	2.80^{e}	450^{abc}	7.76^{bc}	0.13^{ab}
170	35	10	57.96^{abcd}	2.80^{e}	450^{abc}	7.76^{bc}	0.13^{ab}
170	40	15	60.57^{bcde}	2.35^{a}	600^{de}	8.50^{bcd}	0.12^{ab}
160	30	10	61.79^{bcde}	2.30^{a}	450^{ab}	7.28^{b}	0.08^{a}
180	30	10	56.29^{abcd}	2.70^{d}	400^{ab}	7.30^{b}	0.14^{ab}
160	35	5	63.54^{cde}	3.15^{f}	600^{de}	9.44^{cde}	0.11^{a}
170	40	5	54.94^{abc}	2.50^{b}	550^{cd}	10.01^{def}	0.10^{a}
170	30	15	65.16^{abcd}	2.30^{d}	700^{bcd}	10.74^{bcd}	0.09^{a}
170	35	10	57.96^{abcd}	2.80^{e}	450^{abc}	7.76^{bc}	0.13^{ab}
170	30	5	56.69^{de}	2.70^{a}	500^{e}	8.87^{efg}	0.11^{a}

TPF: Tigernut pomace flour; values are means of triplicate determination. Mean values with different superscripts within the same column are significantly different ($p < 0.05$)

Table 4. Regression coefficient of physical attribute of cake produced from wheat-tigernut pomace flour blends

Parameters	Weight	Oven spring	Volume	Specific volume	Density
β_o	57.96	2.80	450.00	7.76	0.13
A	-2.38*	0.044	-50.00	-0.52	0.019*
B	-2.49*	6.250E-003	31.25	0.91	-.2.500E-003
C	3.38*	-0.17*	18.75	-0.35	8.750E-003
A^2	1.00	0.038	-18.75	-0.025	1.250E-003
B^2	-3.33*	-0.29*	68.75	1.66	-0.031*
C^2	4.71*	-0.050	68.75	0.11	6.250E-003
AB	0.63	-0.100	25.00	0.16	-0.015

AC	0.47	0.14*	-25.00	-0.42	0.013
BC	-0.71	0.063	-37.50	-0.85	1.000E-002
R^2	0.978	0.936	0.623	0.602	0.89
F-VALUE	35.02	11.38	1.29	1.18	6.36

* = Significant difference ($p<0.05$); β_o= Intercept, A =main effect of temperature, B = main effect of baking time, C = main effect tigernut pomace flour, A^2 = Quadratic effect of temperature, B^2= Quadratic effect of time, C^2 = Quadratic effect of tigernut pomace flour, AB = Interaction effect of temperature and time, AC =Interaction effect of temperature and tigernut pomace flour, BC = Interaction effect time and tigernut pomace flour, R^2= coefficient of determination

Volume is one of the main quality characteristics of baked product and it is negatively affected when wheat is replaced with composite flour. Volume is affected by the amount and quality of protein in the flour (Ragaee and Abdel-Aal, 2006) as well as proofing time (Zghal*et al.,* 2002). The cake volume ranged from 350cm^3 to 700cm^3.

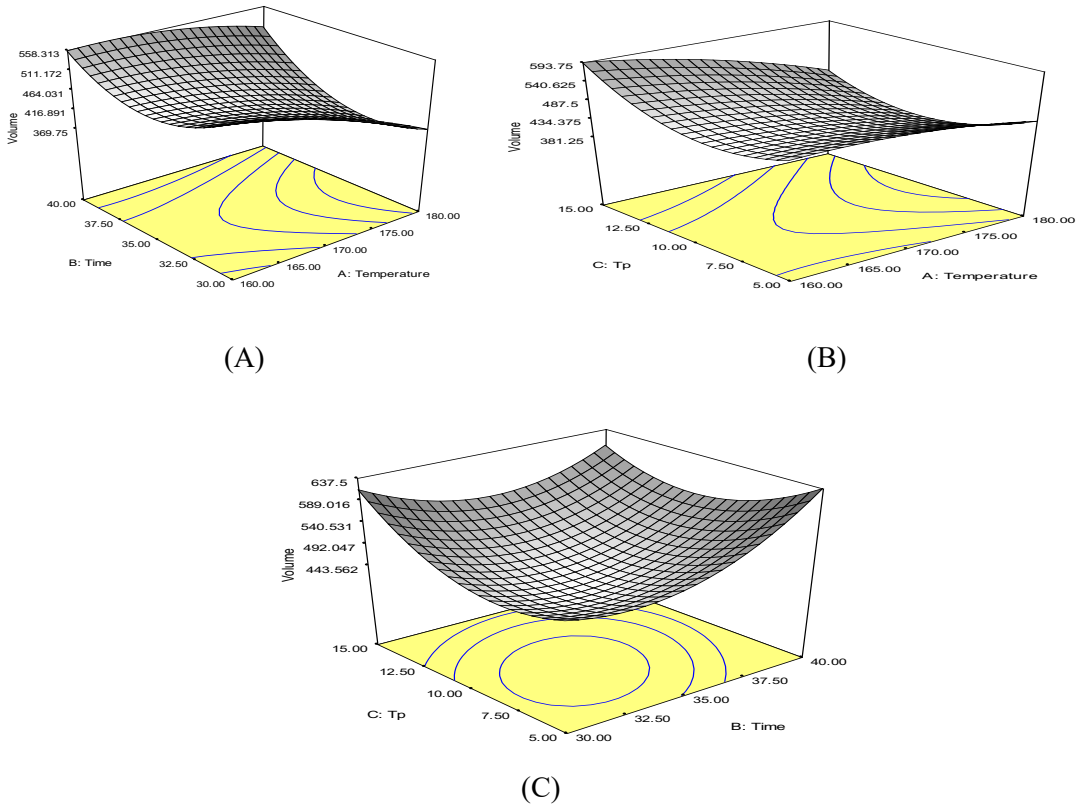


Figure 2. Response surface plot for volume of cake from wheat-tigernut pomace flour blends

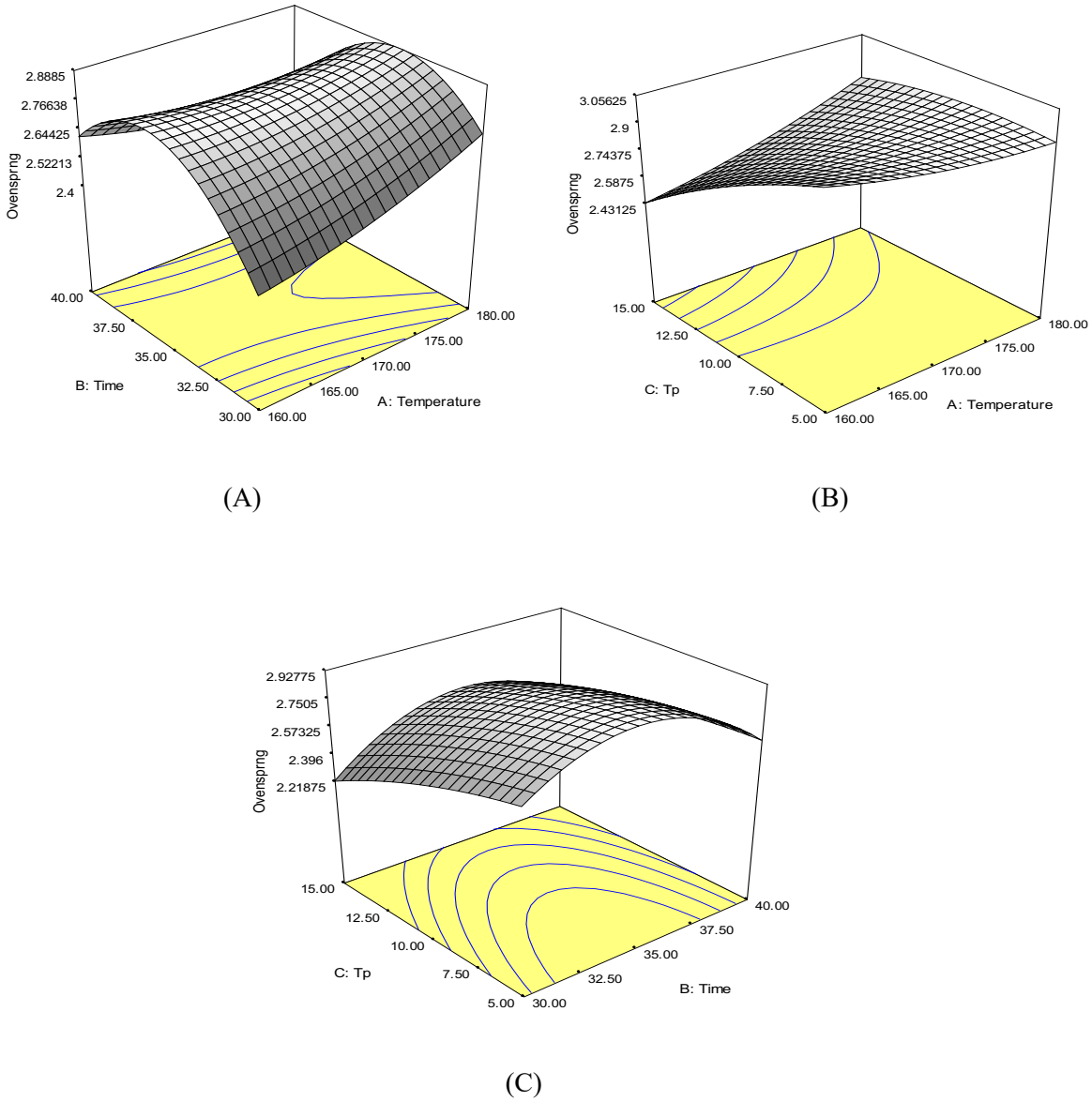


Figure 3. Response surface plot for oven spring of cake from wheat-tigernut pomace flour blends

The volume of the wheat-tigernut pomace cake showed no definite trend but varied significantly ($p<0.05$) from each other. However, the response surface plot for the cake volumes (Figure 2) reveals that increase in the baking time and baking temperature when tigernut pomace was held constant led to a decrease in cake volume. Increasing baking temperature and tigernut pomace led to decrease in cake volume when baking time was held constant while increasing baking time and tigernut pomace when baking temperature was held constant led to an increase in the volume of the cake. From the regression coefficient table, the main, quadratic and interaction effect had no significant effect ($p>0.05$) on the volume of wheat-tigernut pomace cake. The variation in the volume of the cake could be as a result of high fibre in the tigernut pomace flour causing gluten dilution and as a result affecting the gluten matrix and baking. However, other researchers such as Wang *et al.*

(2002) reported that fibre addition in baked product such as bread could cause reduction in loaf volume.

Cake oven spring is a measure of the cake expansion in volume (difference in the height of dough just before and after baking) (Idowu *et al.,* 1996). It measures the dough strength against heat related changes that take place in the oven (Akobundu *et al.,* 1998).The cake oven spring ranged from 2.3 cm to 3.5cm. From the regression coefficient (Table 4), the quadratic model developed for the oven spring had coefficient of determination (R^2) of 0.939 and F-value of 11.38 as shown in Table 4. The interaction effect of baking temperature and tigernut pomace had a significant effect ($p<0.05$) on the cake oven spring while the main effect of tigernut pomace and the quadratic effect of baking time had a negative effect on the oven spring. The response surface plot for cake oven spring (Figure 3) shows that when tigernut pomace was held constant, increase in baking temperature and baking time led to increase in oven spring. Increase in baking temperature and tigernut pomace when baking time was held constant led to a decrease in the oven spring while increasing baking time and tigernut pomace when baking temperature was held constant led to a decrease in oven spring. However, oven spring obtained in this study was higher than the values of 0.36 to 1.09 reported by Oke *et al.* (2017a) for wheat flour substituted with tigernut flour for bread. As the substitution increased, there was decrease in the oven spring, and this can be attributed to the reduction in the amount of gluten and a lower ability of the dough to enclose air (Akobundu *et al.,* 1998).

3.2. Colour attribute of cake produced from wheat-tigernut pomace flour blends

3.2.1. Crumb and crust colour

The values of the tristimulus colour parameters L*, a* and b* of the cake crumb as affected by various temperature–time combination in baking are shown in Table 5.

Table 5. Crumb colour of cake produced from wheat- tigernut pomace flour blends

Baking Temperature (°C)	Baking Time (mins)	TPF (%)	(CRUMB) L*	a*	b*
170	35	10	59.69^{e}	1.47^{f}	27.85^{b}
180	40	10	55.91^{b}	2.31^{i}	27.01^{b}
160	40	10	57.28^{c}	2.04^{h}	27.34^{b}
180	35	5	61.03^{f}	1.30^{e}	30.92^{c}
170	35	10	59.69^{e}	1.47^{f}	27.85^{b}
180	35	15	54.86^{a}	2.54^{j}	25.62^{a}
160	35	15	55.84^{b}	2.00^{h}	24.65^{a}
170	35	10	59.69^{e}	1.47^{f}	27.85^{b}
170	35	10	59.69^{e}	1.47^{f}	27.85^{b}
170	40	15	56.88^{c}	2.26^{i}	25.55^{a}
160	30	10	60.91^{f}	1.23^{d}	27.36^{b}
180	30	10	58.23^{d}	1.82^{g}	27.19^{b}
160	35	5	65.84^{h}	0.08^{a}	31.77^{c}
170	40	5	64.25^{g}	0.70^{c}	31.32^{c}
170	30	15	57.24^{i}	2.04^{b}	25.71^{c}
170	35	10	59.69^{e}	1.47^{f}	27.85^{b}
170	30	5	66.77^{c}	0.16^{h}	30.80^{a}

TPF: Tigernut pomace flour, values are means of triplicate determination. Mean values with different superscripts within the same column are significantly different ($p < 0.05$); L*= lightness, a*= Redness, b*= yellowness

Table 6. Regression coefficient of crumb colour of cake from wheat- tigernut pomace flour blends

CRUMB			
Parameter	L*	a*	b*
β_o	59.69	1.47	27.85
A	-1.23*	0.33*	-0.047
B	-1.10*	0.26*	0.020
C	-4.13*	0.83*	-2.91*
A^2	-1.75*	0.29*	-0.37*
B^2	0.14	0.095	-0.26
C^2	1.45*	-0.27*	0.76*
AB	0.33	-0.080	-0.740
AC	0.96*	-0.17	0.46*
BC	0.54	-0.080	-0.17
R^2	0.991	0.981	0.995
F-VALUE	88.45	4.43	142.67

* = Significant difference ($p<0.05$); β_o= Intercept, A =main effect of temperature, B = main effect of baking time, C = main effect tigernut pomace flour, A^2 = Quadratic effect of temperature, B^2= Quadratic effect of time, C^2 = Quadratic effect of tigernut pomace flour, AB = Interaction effect of temperature and time, AC =Interaction effect of temperature and tigernut pomace flour, BC = Interaction effect time and tigernut pomace flour, R^2= coefficient of determination, L*= lightness, a*= Redness, b*= yellowness

Table 7. Crust colour of cake produced from wheat - tigernut pomace flour blends

Baking Temperature (°C)	Baking Time (minutes)	TPF (%)	(CRUST)		
			L*	a*	b*
170	35	10	53.31^{i}	11.00^{c}	32.32^{i}
180	40	10	36.71^{a}	12.6^{g}	19.96^{a}
160	40	10	49.62^{g}	12.96^{h}	31.04^{g}
180	35	5	37.9^{b}	13.28^{i}	21.33^{c}
170	35	10	53.31^{i}	11.00^{c}	32.32^{i}
180	35	15	36.97^{a}	12.61^{g}	20.68^{b}
160	35	15	45.4^{d}	12.35^{f}	27.72^{d}
170	35	10	53.31^{i}	11.00^{c}	32.32^{i}
170	35	10	53.31^{i}	11.00^{c}	32.32^{i}
170	40	15	47.45^{e}	11.9^{e}	28.94^{e}
160	30	10	48.63^{f}	11.55^{d}	29.01^{e}

180	30	10	39.26^{b}	13.00^{h}	21.73^{c}
160	35	5	50.07^{g}	12.39^{f}	31.64^{h}
170	40	5	47.96^{ef}	13.32^{i}	30.33^{f}
170	30	15	51.51^{j}	10.67^{a}	30.18^{j}
170	35	10	53.31^{i}	11.00^{c}	32.32^{i}
170	30	5	86.36^{h}	9.76^{b}	34.51^{f}

TPF: Tigernut pomace, values are means of triplicate determination.Mean values with different superscripts within the same column are significantly different ($p < 0.05$), L*= lightness, a*= Redness, b*= yellowness.

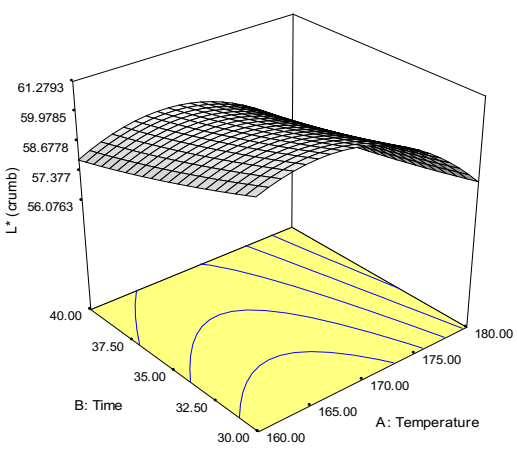
Table 8. **Regression coefficient of crust colour of cake from wheat- tigernut pomace flour blends**

Parameter	CRUST		
	L*	a*	b*
β_o	53.31	11.00	32.32
A	-5.36	0.28	-4.46*
B	-5.50	0.73*	-0.64
C	-5.12	-0.15	-1.29*
A^2	-12.76*	1.39*	-6.27
B^2	2.99	0.14	-0.62
C^2	2.02	0.27	-0.71
AB	-0.89	-0.45	-0.95*
AC	0.93	-0.16	0.82
BC	8.59*	-0.58	0.74
R^2	0.841	0.900	0.988
F-VALUE	4.13	7.00	64.48

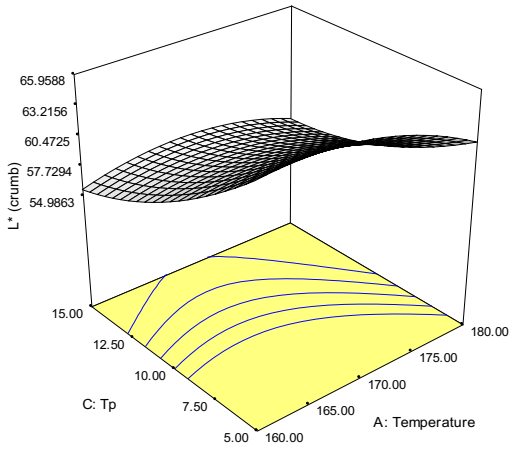
* = Significant difference ($p<0.05$); β_o= Intercept, A =main effect of temperature, B = main effect of baking time, C = main effect tigernut pomace flour, A^2 = Quadratic effect of temperature, B^2= Quadratic effect of time, C^2 = Quadratic effect of tigernut pomace flour, AB = Interaction effect of temperature and time, AC =Interaction effect of temperature and tigernut pomace flour, BC = Interaction effect time and tigernut pomace flour, R^2= coefficient of determination, L*= lightness, a*= Redness, b*= yellowness

Colour is an important quality attribute in the food and bioprocess industries, and it influences consumer's choice and preferences. Food colour is governed by the chemical, biochemical, microbial and physical changes which occur during growth, maturation, postharvest handling and processing (Pathare *et al.,* 2013). It is one of the parameters used for process control during baking and roasting, because brown pigments appear as browning and caramelization reactions progress (Pereira *et al.,* 2013). The values for lightness, redness and yellowness of wheat-tigernut pomace cake crumb ranged from 55.84 to 66.77, 0.16 to 2.54 and 24.65 to 31.77 respectively. The values obtained in this study were within the range obtained in previous works for various baked products such as those reported by Oke *et al.* (2017a) which ranged from 74.63 to 83.50, 1.39 to 3.39 and 27.08 to 30.79 respectively. From the regression coefficient table (Table 6), the main effect of baking temperature, baking time and tigernut pomace and also the quadratic effect of baking temperature negatively

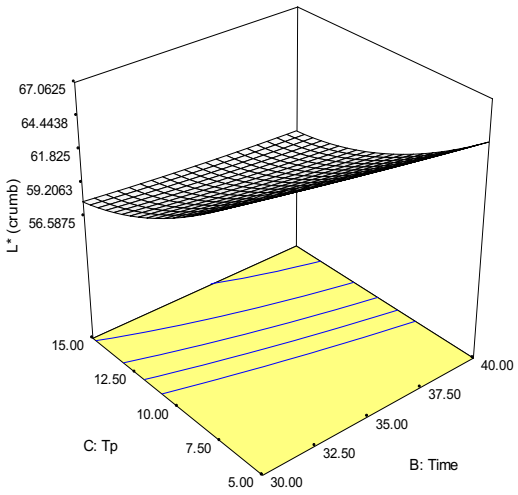
affected the crumb lightness. The quadratic effect of tigernut pomace and also the interaction effect of baking temperature and tigernut pomace were significant ($p<0.05$) on the crumb lightness. The regression coefficient table also reveals that the main effect of baking temperature, baking time and tigernut pomace and the quadratic effect of baking temperature were significant ($p<0.05$) while the quadratic effect of tigernut pomace had a negative effect on the crumb redness. Figure 4-6 reveals the response surface plots for the crumb colour of cake produced from wheat-tigernut pomace blends.



(A)

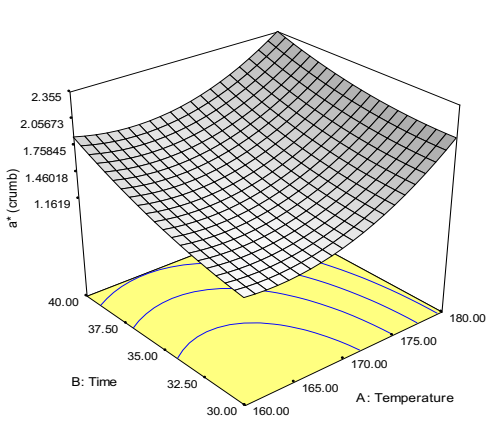


(B)

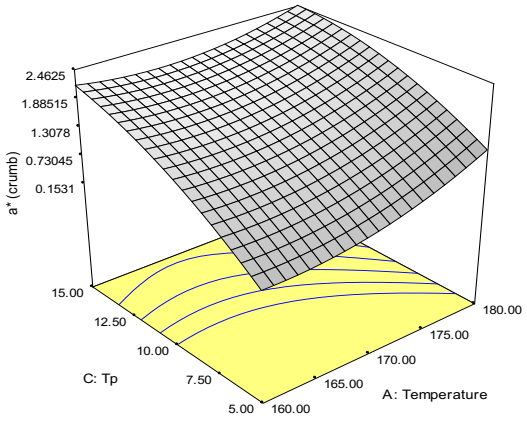


(C)

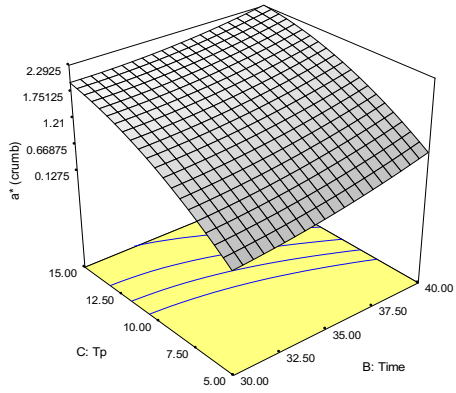
Figure 4. Response surface plot for lightness (L*) of crumb cake from wheat-tigernut pomace flour blends



(A)

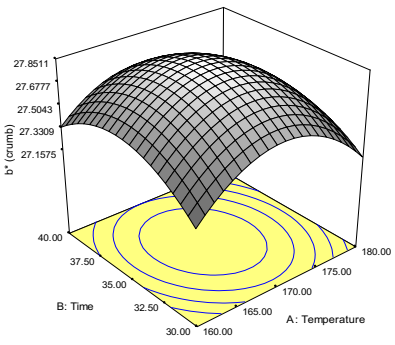


(B)

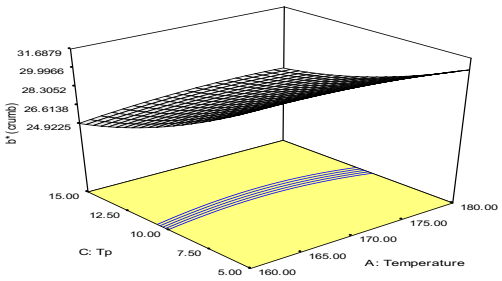


(C)

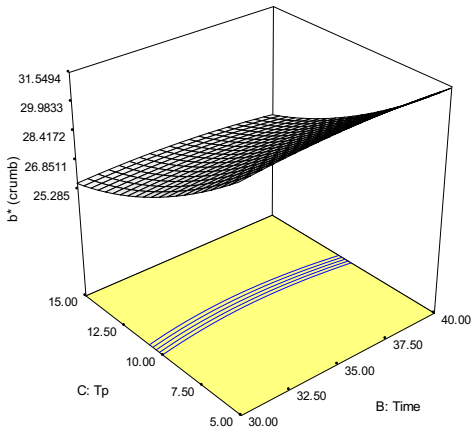
Figure 5. Response surface plot for redness (a*) of crumb cake from wheat-tigernut pomace flour blends



(A)

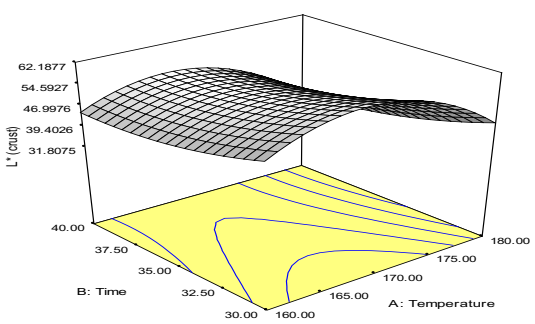


(B)

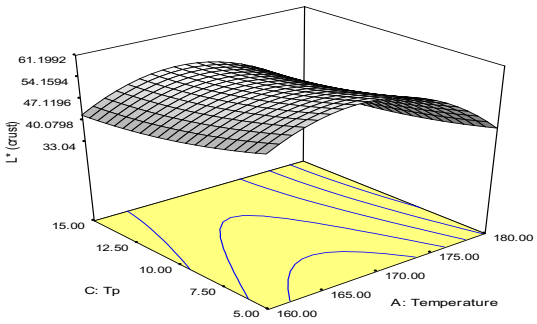


(C)

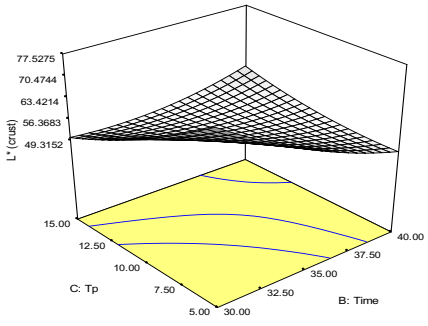
Figure 6. Response surface plot for yellowness (b*) of crumb cake from wheat-tigernut pomace flour blends



(A)

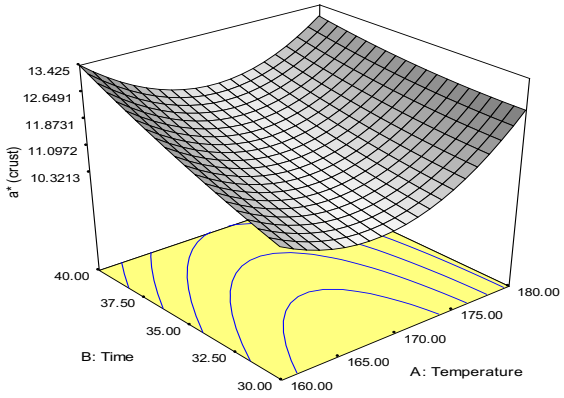


(B)

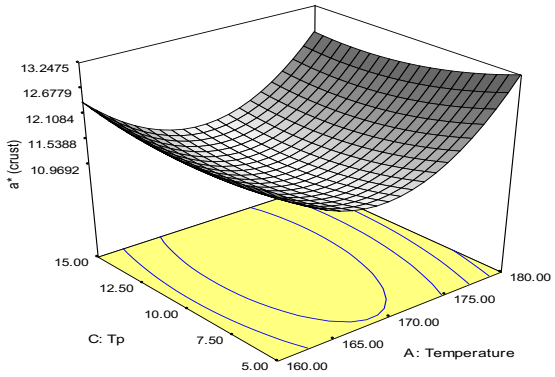


(C)

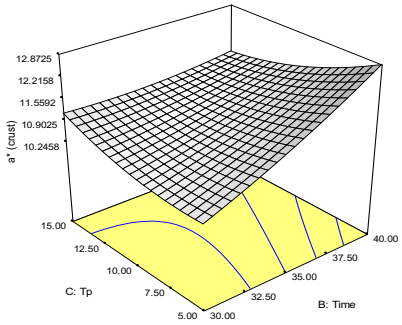
Figure 7. Response surface plot for lightness (L*) of crust cake from wheat-tigernut pomace flour blends



(A)

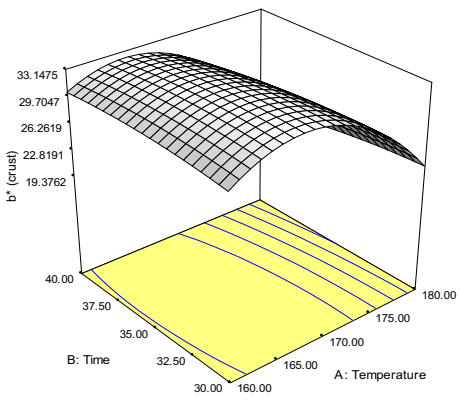


(B)

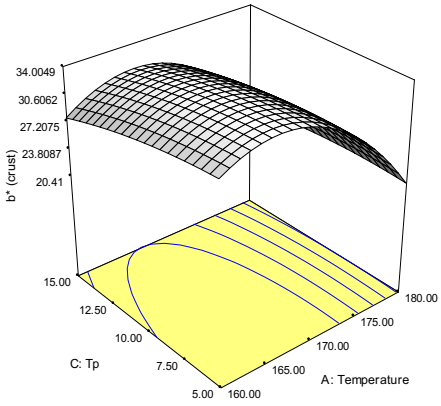


(C)

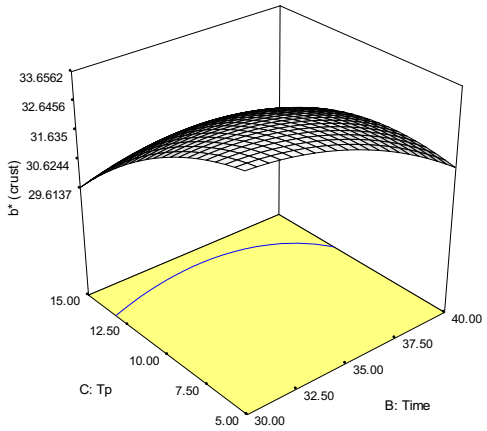
Figure 8. Response surface plot for redness (a*) of crust cake from wheat-tigernut pomace flour blends



(A)



(B)



(C)

Figure 9. Response surface plot for yellowness (b*) of crust cake from wheat-tigernut pomace flour blends

The mean values of crust lightness, redness and yellowness of cake produced from wheat - tigernut pomace colour ranged from 36.71 to 86.36, 9.76 to 13.32 and 19.96 to 34.51 respectively as shown in Table 7. From the regression (Table 8), the interaction effect of time and tigernut pomace had a significant ($p<0.05$) effect on the crust lightness while the quadratic effect of temperature had a negative effect on the crust lightness. The main effect of temperature and tigernut pomace and also the interaction effect of temperature and time had a significant ($p<0.05$) effect on the yellowness of the cake crust negatively. However, as tigernut pomace level increased, the crust colour of the cake became dark brown. The dark brown colour of the cake could be attributed to an increased maillard reaction taking place during baking due to higher lysine content (Abdelrahman, 2014). In the maillard reaction reducing carbohydrates react with free amino acid side chain of protein mainly lysine and lead to amino acid–sugar reaction products (polymerized protein and brown pigments). However, Amir *et al.* (2013) reported that temperature, time and moisture could influence the maillard reaction. Figure 7,8,9 shows the response surface plots for lightness, redness and yellowness of crust colour of cake produced from wheat - tigernut pomace

3.3. Sensory scores of cake produced from wheat-tigernut pomace blends

The mean sensory scores for the cake produced from the wheat-tigernut pomace blends are presented in Table 9. The colour ranged from 5.92 to 7.62. This shows that cake baked from the combination was more appealing to the panelists. The crumb texture of the cake ranged from 5.60 to 7.74 with cake baked at 170°C for 40mins and inclusion of 15% tigernut pomace having the highest score while cake baked at 160°C for 35mins and inclusion of 5% tigernut pomace had the least score. The aroma values for the cakes were significantly different ($p<0.05$). The result showed that cake baked at 160°C for 35mins and inclusion of 5% tigernut pomace had the best aroma and was more preferred by the panelists. The taste ranged from 5.96 to 6.94 with cake baked at 160°C for 40mins and inclusion of 10% tigernut pomace having the highest score for taste and cake baked at 170°C for 40mins and inclusion of 15% tigernut pomace having the lowest score. The overall acceptability of the cake ranged from 5.86-7.86 with cake baked at 160ºC for 35 mins at 5%

tigernut pomace inclusion having the highest while cake baked at 170ºCfor 40 minutes at 15% tigernut pomace inclusion having the lowest in terms of overall acceptability. The result of the sensory evaluation revealed that cakes from wheat flour with low amount of tigernut pomace and those baked at lower temperature and time were rated alike in almost all the sensory attributes evaluated, indicating the feasibility of adding tigernut to baked goods.

Table 9. Sensory score of cake from wheat- tigernut pomace flour blends

Baking Temperature (°C)	Baking Time (minutes)	TPF (%)	Colour	Texture	Aroma	Taste	Overall acceptability
170	35	10	6.28^{abc}	6.06^{abcd}	5.92^{abc}	6.36^{abc}	6.28^{abc}
180	40	10	6.18^{abc}	5.76^{ab}	5.51^{ab}	6.41^{abc}	6.02^{ab}
160	40	10	6.98^{ef}	6.46^{defg}	6.50^{d}	6.94^{d}	6.64^{cd}
180	35	5	6.44^{bcd}	6.24^{bcdef}	5.62^{ab}	6.22^{ab}	6.00^{ab}
170	35	10	6.54^{bcd}	6.32^{cdefg}	5.68^{ab}	6.26^{ab}	6.08^{ab}
180	35	15	6.14^{ab}	5.94^{abc}	5.98^{bc}	6.52^{bcd}	6.22^{abc}
160	35	15	7.16^{fg}	6.66^{fg}	6.34^{cd}	6.88^{cd}	6.92^{d}
170	35	10	6.22^{abc}	5.98^{abcd}	5.98^{bc}	6.52^{bcd}	6.22^{abc}
170	35	10	6.20^{abc}	5.76^{ab}	5.46^{ab}	6.40^{abc}	6.02^{ab}
170	40	15	5.92^{a}	5.60^{a}	5.56^{ab}	5.96^{a}	5.86^{a}
160	30	10	7.52^{gh}	7.32^{h}	7.54^{e}	7.54^{e}	7.58^{e}
180	30	10	5.92^{a}	5.74^{ab}	5.66^{ab}	6.20^{ab}	6.00^{ab}
160	35	5	7.62^{h}	7.74^{h}	7.64^{e}	7.60^{e}	7.86^{e}
170	40	5	6.44^{bcd}	6.16^{bcde}	5.90^{abc}	6.40^{abc}	5.94^{ab}
170	30	15	6.64^{cde}	6.56^{efg}	6.30^{cd}	6.62^{bcd}	6.46^{bc}
170	35	10	6.34^{abcd}	6.06^{abcd}	5.40^{a}	6.12^{ab}	5.90^{a}
170	30	5	6.76^{def}	6.78^{g}	6.40^{cd}	6.52^{bcd}	6.44^{bc}

TPF: Tigernut pomace flour; Mean values with different superscripts within the same column are significantly different

4. Conclusions

The addition of tigernut pomace into wheat flour for cake baking had significant effect on the baking and sensory qualities of cake. The resultant effect of the baking temperature, baking time and tigernut pomace significantly affected the cake oven spring and led to decrease in cake weight. The lightness, redness and yellowness of the cake samples varied significantly among different levels of the substitution. The sensory evaluation revealed that cakes produced from the combination had desirable aroma, taste, crust colour, crumb texture and overall acceptability. However, cake can be baked from wheat-tigernut pomace flour blends between160-170°C for 30-35 minutes with inclusion of 10% tigernut pomace flour to yield cake of enhanced quality attributes especially volume, colour, texture and overall acceptability. Further studies should be carried out on the proximate and storage stability of cake from wheat-tigernut pomace.

5. References

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EFFECT OF UV-C IRRADIATION ON MICROBIAL LOAD AND PHENOLIC CONTENT OF POTATO TUBERS AND SLICES

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ABSTRACT

The effect of different UV-C irradiation doses on tubers and sliced potato (with or without sample's turning during irradiation) on aerobic mesophilic bacteria count (AMB), phenolic composition and visual appearance of potatoes after 24 h storage at 10 °C was examined. Doses of UV-C irradiation were 1.08, 2.70, 5.40 and 10.08 kJ m^{-2} for tubers and 1.08, 1.62 and 2.70 kJ m^{-2} for potato slices. UV-C irradiation, particularly with slice turning, caused the significant reduction of the AMB in comparison with untreated potatoes in accordance to the irradiation dose. Further, it caused a significant increase of phenolic substances in all samples, especially in tubers, although inversely to the irradiation dose. Independently of the applied treatment, chlorogenic acid, caffeic acid and rutin were identified in all samples where chlorogenic acid was predominant. Generally, the turning of potatoes during irradiation had a significant effect on the increase of chlorogenic acid in potato slices. Samples with higher phenolic content were more prone to browning. Based on all obtained results UV-C treatment of potato slices with dose 1.62 - 2.70 kJ m^{-2} and with turning could be recommended as the most promising treatment.

1. Introduction

Nowadays consumers prefer minimally processed, highly nutritional, safe, and attractive food, in the same time being convenient for easy and fast use and meals preparation, e.g., „ready to eat" or „ready to use" foods. Therefore, popularity and market demand for minimally processed, fresh-cut fruits and vegetables (FCFV) have been constantly increased. Minimal processing includes operations such as peeling and cutting, which cause tissue damage, thus lead to the quality deterioration, and reduced shelf-life (Rico *et al*., 2007). Consequently, fresh-cut products have shorter shelf-life compared to the intact fruits or vegetables (Gutierrez *et al*., 2017), where potato presents an excellent example regarding this matter. The loss of the natural protection as well as the high water content along with the presence of several nutrients are responsible for the main undesirable alterations in FCFV such as increased metabolic activity, enzymatic browning and microbiological growth (Gutierrez *et al.,* 2009), which additionally could raise the risk of foodborne diseases (Manzocco *et al*., 2011). The main challenge of minimal processing industry is to obtain a safe and high-quality product as well as its prolonged shelf-life (Gutierrez *et al.* 2017), therefore the interest for the application of new efficient technologies is being raised (Gonzales-Aguilar *et al*., 2010). Currently, the main tools in maintaining the quality and safety of FCFV are the utilization of appropriate packaging in modified atmosphere and refrigeration (Gutierrez *et al.*, 2017), but various approaches have been examined to improve the shelf-life of FCFV, including many non-thermal

technologies (Ma *et al.,* 2017). Primary advantages of ultraviolet irradiation (UV-C), responsible for considering it as the promising non-thermal technique, are its efficacy on various types of microorganisms, no residue in the treated food, easy manipulation and not too large equipment investments (Yaun *et al*., 2004; Ma et al. 2017; Koutchama, 2019). UV-C irradiation can reduce the microorganism's growth, control browning (Guan *et al*. 2013), and inhibits peroxidase (POD) activity (Teoh *et al*. 2016) as well as increases the resistance to rot and extend the shelf life (Jakubowski, 2019). Further, UV-C treatment has been associated with an increase in bioactive compounds such as vitamins, carotenoids and phenolic compounds in several tested fruits and vegetables, e.g., tomatoes (Jagadeesh *et al*., 2011; Maharaj *et al*. 2014), strawberries (Erkan *et al.,* 2008; Xie *et al*., 2014), carrots (Alegria *et al.,* 2012) and broccoli (Costa *et al.*, 2006). However, several authors observed the UV-C negative influence with elongated UV-C exposure time, e.g., browning in pineapple slices during storage and decrease of vitamin C content (Pan & Zu, 2012) as well as plant tissue damage, increase stress and respiration rate (Rico *et al*., 2007). Hence, the use of non-ionizing, germicidal UV-C irradiation can be effective if the optimal radiation dose for each product is previously examined and determined.

Therefore, the aim of this study was to examine the influence of germicidal UV-C irradiation dose and treatment conditions on microbial load, phenolic compounds and susceptibility to browning of potato tubers and slices.

2. Materials and methods

2.1. Samples

Tubers of cultivar Birgit (*Solanum tuberosum* L.), grown in Slavonia region (Croatia) during 2018 were used for the experiment. Prior to analysis, tubers were stored in dark room at 8 °C with a relative humidity approximately 100%.

2.1.1. Sample preparation

Undamaged potato tubers were selected, washed with tap water and dried with paper towels. One batch was peeled, again washed, surface moisture was removed by paper towels and then cut into slices 0.4 cm wide (potato slices, PS) using an electric cutter (SFS 1001 GR, Sencor, Czech Republic). Prepared samples of potato tubers (PT) and PS were further subjected to UV-C treatment in an UV-C chamber (UVpro EKB 100, assembled by Aura Steril, Jalkovec, Varaždin, Croatia) equipped with 2 pairs of UV-C lamps (UVpro, 4xHNSL 24W) situated above and below perforated shelf (Figure 1). The dose of irradiation was measured using UV-C radiometer (UVCpro UVC-LOG, Orca GmbH, Germany). PT were exposed to UV-C irradiation for 0 (control), 2, 5, 10 and 20 min (doses were 0, 1.08, 2.70, 5.40 and 10.08 kJ m^{-2}, respectively) and PS for 0 (control), 2, 3 and 5 min (doses were 0, 1.08, 1.62 and 2.70 kJ m^{-2}, respectively) without or with turning halfway through the treatment (without opening the chamber) to ensure uniform radiation exposure. The samples were arranged on the perforated shelfs on the positions with uniform radiation, what was previously established. Each treatment was performed in triplicates. After the treatment, PT were peeled and sliced, and PT slices as well as PS were placed on a plate and stored in refrigerator at 10 °C for 24 h. They were photographed before and after storing to visually monitor the color stability. For phenolic extraction and analysis all samples were freeze-dried, grounded into powder and kept on -20 °C until extraction.

2.2. *Microbiological analysis*

Total mesophilic count, AMB, was performed according to the HRN EN ISO 4833-1:2013 (ISO 4833-1: 2013, EN ISO 4833-1:2013). Immediately after the treatment 10 g of sample (PT or PS) was mixed with 90 ml peptone saline solution in a sterile stomacher bag and homogenized for 1 min using a Stomacher. Dilutions were made in peptone water as needed for plating.

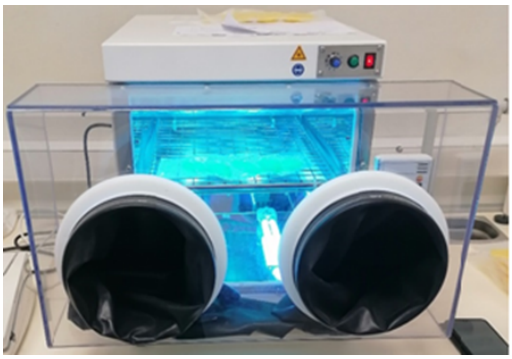


Figure 1. UV-C (UVpro EKB 100, assembled by Aura Steril, Jalkovec, Varaždin, Croatia)

Plate Count Agar (Biolife, Milan, Italy) was used as the media for AMB pour plate, incubated at 30±1 °C for 72±3 h.

2.3. Phenolic compounds

2.3.1. Chemicals and standards

Methanol and formic acid were HPLC grade (Sigma Aldrich, Milano, Italy). Chlorogenic acid (CHA) and caffeic acid (CA) were purchased from Sigma–Aldrich (Steinheim, Germany) and rutin (RUT) from Acros Organics (Thermo Fisher Scientific, Geel, Belgium).

2.3.2. Extraction of phenolic compounds

Extraction was performed according to Elez Garofulić and co-workers (2018) with modifications. Briefly, 0,5 g±0,01 g of potato powder was homogenized on Vortex with 5 ml 80% (v/v) aqueous methanol + 1% formic acid. Extraction was hold in the ultrasonic bath (Elmasonic 40H, Elma, Germany) at 50 °C for 30 min, followed by centrifugation at 3000 rpm for 10 min. Supernatant was filtrated in volumetric flask and made up to 10 ml with a matching solvent. Extract (1 ml) was filtered through 0.45-µm pore size membrane filter and stored at -20 °C until analysis.

2.3.3. UPLC MS^2 analysis

An Agilent series 1290 RRLC instrument (Agilent Technologies, Santa Clara, CA, USA) with an Agilent triple quadrupole mass spectrometer (6430) and an ESI ion source in the negative and positive mode (m/z 100 to 1000) with the data collected in the dMRM mode was used for the identification and quantification of phenolic content with followed settings: Agilent Eclipse Plus C18 column (100 mm × 2.1 mm, 1.8 µm particle size), temperature 35 °C, the injection volume 2.5 µL, flow rate 0.3 mL min-1 at chromatographic conditions previously described by Elez Garofulić and co-workers (2018). The eluent A contained 0.1% of formic acid and eluent B contained 0.1% formic acid in acetonitrile. Identification and quantification were done by related calibration curves.

2.4. Statistical analysis

Statistical analysis was performed using Statistica ver. 8.0 software (Statsoft Inc., Tulsa, USA). Variables were analyzed by multivariate analysis of variance (MANOVA), while differences between specific group means were determined with Tukey's HSD test. The significance level for all tests was $p \leq 0.05$.

3.Results and discussion

3.1. Microbiological analysis

Prior to UV-C irradiation, the microbial load of AMB was 3.41 $\log_{10}$ CFU g^{-1} in PT and 3.36 $\log_{10}$ CFU g^{-1} in PS (Figure 2). The AMB reduction during irradiation was remarkably higher in PS in comparison with PT. Specifically, to achieve AMB reduction similar to PS, application of higher doses was required for PT. The irradiation time required to reduce AMB by 1.5 $\log_{10}$ CFU g^{-1} in PS was 2 min, while in PT a reduction of 1 $\log_{10}$ CFU g^{-1} was achieved during 10 min irradiation, probably as a consequence of the surface topography (Koutchma *et al*., 2019). Turning samples during UV-C irradiation significantly influenced on the AMB in PS, but not in PT. One of the main advantages of UV-C treatment is inactivation/reduction of microbial load, attributed to the photochemical lesion induced on microorganisms' DNA and RNA, which results in the inability to regularly transcribe and replicate nucleic acids and microorganisms' cells die (Koutchma *et al*., 2019).

3.2. Phenolic content

CHA was the most abundant phenolic compound in all samples, followed by remarkably lower content of CA and RUT (Table 1), what was in accordance to Deußer and co-workers (2012) results as well as its concentrations which were in reported ranges. UV-C treatment significantly influenced on the content of CHA and CA in PT and PS. Generally, increase of phenolics with the treatment time was observed, with an exception of CA in PS (Table 1). Teoh and co-workers (2016) also reported increase of total phenolic content by UV-C treatment time, which they linked with the activated phenolic biosynthesis caused by the cell damage as well as the UV-C irradiation as a stress conditions for potato tissue. On the other hand, slight CHA decrease was observed with elongation of UV-C treatment, but still its contents were significantly higher compared to the untreated samples. Different enzymes showed different sensitivity to UV-C irradiation and have different roles in biochemical changes of phenolic compounds, where phenylalanine ammonia lyase could be activated by UV-C, and has an important role in phenolic biosynthesis. The polyphenol oxidase and peroxidase have a great role in enzymatic browning, particularly in the first reaction where oxidation of phenolic compounds occurs and its resistance to UV-C treatment depend upon applied doses, especially for peroxidase (Teoh *et al*., 2016). Also, CHA is a known substrate for enzymatic browning (Li *et al*., 2018).

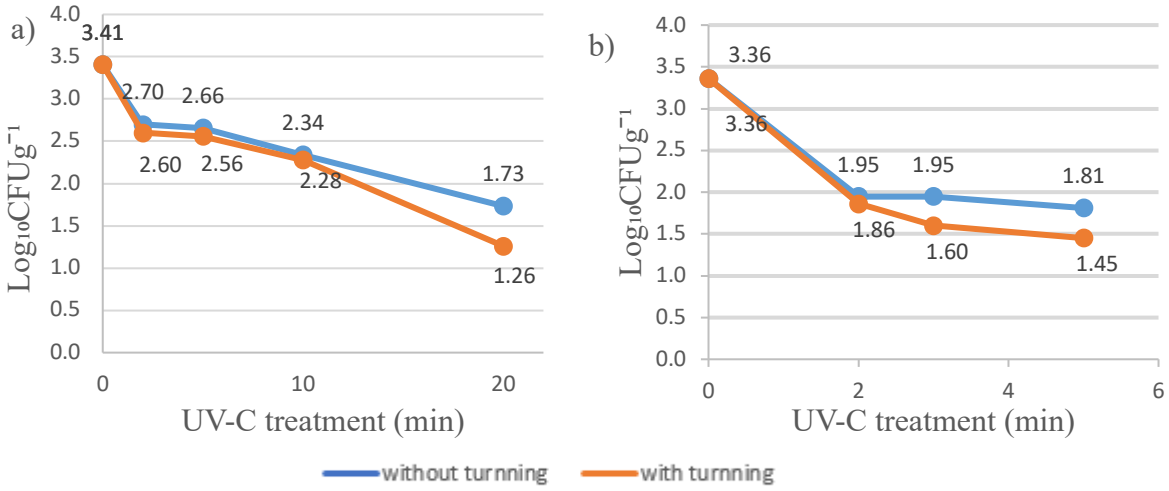


Figure 2. The effect of UV-C treatment on aerobic mesophilic bacteria (Log_{10} CFU g^{-1}) in potato tubers (a) and potato slices (b)

Further, phenolic content in PT was expectedly higher in comparison with PS, since potato peel contains higher amounts of phenolics (Deußer *et al*., 2012), and absorbed energy of UV-C light could affect certain reactions and changes of molecules (Koutchma *et al*., 2019) what consequently could enhance phenolic content in PT although peeled tubers were analyzed.

Turning samples halfway through the treatment significantly influenced on CA in PT and CHA in PS (Table 1), where an increase was noticed what is in accordance with previous observations that UV-C irradiation induces phenolic biosynthesis.

Nevertheless, considering the both sources of variation, treatment time and turning halfway through the treatment, contents of CHA, CA and RUT in PT, and CHA in PS were significantly affected but without uniform pattern (Table 1). Generally, PT turning caused a slight decrease of CHA and RUT as well as an increase of CA in comparison with unturned samples. The decrease of CHA was more obvious in 5 and 10

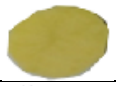
min UV-C treatment. Content of CHA in PS increased with PS turning, where the highest increase was observed in 2 min UV-C treatment. Such different pattern could caused by the difference in topography of the tubers surface and consequently absorbed energy of UV-C light (Koutchma et al., 2019) and probably the thickness of the samples could have a certain effect on susceptibility of different enzymes included in the synthesis or decomposition of phenolic compounds (Teoh *et al*., 2016).

Table 1. Individual phenols (mg 100 g^{-1} total solids) in UV-C treated potato influenced by UV-C treatment time and turning

Sample	Source of variation	Chlorogenic acid	Caffeic acid	Rutin
	UV-C treatment (min)	p<0.01*	p=0.03*	p=0.13
Potato tubers	0	14.23±0.36^{a}	1.20±0.09^{ab}	1.93±0.34^{a}
	2	22.45±0.36^{c}	1.51±0.09^{b}	2.30±0.34^{a}
	5	20.75±0.36^{b}	1.20±0.09^{ab}	2.79±0.34^{a}
	10	19.98±0.36^{b}	1.29±0.09^{ab}	1.57±0.34^{a}
	20	20.12±0.36^{b}	1.06±0.09^{a}	2.57±0.34^{a}
		p=0.05*	p<0.01*	p=0.08
Potato slices	0	8.92±1.03^{a}	0.92±0.04^{b}	0.89±0.24^{a}
	2	13.58±1.03^{c}	0.32±0.04^{a}	1.83±0.24^{a}
	3	12.11±1.03^{b}	0.27±0.04^{a}	1.58±0.24^{a}
	5	11.78±1.03^{b}	0.18±0.04^{a}	1.27±0.24^{a}
	Turning	p=0.22	p=0.02*	p=0.08
Potato tubers	without	21.21±0.43^{a}	1.13±0.07^{a}	2.66±0.27^{a}
	with	20.44±0.43^{a}	1.40±0.07^{b}	1.95±0.27^{a}
		p<0.01*	p=0.49	p=0.22
Potato slices	without	10.56±0.48^{a}	0.24±0.03^{a}	1.36±0.22^{a}
	with	14.42±0.48^{b}	0.27±0.03^{a}	1.76±0.22^{a}
	UV-C treatment (min) × turning	p<0.01*	p<0.01*	p<0.01*
Potato tubers	2 × without	22.88±0.08^{e}	1.37±0.04^{b}	3.22±0.07^{c}
	2 × with	22.02±0.08^{d}	1.64±0.04^{c}	1.37±0.07^{a}
	5 × without	21.45±0.08^{c}	1.07±0.04^{a}	2.26±0.07^{b}
	5 × with	20.05±0.08^{b}	1.34±0.04^{b}	3.32±0.07^{c}
	10 × without	21.08±0.08^{c}	1.02±0.04^{a}	2.11±0.07^{b}
	10 × with	18.88±0.08^{a}	1.56±0.04^{bc}	1.04±0.07^{a}
	20 × without	20.30±0.08^{b}	1.06±0.04^{a}	3.06±0.07^{c}
	20 × with	19.95±0.08^{b}	1.05±0.04^{a}	2.09±0.07^{b}
		p<0.01*	p=0.48	p=0.14
Potato slices	2 × without	10.67±0.14$_{a}$	0.30±0.02^{a}	1.89±0.31^{a}
	2 × with	16.50±0.14^{c}	0.35±0.02^{a}	1.78±0.31^{a}
	3 × without	10.67±0.14^{a}	0.27±0.02^{a}	1.54±0.31^{a}
	3 × with	13.53±0.14^{b}	0.27±0.02^{a}	1.62±0.31^{a}
	5 × without	10.35±0.14^{a}	0.16±0.02^{a}	0.65±0.31^{a}
	5 × with	13.20±0.14^{b}	0.20±0.02^{a}	1.89±0.31^{a}

Results are expressed as mean±SE. *Statistically significant variable at p≤0.05. Different letters mean statistically different values at p≤0.05.

Table 2. Visual appearance of the samples

Turning	Potato tubers			Potato slices		
	UV-C treatment (min)	Immediately after treatment	After 24 h at 10 °C	UV-C treatment (min)	Immediately after treatment	After 24 h at 10 °C
	0			0		
without	2			2		
with						
without	5			3		
with						
without	10			5		
with						
without	20					
with						

3.3.Browning susceptibility as a visual appearance

Immediately after the treatment (PT, additionally, after peeling and cutting) and after 24 h storage at 10 °C samples were photographed to visually monitor the influence of UV-C light on browning susceptibility of PT and PS considering the increase of phenolic content (Table 2). The visual appearance of the samples stored for 24 h were in accordance with the results of the phenolic content, where samples with higher phenolic content, particularly CHA, had more visually noticeable browning, respectively (Table 2). All PT samples showed more intense browning and had higher content of phenolic compounds in comparison with PS. The appearance of PT was generally the same for all applied UV-C doses, except samples without turning which showed a little bit more pronounced browning. Also, the appearance of PS was pretty similar for all doses, but it could be observed that the slightly greater browning occurred in 2 min UV-C treatment.

4. Conclusions

The results of microbiological analysis confirmed that UV-C treatment was effective for the reduction of aerobic mesophilic bacteria in tubers and potato slices, but irradiation affected on the increase in phenolic content and consequently the sensitivity of treated potatoes to browning. Considering all obtained results (aerobic mesophilic bacteria count, phenolic content and susceptibility to browning), UV-C treatment of potato slices with the dose 1.62 - 2.70 kJ m^{-2} and with turning could be suggested

as the most promising treatment, but further research is required.

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EFFECT OF HYDROTHERMAL TREATMENTS ON PASTING PROPERTIES OF PARBOILED BROWN RICE

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ABSTRACT

The rice varieties namely *PR-115*, *PR-118* and *Punjab mehak* were subjected to three different treatments to improve quality and shelf life of brown rice. Paddy was milled to brown rice and stored at room temperature in four different types of packaging materials. Brown rice was assessed periodically for changes in pasting qualities. Milling quality improved with treatments. Pasting quality improved with treatments leaving better quality brown rice. Hot water treatment followed by steaming for 15 min was found to be best among all treatments. Peak viscosity varied significantly as influenced by variety, treatment, and storage. Peak viscosity decreased with storage. Peak viscosity decreased with treatments. Packaging material showed non-significant effect on peak viscosity. *Punjab mehak* had higher hold viscosity followed by *PR-118*and *PR-115* in the order. Breakdown viscosity varied significantly with respect to all factors except packaging material. Breakdown values decreased with storage period. Setback viscosity followed reverse pattern as that for breakdown viscosity. Setback viscosity decreased with treatments and increased with storage period. Low setback viscosity values of hydrothermally treated flour samples indicated lesser tendency to retrograde or syneresis upon cooling. Packaging in plastic bag under vacuum was found to be the best packaging material for control however for treated samples experimental data showed that packaging material play no significant role. Overall treatments proved to be functional in improving quality and shelf life of brown rice.

1. Introduction

Hydrothermal treatments altered the pasting and gelling properties of rice starch, resulting in lower peak viscosity heights, lower setbacks and greater swelling consistency as investigated by Shih *et al.* (2007). The modified starch showed increased gelatinization temperature and narrower gelatinization temperature ranges on ANN (annealed) or broader ones on HMT (heat-moisture treatment). The effect was more pronounced for HMT than for ANN.

The investigation that with drying process (high-temperature fluidized bed drying, tempering and ventilation) starch granules lost their polygonal shape as revealed by scanning electron microscopy and gelatinization of rice starch had partially taken place was undertaken by Jaisut *et al.* (2008). DSC thermogram showed the amylase lipid complex formation for the treated brown rice, resulting in lowering starch hydrolysis. The head rice yield of the treated samples was slightly lower than that of the reference rice, which was dried in shade. The treated brown rice was harder than the reference rice as indicated by the RVA analysis. Consistency of cooked rice was negatively correlated with stickiness was reported by Kumar *et al.* (1976). The water

insoluble amylose content of cooked rice seemed to be related to stickiness and consistency.

Improvement of cooking quality by applying steam to the freshly harvested paddy was made by Desikachar and Subramanyan (1957). Steamed sample showed fewer tendencies to pastiness. Steaming fresh paddy for 15-20 minutes and keeping the paddy hot for 1-2 hours before shade drying, rice which possessed the appearance and cooking quality of old rice was obtained. The consequences of parboiling treatment on the behavior of rice on cooking and other end use applications were important and merit some thorough investigations (Patindol *et al.,* 2008). The functional properties of milled rice obtained from parboiling rough rice and brown rice need to be clearly documented; hence, this study was undertaken to find the effect of hydrothermal treatments on pasting properties of flour obtained from grinding of parboiled brown rice.

2. Materials and methods

The present study was carried out in Department of Food Science and Technology, College of Agriculture, Punjab Agricultural University, Ludhiana.

2.1. Raw materials

Three varieties of paddy namely *PR-115*, *PR-118* and *Punjab mehak* were procured from Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana.

2.2. Hydrothermal treatments

Three treatments were given to paddy to study their pasting properties obtained after dehusking of paddy. The treatments were as follows:

1. Soaking paddy in water at room temperature for 2 hours followed by steaming for 15 minutes under atmospheric pressure and drying by conventional and microwave method.
2. Soaking paddy in water at room temperature for 2 hours followed by steaming for 5-10 minutes under 15-20 PSI pressure and drying by conventional and microwave method.
3. Soaking paddy in hot water (70±2°C) for 2-3 hours followed by steaming for 15 minutes under atmospheric pressure and drying by conventional and microwave method.

2.3. Milling

Paddy was shelled to obtain brown rice in Satake Rice Sheller. For each milling test, paddy samples (100 g each) were cleaned before passing through a Satake rubber roll huller (Model THU 35A, Japan). Broken rice was separated from head rice before packaging using a Satake grader (Model TRG05B, Japan) process. Head rice yield was determined three times.

2.4. Packaging

Brown rice were packed in PET jars, cloth bags, sealed plastic bags (HDPE) and vacuum packaging in plastic bags and studied for the pasting properties over a period of 4 months.

2.5. Pasting characteristics

A Rapid Visco Analyzer (RVA) model (AACC, 2000) was used to determine the pasting properties of brown rice using following procedure:

1. Switched on the RVA and allowed it to warm for 30 minutes prior to the experiment.
2. Weigh 3g (14 per cent moisture basis) of flour in canister.
3. Place the paddle into the canister and vigorously jogged the blade through the sample up and down 10 times or until it mixes uniformly.
4. Insert the canister into pre-adjusted instrument.
5. Initiate the measurement by depressing the motor tower of the instrument.
6. Remove the canister on completion of test and discard.

2.6. Storage studies

The brown rice samples were stored in different packages at ambient conditions to estimate the pasting behavior of treated brown

rice over a period of 4 months and samples were evaluated for pasting temperature, peak viscosity, hold viscosity, final viscosity, breakdown viscosity and setback viscosity at the interval of 1 month, during the storage period.

2.7. Statistical analysis of data

Data collected from aforesaid experiments was subjected to statistical analysis with the help of factorial design in CRD using CPCS1 computer program (Singh *et al.,* 1998). The readings were taken in a set of triplicate and data were presented in form of Mean±S.D.

3. Results and discussions

Samples of three varieties of rice viz. *PR-115*, *PR-118* and *Punjab mehak* were procured from Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. Paddy was shelled to obtain brown rice in Satake Rice Sheller. Hydrothermal treatments were given to paddy. Treated paddy were milled to brown rice and packed in four different types of packages namely polythene packaging, jute/cloth bags, vacuum plastic bags and PET jars and stored under room temperature for 4 months. Brown rice was periodically assessed for changes in pasting characteristics (Rapid Visco Analyser). For convenience and proper presentation of data, abbreviations have been used in the subsequent part of results and discussion. Details of these abbreviations were given in Table 1.

Table 1. Description of experimental samples and the abbreviations used

Varieties (V)	**Abbreviation**
PR 115	V_1
PR 118	V_2
Punjab mehak	V_3
Treatments (T)	**Abbreviation**
Control	T_1
Soaking paddy in water at room temperature for 2 hours followed by steaming for 15 minutes under atmospheric pressure	T_2
Soaking paddy in water at room temperature for 2 hours followed by steaming for 5-10 minutes under 15-20 PSI	T_3
Soaking paddy in hot water (70±2°C) for 2-3 hours followed by steaming for 15 minutes under atmospheric pressure	T_4
Packaging materials	**Abbreviation**
Polythene packaging	**P_1**
Jute/cloth bags	**P_2**
Vaeeum plastic bags	**P_3**
PET jars	**P_4**
Storage period (S)	**Abbreviation**
0 day	**S_1**
1 month	**S_2**
2 months	**S_3**
3 months	**S_4**
4 months	**S_5**
Interactions	**Abbreviation**
Variety ×Treatment	**V×T**
Treatments ×Packaging	**T×P**
Treatments ×Storage period	**T×S**

Variety× Packaging	**V×P**
Variety× Storage period	**V×S**
Packaging × Storage period	**P×S**

Table 2. Effect of variety, treatments, packaging material and storage period on pasting temperature (°C) of brown rice

Variety	Treatment	Packaging Material	Storage Period				
			S_1	S_2	S_3	S_4	S_5
V_1	T_1	P_1	92.40±1.74	89.80±1.27	88.10±0.57	NR	NR
		P_2	92.10±1.74	89.90±1.56	88.50±0.64	NR	NR
		P_3	92.50±1.74	89.60±1.45	88.20±0.59	NR	NR
		P_4	92.30±1.74	89.80±1.25	88.30±0.51	NR	NR
	T_2	P_1	92.90±0.91	92.50±0.59	91.90±0.48	91.40±0.34	89.10±0.12
		P_2	92.80±0.91	92.40±1.86	91.70±0.44	91.20±0.38	89.00±0.58
		P_3	92.90±0.91	92.50±0.68	91.50±1.21	91.00±0.54	89.10±0.55
		P_4	92.10±0.91	92.60±1.23	91.70±0.24	91.20±0.61	89.00±0.61
	T_3	P_1	93.80±1.89	92.50±0.49	91.90±0.57	91.20±0.57	90.90±0.78
		P_2	93.90±1.89	92.40±0.53	91.50±0.67	91.00±0.61	90.70±0.49
		P_3	93.50±1.89	92.50±0.81	91.70±0.68	91.10±0.38	90.60±1.21
		P_4	93.60±1.89	92.50±0.89	91.60±0.68	91.00±0.46	90.70±0.49
	T_4	P_1	94.80±0.59	92.00±0.71	91.00±0.64	90.50±0.55	88.20±1.04
		P_2	94.70±0.59	92.10±0.76	91.10±1.24	90.20±0.68	88.20±1.02
		P_3	94.70±0.59	92.20±1.24	91.00±1.34	90.30±0.61	88.301±0.01
		P_4	94.80±0.59	91.80±1.54	91.20±1.57	90.10±0.49	88.10±0.59
V_2	T_1	P_1	85.70±0.44	85.00±1.35	84.50±0.84	84.00±0.52	83.60±0.81
		P_2	85.80±0.44	85.10±0.68	84.30±0.89	84.10±1.11	83.50±0.48
		P_3	85.70±0.44	85.30±1.27	84.20±1.24	84.20±1.34	83.20±0.49
		P_4	85.60±0.44	85.00±1.68	84.30±1.29	84.10±0.69	83.10±0.57
	T_2	P_1	89.50±0.57	89.00±1.38	88.40±1.37	88.10±0.97	87.00±0.29
		P_2	89.60±0.57	89.00±1.74	88.50±1.38	88.00±1.24	87.20±0.58
		P_3	89.50±0.57	89.10±0.88	88.40±0.58	88.00±0.59	87.20±1.16
		P_4	89.70±0.57	89.10±1.48	88.30±0.67	88.20±0.57	87.10±1.14
	T_3	P_1	92.70±1.24	91.70±0.59	90.90±1.27	87.00±0.64	85.90±0.91
		P_2	92.80±1.24	91.50±0.87	90.80±1.54	87.20±0.61	85.20±1.85
		P_3	92.90±1.24	91.70±1.24	90.50±1.38	87.00±1.23	85.30±0.95
		P_4	92.70±1.24	91.60±1.25	90.70±1.61	87.20±1.55	85.40±1.21
	T_4	P_1	92.40±1.32	92.00±0.76	91.00±1.28	89.50±0.87	88.20±1.35
		P_2	92.60±1.32	92.10±1.49	91.30±1.25	89.50±0.59	88.20±0.59
		P_3	92.50±1.32	92.00±1.57	91.00±1.36	89.20±1.08	88.00±0.69
		P_4	92.50±1.32	92.00±1.26	91.10±0.58	89.30±0.66	88.10±0.58
V_3	T_1	P_1	87.50±1.65	87.50±1.74	87.50±1.61	88.60±1.09	88.40±0.67
		P_2	87.50±1.65	87.60±0.58	87.40±0.83	88.50±1.15	88.10±0.59
		P_3	87.60±1.65	87.10±0.67	87.40±1.64	88.40±0.59	88.20±1.59
		P_4	87.60±1.65	87.10±1.27	87.50±0.79	88.50±0.47	88.10±1.22
	T_2	P_1	88.70±1.27	87.50±0.84	86.80±1.67	86.00±0.84	85.50±1.46
		P_2	88.90±1.27	87.40±1.67	86.60±1.59	86.20±1.32	85.40±0.59

		P_3	88.70±1.27	87.60±1.38	86.80±0.55	86.00±0.91	85.30±0.44
		P_4	88.60±1.27	87.50±1.67	86.50±0.59	86.30±0.57	85.40±0.68
	T_3	P_1	90.70±0.85	90.10±1.54	89.40±0.61	89.00±0.62	88.40±0.57
		P_2	90.60±0.85	90.00±1.61	89.20±0.47	89.30±0.55	88.30±1.23
		P_3	90.80±0.82	90.00±1.59	89.20±1.14	89.00±1.12	88.30±0.77
		P_4	90.70±0.85	90.10±1.49	89.30±1.12	89.10±0.69	88.20±0.81
	T_4	P_1	91.601±0.23	90.10±1.58	88.20±1.19	88.00±1.31	87.80±1.24
		P_2	91.70±1.23	90.30±1.64	88.10±0.67	88.20±0.51	87.80±1.51
		P_3	90.70±1.23	90.20±0.76	88.20±0.58	88.20±0.84	87.60±0.68
		P_4	90.60±1.23	90.20±0.81	88.00±0.54	88.00±0.86	87.90±0.59
CD (p≤0.05): V: 1.79, T: 1.26, S: 1.43, P: 0.38, VT: 1.13, VS: 1.06, TS: 0.79, SP: 0.27							

Table 3. Effect of variety, treatments, packaging material and storage period on peak viscosity (cP) of brown rice

Variety	Treatment	Packaging Material	Storage period				
			S_1	S_2	S_3	S_4	S_5
V_1	T_1	P_1	2415±2.11	2345±3.31	1386±3.35	448±1.25	440±1.04
		P_2	2415±2.11	2344±2.35	1387±3.16	449±1.35	441±1.22
		P_3	2415±2.11	2345±3.56	1386±3.96	447±1.18	442±1.18
		P_4	2415±2.11	2345±4.25	1386±4.15	448±1.37	440±1.31
	T_2	P_1	1988±6.24	1603±2.12	1580±1.88	1554±2.25	1550±2.15
		P_2	1988±6.24	1602±2.54	1580±1.64	1556±2.34	1552±2.27
		P_3	1988±6.24	1603±2.84	1581±2.54	1555±2.19	1551±3.18
		P_4	1988±6.24	1603±2.14	1580±2.35	1554±2.54	1550±2.51
	T_3	P_1	1965±3.21	1796±3.25	1750±6.01	1628±3.15	1601±3.27
		P_2	1965±3.21	1795±3.27	1751±1.28	1629±2.24	1600±2.59
		P_3	1965±3.21	1796±3.62	1750±3.35	1628±3.28	1600±2.58
		P_4	1965±3.21	1797±3.85	1750±2.64	1628±3.61	1599±2.41
	T_4	P_1	1954±4.53	1560±2.84	1550±2.57	1500±2.59	1459±2.38
		P_2	1954±4.53	1560±1.99	1548±1.84	1500±2.57	1458±2.59
		P_3	1954±4.53	1563±2.54	1550±2.51	1504±3.18	1459±2.34
		P_4	1954±4.53	1562±2.84	1551±3.12	1502±4.15	1460±1.28
V_2	T_1	P_1	2456±3.51	2384±2.15	2214±3.24	2049±2.94	1954±1.53
		P_2	2456±3.51	2385±3.14	2215±3.21	2048±3.27	1954±1.51
		P_3	2456±3.51	2386±3.05	2214±3.11	2045±2.59	1956±1.48
		P_4	2456±3.51	2384±3.09	2213±1.55	2048±2.51	1955±1.94
	T_2	P_1	1414±5.25	1401±3.54	1385±2.64	1354±2.48	1209±2.28
		P_2	1414±5.25	1400±3.18	1386±2.24	1355±2.61	1206±2.68
		P_3	1414±5.25	1400±3.24	1385±2.28	1357±2.52	1205±3.27
		P_4	1414±5.25	1402±2.19	1384±2.61	1354±2.38	1208±2.35
	T_3	P_1	1130±2.16	1117±3.14	1100±1.84	1094±3.15	1071±3.25
		P_2	1130±2.16	1115±2.25	1104±1.67	1095±3.29	1072±4.15
		P_3	1130±2.16	1114±1.49	1102±2.04	1095±3.48	1071±4.04
		P_4	1130±2.16	1118±1.08	1101±3.24	1094±4.12	1073±2.28
	T_4	P_1	1109±5.24	1101±2.18	1054±4.19	1004±4.08	958±1.35
		P_2	1109±5.24	1102±2.62	1055±1.83	1001±4.01	959±1.81
		P_3	1109±5.24	1099±2.51	1058±4.27	1000±2.35	957±2.29

		P_4	1109±5.24	1100±2.48	1055±5.08	1005±2.38	958±4.28
V_3	T_1	P_1	2345±6.14	1869±2.19	1850±4.29	1654±2.15	1524±1.29
		P_2	2345±6.14	1865±3.12	1851±3.25	1655±2.48	1525±4.28
		P_3	2345±6.14	1868±2.25	1852±3.17	1652±3.15	1524±4.29
		P_4	2345±6.14	1867 3.34	1855±3.28	1651±3.27	1523±2.28
	T_2	P_1	1603±4.53	1505±3.25	1484±3.41	1325±1.29	1124±1.59
		P_2	1603±4.53	1505±3.17	1485±3.37	1325±2.22	1125±2.58
		P_3	1603±4.53	1507±2.11	1484±3.27	1326±2.39	l124±2.39
		P_4	1603±4.53	1508±2.35	1484±2.52	1324±1.29	1124±2.18
	T_3	P_1	1364±3.54	1214±3.28	11012.61	1017±1.51	1005±2.48
		P_2	1364±3.54	1215±4.15	1100±2.38	1018±2.28	1005±3.15
		P_3	1364±3.54	1214±3.29	1101±2.41	1017±3.24	1004±3.04
		P_4	1364±3.54	1213±2.18	1102±2.62	1016±2.38	1006±3.08
	T_4	P_1	1192±2.36	1154±4.15	1148±3.12	1041±1.26	1011±2.09
		P_2	1192±2.36	1155±4.28	1149±2.35	1042±1.28	1012±2.12
		P_3	1192±2.36	1154±4.05	1147±4.12	1041±3.28	1011±2.18
		P_4	1192±2.36	1153±3.12	1148±2.35	1041±3.27	1011±2.28
CD (p≤0.05): V: 0.42, T: 3.32, S: 1.54, P: NS, V×T: 4.53, V×S: 1.78, T×S: 2.67, P×S: NS							

3.1. Effect of variety, treatment, packaging material and storage period on pasting temperature of parboiled brown rice

Studies were carried out to observe and analyze the effect of varieties, treatments, storage period and packaging material on pasting properties of parboiled brown rice flour. Samples of treated varieties were prepared, and the pasting properties were determined using the rapid visco analyser (RVA), starch master R&D pack V-3.0 (Newport Scientific, Narrabean, Australia). The parameters measured were pasting temperature (the temperature at which the viscosity of the paste starts to increase), peak viscosity (the maximum viscosity that the slurry attains), holding viscosity (the trough at minimum hot paste viscosity), final viscosity (the viscosity of the slurry after cooling to 50°C and holding the temperature), breakdown viscosity (peak-trough viscosity) and setback (final-trough viscosity) in accordance with the method given by Walker *et al.* (1988) and Batey *et al.* (1997). Hydrothermal treatments affect on the pasting temperature of parboiled brown rice flour significantly (Table 2). The pasting temperature increased with increase in hydrothermal treatments therefore pasting temperature was maximum for T_4 and minimum for T_1 i.e., control. Pasting temperature decreased with storage period from 94.8 cP on 0 day to 88.2 cP at the end of the storage period in case of variety V_1 and treatment T_4. The individual effect of varieties, treatments, storage period and packaging material were significant on pasting temperature of parboiled brown rice flour. Interactions of varieties with treatment, varieties with storage period, treatment with storage period and storage period with packaging material were found significant pasting temperature of parboiled brown rice flour. The higher hydrothermal treatment causes the increase in pasting temperature with processing.

3.2. Effect of variety, treatment, packaging material and storage period on peak viscosity (cP) of parboiled brown rice

The individual effect of varieties, treatments, storage period were significant while the individual effect of packaging material was insignificant on peak viscosity of parboiled brown rice flour (Table 3). Peak viscosities attained during the heating portions of the tests indicate that water binding capacity of starch. Peak viscosity decreased with storage from 2415 cP on zero day to 440 cP at the end of the storage in case of control of variety V_1

and from 1954 cP to 1459 cP in case of T_4. Peak viscosity decreased with treatments as control of V_2 has 2456 cP value but for T4 value decreased to 1109 cP. Packaging material insignificantly affect on peak viscosity of parboiled brown rice. Interactions of varieties with treatment, varieties with storage period and treatment with storage period were significant while interaction of storage period with packaging material were insignificant on peak viscosity (cP) of parboiled brown rice. The decrease in peak viscosity during aging of rice showed that the starch granules of aged rice were more resistant to swelling than that of fresh rice.

Dengate (1984); Dengate and Meredith (1984) reported that peak viscosity was dependent on swelling, exudation and fragmentation of starch. Peak viscosity is indicative of water binding capacity and ease with which starch was disintegrated and it was often correlated with final product quality (Thomas and Atwell, 1999). Mir *et al.* (2013) studied the effect of soaking temperature (60°C, 70°C and 80°C) on pasting properties using Rapid Visco Analyzer (RVA). The comparison of pasting profile of raw rice from different cultivars with parboiled rice showed that pasting profile of parboiled rice decreased as result of increased damaged starch which absorbs the water content and decreased peak viscosity resulting from the resistance of starch granules for swelling due to the gelatinization process takes place in parboiling.

Symons and Brennan (2004) suggested that a reduction in pasting characteristics could be associated with a reduced enthalpy of starch gelatinization and with retention of the integrity of starch granule, the reduction in peak viscosity being associated to reduced degree of starch granule swelling. It had been stressed that the endogenous presence and external addition of dietary fiber to starch based food systems involved nutritional benefits (Brennan and Samyue, 2004).

3.3. Effect of variety, treatment, packaging material and storage period on hold viscosity (cP) of parboiled brown rice

The individual effect of varieties, treatments and storage period were found significant while the individual effect of packaging material was insignificant on peak viscosity of parboiled brown rice flour (Table 4). Hold viscosity decreased with hydrothermal treatments as well as with storage. V_3 had higher values for hold viscosity followed by V_2 and V_1 in the order. Hold viscosity decreased from 1018 cP to 875 cP in case of V_3 under T_4. This showed that there was decrease in hold viscosity with increased storage period, thus fresh brown rice had higher hold viscosity than aged brown rice. The interactions of varieties with treatment, varieties with storage period, treatment with storage period and storage period with packaging material were found significant on hold viscosity (cP) of parboiled brown rice.

3.4. Effect of variety, treatment, packaging material and storage period on final viscosity (cP) of parboiled brown rice

Final viscosity of treated samples was less than control which depicts that final viscosity decreased with hydrothermal treatments however final viscosity increased with storage period (Table 5). Variety V_1 had higher final viscosity at the end of the storage followed by V_2 and V_3. Final viscosity varied significantly with respect to varieties, treatments, and storage. Packaging material did not affect the final viscosity significantly. The interactions of varieties with treatment, varieties with storage period, treatment with storage period and storage period with packaging material were found significant on final viscosity (cP) of parboiled brown rice. *PR-115* had higher final viscosity at the end of the storage followed by *PR-118*and *Punjab mehak.* The decreased final viscosity of sample with added fiber suggested that the three-dimensional network was weekend by the presence of fiber in matrix particularly by those of larger particle size and water in solubility.

Table 4. Effect of variety, treatments, packaging material and storage period on hold viscosity (cP) of brown rice

Variety	Treatment	Packaging Material	Storage period				
			S_1	S_2	S_3	S_4	S_5
V_1	T_1	P_1	1051±3.12	1024±2.38	1008±3.33	986±2.71	942±3.12
		P_2	1051±3.12	1023±2.38	1007±2.59	985±2.53	943 ±3.16
		P_3	1051±3.12	1024±2.63	1006±1.58	985±2.69	943±2.54
		P_4	1051±3.12	1023±2.63	1008±1.69	986±1.34	942±2.59
	T_2	P_1	987±4.05	974±1.35	955±1.88	941±1.29	847±1.59
		P_2	987±4.05	975±1.69	956±2.54	940±1.58	846±1.67
		P_3	987±4.05	976±1.48	956±2.69	943±2.65	845±1.18
		P_4	987±4.05	975±1.57	955±2.11	942±2.84	845±2.31
	T_3	P_1	884±3.24	851±2.38	846±3.27	789±1.59	748±2.61
		P_2	884±3.24	850±2.19	847±3.58	788±1.58	747±3.12
		P_3	884±3.24	850±3.29	846±1.59	789±2.59	747±3.05
		P_4	884±3.24	852±3.28	845±1.86	789±2.54	748±4.01
	T_4	P_1	761±2.59	754±3.15	737±2.54	712±1.59	701±1.25
		P_2	761±2.59	755±3.08	736±3.28	713±2.58	700±1.37
		P_3	761±2.59	754±2.19	735±2.66	713±3.24	700±1.67
		P_4	761±2.59	756±1.67	737±1.47	714±3.57	703±1.84
V_2	T_1	P_1	1331±3.31	1351±2.58	1254±1.69	1126±1.61	1109±1.66
		P_2	1331±3.31	1352±3.19	1255±2.59	1123±2.59	1108±2.54
		P_3	1331±3.31	1350±2.38	1256±1.94	1124±1.61	1107±2.61
		P_4	1331±3.31	1350±2.18	1256±1.68	1123±1.25	1105±3.16
	T_2	P_1	1314±2.24	1287±1.57	1200±2.98	1189±2.15	1158±3.24
		P_2	1314±2.24	1285±1.36	1202±3.48	1188±2.19	1160±3.25
		P_3	1314±2.24	1287±2.15	1200±3.24	1189±2.51	1162±1.67
		P_4	1314±2.24	1286±3.13	1201±1.86	l190±2.64	1161±2.46
	T_3	P_1	1128±1.58	1l09±2.65	1084±2.44	1057±5.01	984±2.59
		P_2	1128±1.58	1108±1.85	1086±3.11	1056±4.29	985±2.67
		P_3	1128±1.58	1109±0.99	1083±1.56	1055±5.03	985±5.31
		P_4	1128±1.58	1109±1.24	1083±1.48	1057±1.28	987± 5.02
	T_4	P_1	1106±2.61	1095±1.29	1081±1.67	1050±4.31	951±3.26
		P_2	I106±2.61	l096±2.38	1080±2.54	1050±2.59	955±2.31
		P_3	1106±2.61	1096±3.16	1082±2.68	1050±2.68	955±2.37
		P_4	1106±2.61	1095±2.65	1080±1.64	1051±2.95	954±2.36
V_3	T_1	P_1	1431±1.08	1239±2.69	1204±2.58	1186±3.54	1149±2.64
		P_2	1431±1.08	1235±3.16	1201±2.26	1185±2.54	1148±3.15
		P_3	1431±1.08	1235±2.18	1202±1.54	1185±2.84	1148±3.04
		P_4	1431±1.08	1236±3.24	1205±2.51	1185±1.87	1149±2.51
	T_2	P_1	1158±2.34	1051±1.55	1042±2.61	1004±1.95	987±2.35
		P_2	1158±2.34	1052±1.59	1045±2.24	1003±1.58	988±2.37
		P_3	1158±2.34	1052±2.58	1042±3.15	1004±1.68	988±3.09
		P_4	1158±2.34	1053±3.27	1043±2.24	1004±2.61	987±3.29
	T_3	P_1	1128±3.25	1114±1.48	1102±1.84	1059±3.24	981±2.54
		P_2	1128±3.25	1115±3.24	1102±1.38	1060±2.15	983±2.61
		P_3	1128±3.25	1116±1.58	1101±3.14	1062±2.34	982±2.29

		P_4	1128±3.25	1113±1.29	1100±2.59	1061±1.26	982±3.25
	T_4	P_1	1018±1.26	986±3.28	953±2.57	948±2.34	875±2.32
		P_2	1018±1.26	985±2.27	954±1.28	947±1.59	875±2.05
		P_3	1018±1.26	987±1.59	954±1.67	947±2.51	875±3.16
		P_4	1018±1.26	986±2.68	955±2.53	948±2.36	874±3.15
CD (p≤0.05): V: 3.85, T: 2.12, S: 5.43, P: NS, V×T: 3.54, V×S: 4.89, T×S: 2.45, P×S: 0.89							

Table 5. Effect of variety, treatments, packaging material and storage period on final viscosity (cP) of brown rice

Variety	Treatment	Packaging Material	Storage Period				
			S_1	**S_2**	**S_3**	**S_4**	**S_5**
V_1	T_1	P_1	2826±6.15	2876±4.36	2894±4.56	2921±6.14	2935±5.26
		P_2	2826±6.15	2875±5.25	2895±6.54	2922±4.12	2937±4.26
		P_3	2826±6.15	2876±7.12	2895±4.35	2921±4.25	2935±5.36
		P_4	2826±6.15	2876±4.35	2894±3.44	2921±4.36	2936±4.15
	T_2	P_1	2041±8.12	2310±8.15	2319±4.55	2514±4.89	2678±6.25
		P_2	2041±8.12	2300±6.25	2319±4.12	2515±5.14	2677±6.35
		P_3	2041±812	2312±4.24	2320±5.12	2516±5.61	2678±4.25
		P_4	2041±8.12	2306±2.59	2319±6.24	2515±7.12	2677±4.15
	T_3	P_1	1897±5.54	1899±8.12	1924±6.59	1957±4.36	2455±5.36
		P_2	1897±5.54	1899±7.09	1924±3.25	1958±5.32	2454±2.36
		P_3	1897±5.54	1898±5.35	1926±4.12	1958±4.65	2455±4.12
		P_4	1897±5.54	1899±4.68	1925±6.35	1957±5.36	2456±5.11
	T_4	P_1	1539±7.15	1654±5.45	1889±5.84	2214±6.14	2300±5.25
		P_2	1539±7.15	1655±4.35	1889±4.59	2215±5.23	2300±5.36
		P_3	1539±7.15	1654±6.15	1889±3.56	2215±4.36	2305±4.65
		P_4	1539±7.15	1655±4.25	1890±6.25	2214±8.24	2301±4.26
V_2	T_1	P_1	2736±4.75	2785±4.12	2814±1.28	2855±6.25	2876±5.25
		P_2	2736±4.75	2785±4.08	2815±6.24	2856±7.15	2875±3.15
		P_3	2736±4.75	2784±7.14	2817±5.36	2854±5.36	2877±6.25
		P_4	2736±4.75	2786±3.25	2818±4.24	2855±6.15	2874±4.58
	T_2	P_1	2438±6.23	2459±6.15	2514±5.26	2657±9.15	2718±7.25
		P_2	2438±6.23	2458±8.12	2513±7.38	2657±4.25	2719±8.15
		P_3	2438±6.23	2461±4.24	2514±7.25	2658±9.25	2718±4.69
		P_4	2438±6.23	2460±5.18	2516±8.15	2655±7.26	2714±4.15
	T_3	P_1	1715±5.25	1751±7.12	1854±4.25	1943±9.26	2018±8.15
		P_2	1715±5.25	1754±6.35	1854±5.38	1945±8.12	2015±4.25
		P_3	1715±5.25	1750±4.15	1856±5.65	1945±4.26	2016±6.35
		P_4	1715±5.25	1750±7.15	1855±5.01	1944±4.35	2018±7.15
	T_4	P_1	1701±7.65	1930±8.12	1988±5.12	2017±4.15	2248±4.59
		P_2	1701±7.65	1932±4.24	1987±5.35	2014±6.35	2245±6.58
		P_3	1701±7.65	1935±5.25	1988±4.65	2016±4.12	2248±6.25
		P_4	1701±7.65	1931±4.25	1985±4.23	2015±4.35	2249±5.36
V_3	T_1	P_1	2500±3.48	2558±4.25	2645±4.78	2697±4.26	2750±4.15
		P_2	2500±3.48	2557±4.36	2644±4.65	2997±4.26	2755±6.25
		P_3	2500±3.48	2555±4.25	2645±4.12	2698±5.01	2754±6.35
		P_4	2500±3.48	2557±4.25	2645±6.01	2699±5.36	2751±4.25

	T_2	P_1	2204±5.55	2230±4.89	2539±4.11	2559±6.25	2584±3.65
		P_2	2204±5.55	2231±5.25	2538±6.23	2560±6.58	2585±3.65
		P_3	2204±5.55	2234±6.32	2538±4.26	2561±5.36	2584±4.15
		P_4	2204±5.55	2232±4.25	2539±7.15	2560±4.15	2584±6.12
	T_3	P_1	2130±6.15	2154±4.65	2567±5.65	2660±2.56	2674±4.36
		P_2	2130±6.15	2155±6.25	2568±9.14	2661±3.25	2675±4.17
		P_3	2130±6.15	2154±5.15	2568±2.65	2660±3.66	2674±4.25
		P_4	2130±6.15	2156±7.12	2564±5.14	2660±4.15	2675±5.14
	T_4	P_1	1862±4.35	1941±2.36	1985±3.65	2417±5.12	2500±5.68
		P_2	1862±4.35	1940±3.25	1984±4.35	2418±4.65	2501±5.36
		P_3	1862±4.35	1940±6.25	1985±5.65	2418±4.15	2500±4.12
		P_4	1862±4.35	1942±4.15	1986±5.47	2416±4.11	2499±5.14
CD (p≤0.05): V: 1.79, T: 3.68, S: 6.23, P: NS, V×T: 3.45, V×S: 4.37, T×S: 6.45, P×S: 1.23							

3.5. Effect of variety, treatment, packaging material and storage period on breakdown and setback viscosity (cP) of parboiled brown rice

Breakdown viscosity is measure of the ease with which the swollen granules could be disintegrated. Higher breakdown viscosity in starches could be attributed to higher crystalline and lower amylose content. The individual effect of varieties, treatments and storage period were found significant while the individual effect of packaging material was found insignificant on breakdown viscosity of parboiled brown rice flour (Table 6). Breakdown values decreased with storage period from 331 cP on zero day to 229 cP at the end of storage period in case of T_4 of variety V_1. The decrease of breakdown viscosity might be due to the failure of complete pasting and swelling of starch granules induced by the reduction of water absorption of starch granules. The interactions of varieties with treatment, varieties with storage period and treatment with storage period had significant effect while interactions of storage period with packaging materials were found insignificant effect on breakdown viscosity of parboiled brown rice. Breakdown viscosity was regarded as measure of degree of disintegration of starch granule or substances. The gel formed at the end of RVA cooling cycle was essentially a three-dimensional network of inter wined amylase molecules incorporating dispersed swollen ruptured starch granules (Langton and Hermansoon, 1989).

Table 6. Effect of variety, treatments, packaging material and storage period on breakdown (cP) of brown rice

Variety	Treatment	Packaging Material	Storage Period				
			S_1	S_2	S_3	S_4	S_5
V_1	T_1	P_1	667±2.12	651±2.36	584±2.15	550±2.59	5l5±2.45
		P_2	667±2.12	650±4.26	585±3.25	548±2.36	5l4±1.25
		P_3	667±2.12	651±2.65	584±2.45	550±2.35	5l5±1.14
		P_4	667±2.12	652±2.36	584±2.68	551±1.14	517±1.11
	T_2	P_1	519±3.01	511±4.01	478±3.01	475±1.36	470±1.36
		P_2	519±3.01	510±2.15	478±3.24	478±1.26	472±1.26
		P_3	519±3.01	511±2.35	478±1.25	475±1.26	470±1.55
		P_4	519±3.01	512±3.36	478±1.36	476±1.45	471±1.36
	T_3	P_1	487±3.13	350±2.35	328±2.36	299±1.12	281±1.65
		P_2	487±3.13	353±3.14	329±1.45	298±2.36	280±1.85
		P_3	487±3.13	351±3.15	328±2.35	294±1.45	280±1.95

		P_4	487±3.13	3501±3.25	328±2.36	298±1.36	282±2.15
	T_4	P_1	331±2.25	324±2.45	285±1.46	264±1.35	229±2.36
		P_2	331±2.25	321±2.15	286±1.56	264±1.14	230±1.14
		P_3	331±2.25	324±2.14	285±1.48	264±1.26	230±1.26
		P_4	331±2.25	325±2.36	284±1.98	265±1.45	231±1.45
V_2	T_1	P_1	445±2.36	418±2.35	401±1.48	382±1.26	357±2.35
		P_2	445±2.36	418±2.35	400±1.68	382±1.15	358±2.36
		P_3	445±2.36	4l8±2.26	400±1.25	382±2.04	359±2.65
		P_4	445±2.36	417±1.24	402±1.44	381±2.02	357±2.14
	T_2	P_1	375±3.35	359±1.55	344±1.32	297±4.15	347±2.15
		P_2	375±3.35	359±1.65	344±1.31	297±2.65	348±2.48
		P_3	375±3.35	358±1.78	345±1.22	294±2.36	349±2.19
		P_4	375±3.35	360±1.45	346±2.02	298±1.26	347±2.36
	T_3	P_1	330±2.45	255±1.65	228±2.04	19±1.24	13±2.15
		P_2	330±2.45	254±1.36	230±1.33	18±3.25	12±1.14
		P_3	330±2.45	256±1.25	230±1.21	18±2.15	11±2.36
		P_4	330±2.45	254±4.01	231±2.05	19±2.35	12±2.25
	T_4	P_1	312±1.23	301±2.35	284±2.06	268±2.36	221±2.15
		P_2	312±1.23	302±1.24	285±1.14	269±2.15	220±2.11
		P_3	312±1.23	301±2.56	285±1.25	268±2.26	221±2.35
		P_4	312±1.23	300±3.01	284±1.35	264±2.15	221±1.56
V_3	T_1	P_1	450±1.54	430±2.15	418±2.04	400±2.35	391±1.44
		P_2	450±1.54	432±2.48	417±2.06	401±1.26	392±1.58
		P_3	450±1.54	430±1.65	417±1.24	402±1.25	395±1.59
		P_4	450±1.54	431±2.35	418±1.06	400±302	392±1.48
	T_2	P_1	445±1.68	437±1.84	421±2.15	338±2.15	321±1.94
		P_2	445±1.68	438±1.35	422±1.26	334±2.26	322±1.44
		P_3	445±1.68	435±1.05	426±1.25	339±2.14	324±1.26
		P_4	445±1.68	437±1.45	422±1.14	338±2.15	321±1.54
	T_3	P_1	236±3.36	220±1.25	214±1.36	187±2.35	181±1.36
		P_2	236±3.36	221±1.56	213±1.26	185±3.25	181±1.48
		P_3	236±3.36	220±1.36	214±1.45	185±1.35	181±1.47
		P_4	236±3.36	220±1.24	215±4.25	186±1.45	180±2.15
	T_4	P_1	174±2.45	170±1.45	168±3.25	165±1.36	157±2.35
		P_2	174±2.45	168±1.36	168±3.15	166±1.35	158±1.14
		P_3	174±2.45	169±1.25	167±1.26	166±1.25	158±1.35
		P_4	174±2.45	170±1.02	167±1.25	165±3.05	154±1.26
CD (p≤0.05): V: 1.34, T: 5.52, S: 3.34, P: NS, V×T: 4.43, V×S: 4.12, T×S: 2.67, P×S: NS							

The individual effect of varieties, treatments, storage period and packaging material were found significant on setback viscosity of parboiled brown rice flour (Table 7). Setback viscosity followed reverse pattern as of breakdown viscosity. Setback viscosity decreased with the treatment and increased with storage period. The setback values indicate the hardness of gel paste upon cooling which is indirect measurement of retrogradation of starches. Low setback viscosity values of hydrothermally treated flour samples indicated lesser tendency to retrograde or syneresis upon cooling. The interactions of varieties with treatment, varieties with storage period and treatment with storage period had

significant effect on setback viscosity while interactions of storage period with packaging materials had insignificant effect on setback viscosity of parboiled brown rice. The increased setback viscosity resulted into more syneresis this indicated higher tendency of starch retrogradation (Hagenimana *et al.*, 2005). High setback value was an indication of the amount of swelling power of starch and it was usually related to the amylase content of the starch. Hydrothermal treatments affect the pasting temperature significantly.

Table 7. Effect of variety, treatments, packaging material and storage period on setback (cP) of brown rice

Variety	Treatment	Packaging Material	Storage Period				
			S_1	S_2	S_3	S_4	S_5
V_1	T_1	P_1	741±2.25	851±1.11	884±1.49	919±2.65	990±1.49
		P_2	741±2.25	852±1.25	885±1.59	920±2.05	991±1.25
		P_3	741±2.25	853±1.36	885±1.49	919±2.18	992±1.36
		P_4	741±2.25	851±1.14	884±1.39	918±2.01	992±1.12
	T_2	P_1	746±2.69	812±1.75	847±1.25	901±2.04	910±1.21
		P_2	746±2.69	811±1.26	847±1.02	900±1.06	912±1.29
		P_3	746±2.69	813±1.25	847±2.09	900±1.25	14±1.47
		P_4	746±2.69	814±1.54	848±2.05	902±1.59	913±1.84
	T_3	P_1	718±2.45	739±1.59	782±2.35	814±1.54	845±1.14
		P_2	718±2.45	738±1.58	785±1.54	185±1.58	846±1.48
		P_3	718±2.45	739±1.48	782±2.48	815±1.25	845±1.29
		P_4	718±2.45	740±1.57	782±2.49	816±1.32	847±1.07
	T_4	P_1	697±1.36	711±1.59	724±2.58	768±1.33	778±1.03
		P_2	697±1.36	712±1.68	726±2.36	765±0.59	777±0.97
		P_3	697±1.36	713±2.15	725±2.14	766±1.24	779±0.48
		P_4	697±1.36	711±1.26	724±1.26	765±1.22	778±0.58
V_2	T_1	P_1	981±2.21	1055±1.54	1071±1.68	1082±1.59	1101±1.36
		P_2	981±2.21	1054±1.05	1070±1.35	1084±1.48	1100±1.24
		P_3	981±2.21	1055±2.22	1074±1.45	1082±0.29	1104±1.11
		P_4	981±2.21	1056±1.24	1071±1.18	1085±1.36	1105±1.48
	T_2	P_1	948±1.24	973±1.36	1004±1.09	1054±0.59	1070±1.59
		P_2	948±1.24	975±1.56	1000±1.02	1054±.68	1073±1.24
		P_3	948±1.24	974±2.35	1004±1.25	1054±0.99	1071±1.29
		P_4	948±1.24	975±2.15	1002±0.78	1055±1.25	1072±1.09
	T_3	P_1	550±3.15	568±2.65	571±0.99	598±1.48	645±1.49
		P_2	550±3.15	569±1.48	570±0.58	598±1.59	644±1.19
		P_3	550±3.15	568±1.85	574±0.69	599±1.68	643±1.35
		P_4	550±3.15	569±1.67	572±1.25	599±2.30	643±1.49
	T_4	P_1	519±2.22	554±1.05	562±1.48	584±1.02	645±1.59
		P_2	519±2.22	559±2.35	562±1.59	585±1.06	646±1.08
		P_3	519±2.22	554±1.04	563±1.48	584±0.84	647±1.27
		P_4	519±2.22	553±1.26	561±2.15	585±1.26	645±1.26
V_3	T_1	P_1	1200±2.5	1254±1.04	1357±2.36	1400±1.45	1401±1.49
		P_2	1200±2.5	1256±1.24	1358±1.69	1399±1.31	1400±1.20
		P_3	1200±2.5	254±1.50	1356±1.58	1401±1.32	1398±1.48
		P_4	1200±2.5	1255±2.15	1355±1.45	1402±1.04	1400±1.26

	T_2	P_1	918±1.24	957±1.04	981±1.29	1008±1.06	1046±0.84
		P_2	918±1.24	957±1.06	980±1.05	1008±1.27	1048±1.49
		P_3	918±1.24	957±2.15	981±2.22	1005±1.29	1047±1.22
		P_4	918±1.24	958±1.24	981±2.06	1007±1.29	1045±1.52
	T_3	P_1	931±2.26	942±1.26	955±2.04	982±1.18	1002±1.49
		P_2	931±2.26	941±1.24	956±2.02	982±0.26	1000±1.47
		P_3	931±2.26	942±1.28	956±1.45	982±0.88	1003±1.58
		P_4	931±2.26	942±1.59	954±1.58	983±1.12	1001±1.29
	T_4	P_1	713±1.24	728±1.47	800±1.26	817±1.25	844±1.47
		P_2	713±1.24	726±1.49	800±1.24	816±1.22	845±1.28
		P_3	714±1.24	725±1.19	801±0.49	816±1.36	847±1.45
		P_4	714±1.24	727±1.85	801±1.12	817±1.49	843±0.95
CD ($p \leq 0.05$): V: 4.56, T: 4.12, P:0.09, S: 3.43, V×T: 4.34, V×S: 4.56, T×S: 3.98, P×S: NS							

During setback the mixture was subsequently cooled, there is reassociation between starch molecules, especially amylose. Insufficient concentration usually caused the formation of gel and viscosity normally increased. Therefore, the control flour without any treatment had higher values of setback. The change in some of the pasting properties during aging could be attributed to starch granule characteristics. The change in breakdown viscosity indicated that the capacity of the starch granules to rupture after cooking was reduced significantly by aging of the starch granules. However, the final and setback viscosity increased with increasing rice storage duration. These results were due to the strong granules after storage, so some starch granules were not disrupted during cooking. Final and setback viscosity might occur by rearrangement of leached amylose and the granules which have not been disrupted (Noomhorm *et al.*, 1997).

4. Conclusions

Hot water treatment followed by steaming for 15 min was found to be best among all treatments. The change in some of the pasting properties during aging could be attributed to starch granule characteristics. The individual effect of varieties, treatments, storage period and packaging material were significant on pasting temperature of brown rice flour. Interactions of varieties with treatment, varieties with storage period, treatment with storage period and storage period with packaging material were found significant on pasting temperature and final viscosity of brown rice flour. The higher hydrothermal treatment causes the increase in pasting temperature with processing. The individual effect of varieties, treatments, storage period were significant while the individual effect of packaging material was insignificant on peak viscosity of brown rice flour. Interactions of varieties with treatment, varieties with storage period and treatment with storage period were significant while interaction of storage period with packaging material were insignificant. Final viscosity of treated samples was less than control which depicts that final viscosity decreased with hydrothermal treatments however final viscosity increased with storage period. The interactions of varieties with treatment, varieties with storage period, treatment with storage period and storage period with packaging material were found significant. The change in breakdown viscosity indicated that the capacity of the starch granules to rupture after cooking was reduced significantly by aging of the starch granules. However, the final and setback viscosity increased with increasing rice storage duration. *Punjab mehak* was best responsive to treatments and hence retained better functional properties upon storage.

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MANAGING QUALITY OF AROMATIZED WINE PREPARED BY CO-FERMENTATION OF GRAPE MUST AND BY-PRODUCTS OF ESSENTIAL ROSE OIL INDUSTRY

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ABSTRACT

Aromatized rosé wines with addition of essential rose oil industry wastes during fermentation of grape must were prepared. Six variants: W1-W6 with added 0.05%, 0.1%, 0.25%, 0.5%, 1%, and 2% *Rosa damascena* Mill. waste, respectively, and control wine were prepared. Slight differences in the color shades were observed: the lower the added rose waste, the more intensive peony color was obtained and this observation was confirmed with the increase of the hue angle value – 46.21±0.84 for the control and 54.95±0.70 for the W6. The polyphenol content increased significantly from 355.01±10.14 to 576.08±12.08 μmol GAE L^{-1} for the control and W6, respectively. The major phenolic acids determined were 3,4-dihydroxy benzoic (up to 65.1±1.1 mg L^{-1} in W6), gallic (up to 25.9±0.9 mg L^{-1} in W6) and chlorogenic acid (up to 11.7±0.6 mg L^{-1} in W5). The GC-FID analysis revealed slight increase of higher alcohols for W5 and W6. β-Caryophyllene, β-citronellol, phenethyl alcohol, rose oxide, and geraniol content increased significantly compared to control. The sensory evaluation revealed most of the panelists preferred W1 and W2 although some of the testers liked better the variants with higher amounts of added waste. The results suggested that rose waste successfully could be utilized for preparation of new aromatized wines with distinctive rose aroma.

1.Introduction

The wine is among the most popular and produced worldwide low alcoholic beverages. The fundamental factors determining the wine quality are geographical region, climate conditions, soils, grape variety, stage of ripeness, yeasts, as well as, vinification (Cioch-Skoneczny *et al.*, 2021; Nardi *et al.*, 2018). The wine aroma is among the most important factors for the wine quality and acceptance (Nardi *et al.*, 2018). The major contributors for the formation of aroma bouquet are the yeast fermentation of grape must and skin contact time (Cabaroglu and Canbas, 2002). Furthermore, the aroma could be modulated by addition of other flavoring substances and these beverages are categorized as aromatized wines. The aromatized wines, according to Regulation 251/2014 of the European Parliament and the Council, are

defined as wines with organoleptic characteristics achieved by addition of natural flavoring substances and/or herbs and spaces, including their extracts, and/or flavor products, and combination thereof. Different flavoring materials were used: wormwood, dwarf gentians (*Gentianella* sp.), mint, cinnamon, green cardamom, elderberry, nutmeg, rosemary, juniper, *Hypericum* sp., clove, flat-leaved vanilla, etc. The utilization of agricultural by-products is a rare practice but some aromatized wines exist, i.e. St. Raphael's aperitif wine prepared with bitter orange peels (Buglass, 2011). By-products from the olive oil industry were used in an attempt for replacing sulfur dioxide in wine models (Ruiz-Moreno *et al.*, 2015), and overripe seeds from white grape by-products were added during red wine fermentation in order to investigate the effect on wine color and phenolic substances (Rivero *et al.*, 2017). Attempts for preparation of aromatized wines with addition of essential oil industry main products were made but problems with solubility and separation of the oils and wine during storage were observed. The literature survey suggested, to the best of our knowledge, that no attempts for preparation of aromatized wines with addition of by-products of the essential-oil industry, which is emblematic and widespread in some European and Asian countries (Bulgaria, France, Turkey, Iran, China, etc.), were described. By-products of the rose oil-industry are usually not further utilized and are discarded, although the waste could serve as a valuable raw material for obtaining of biologically active substances (Slavov *et al.*, 2017). For this reason, based on the above-mentioned observations, literature survey and experimental data, the present study aimed to investigate the possibility for preparation and managing quality of aromatized wines with addition of rose oil industry waste in the course of Mavrud must fermentation.

2. Materials and methods

2.1.Materials

2.1.1.Samples and reagents

The *Rosa Damascena* Mill. waste was provided by EKOMAAT Ltd. distillery (Mirkovo, region of Sofia, Bulgaria; 2016 harvest; waste obtained from certified bio roses was used). The grape used for wine preparation was *Vitis vinifera* L. cv Mavrud (Brestovica, region of Plovdiv, Bulgaria; 2016 harvest) with 23.8% sugars and 8.2% titratable acids. The Lallzyme cuvée blanc and the yeast strain Lalvin D47 were obtained from Lallemand (France). The Polymust press and bentonite were obtained from Laffort (France).

Acetonitrile, acetic acid, dichloromethane, sodium acetate, pyridine, N,O-Bis-(trimethylsilyl)-trifluoroacetamide, gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, rosmarinic acid, cichroric acid and cinnamic acid were obtained from Sigma-Aldrich (USA). The DPPH (2,2-diphenyl-1-picrylhydrazyl) was from Merck (Germany).

2.2. Methods

2.2.1.Preparative

The wines were prepared in the facilities of Villa Vinifera (Brestovitsa, Plovdiv, Bulgaria). The grape was pressed in a hydraulic press and 60 mg L^{-1} SO_2 and 2g kg^{-1} Lallzyme cuvée blanc was added. The must was cooled down to 8°C and when clarification occurred the precipitates were removed by filtration. The filtrated must was transferred to a fermentation vessel, warmed to 15°C and inoculated with LalvinD47 (0.25 g L^{-1}). The must was divided in seven vessels – one control and six variants (17 L each) and to each vessel (without the control one) was added rose waste: W1–8.5g (0.05%); W2–17g (0.1%), W3–42.5g (0.25%), W4–85g (0.5%), W5–170g (1%) and W6–340g (2%). The fermentation continued 22 days at 16±1°C and the solid substances were removed by filtration. A combined agent for wine treatment consisted of plant protein, bentonite and polyvinylpolypyrrolidone (Polymust press) was added (6 g per each). The wines were filtered,

bottled with cork stoppers and stored in dark at 18±1°C.

2.2.2.Analytical

The ethanol content was determined by the pycnometric method (Cioch-Skoneczny *et al.*, 2021). Total polyphenols were determined according to Singleton and Rossi (1965) with Folin-Ciocalteu's reagent. Gallic acid was employed as calibration standard and the results were expressed as gallic acid equivalents (GAE) per liter of wine. The antioxidant activities were evaluated by [2,2-diphenyl-1- picrylhydrazyl] radical (DPPH) and Ferric Reducing Antioxidant Power (FRAP) methods as described by Slavov *et al.* (2017). The amount of total monomeric anthocyanins was determined by the pH-differential method (Giusti and Wrolstad, 2001). Briefly, the wine samples were diluted in parallel with two buffer solutions: 0.025 M KCl with pH 1.0 and 0.4 M sodium acetate with pH 4.5. After one hour at room temperature (22±1°C) absorption at 520 and 700 nm were measured (1 cm cuvette; spectrophotometer Helios Omega UV-Vis with VISION*lite* software (Thermo Fisher Scientific, Madison, USA)). The results were calculated using molar absorption coefficient 26900 L mol^{-1} cm^{-1}, molecular mass of 449.2 g mol^{-1} and were expressed as equivalents cyaniding-3-glucoside per liter.

The color characteristics of wines were determined with a Helios Omega UV-Vis spectrophotometer equipped with VISION*lite* ColorCalc Basic software (Thermo Fisher Scientific, USA) using 1 cm cuvettes. Spectra were recorded in a 380–780 nm range at intervals Δλ=2 nm. CIELCh color coordinates were calculated using standard illuminant D 65 and 10° observer angle.

The relative proportion of red color from anthocyanes' flavylium cations, *d*A(%), was calculated using the equation (1), according to Azar *et al.* (1990):

$$dA(\%) = \left(1 - \frac{A420 - A620}{2 \times A520}\right) x\ 100 \quad (1)$$

where, A420, A520 и A620 are the values of absorption at 420, 520 and 620 nm, respectively.

Individual phenolic acids were determined as described by Terzieva *et al.* (2017) with an HPLC system ELITE LaChrome (Hitachi, Japan) equipped with diode array detector Elite LaChrome L-2455. The separation was performed on Supelco Discovery HS C_{18} column (5 μm × 25 cm × 4.6 mm) operated at 30°C under gradient conditions with mobile phase consisting of 2% (v/v) acetic acid (mobile phase A) and acetonitrile (mobile phase B) at a flow rate 0.8 mL min^{-1}. The gradient used was: 0–1 min: 95% A and 5% B; 1-40 min: 50% A and 50% B; 40-45 min: 100% B; 46-50 min: 95% A and 5% B. The gallic, protocatechuic and cinnamic acids were detected at 280 nm and the chlorogenic, caffeic, ferulic, p-coumaric, sinapic, rosmarinic and chicoric acids – at 320 nm.

The composition of aromatized wines was investigated by gas chromatography with flame ionization detector (GC-FID) and gas chromatography with mass selective detector (GC-MS). The GC-FID analyses were performed on Shimadzu GC-17A (Shimadzu, Japan) equipped with TEKNOKROMA TRB-WAX column (30m×0.32mm×0.25μm) and software GC Solution (Shimadzu, Japan). Sample amount: 1μL; injector temperature: 229°C; career gas pressure: 32 κPa; career gas speed: 1 mL min^{-1}; detector temperature: 250°C; temperature regimen of the column: starting from 40°C, hold for 1 min, increase with 5°C min^{-1} until 100°C, hold for 10 minutes and increase with 15°C min^{-1} until 220°C.

The GC-MS analyses were performed as follow:

1). Non-volatile polar substances: 0.2 mL ethanolic extract was lyophilized and 50 μL pyridine and 50 μL N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were added. The sample was incubated at 70°C for 40 min. For analysis 1.0μL from the solution was injected on gas chromatograph Agilent GC 7890 (Agilent Technologies, Palo Alto, CA, USA) with mas-selective detector Agilent MD 5975 and column HP-5ms (30m × 0.32mm ×0.25μm thicknesses). The following temperature regimen was used: initial temperature 100°C

(hold for 2 min) then increased to 180°C with 15°C min^{-1} (hold for 1 min) and increase of the temperature to 300°C with 5°C min^{-1} (hold for 10 min); injector and detector temperatures – 250°C, helium was used as carrier gas at flow rate 1.0 mL/min. The scanning range of mass-selective detector was m/z = 50 – 550 in split-split mode (10:1).

2). Volatile substances: The aroma substances were extracted according to the procedure described by Uekane et al. (2017). The analyses were performed with gas chromatograph Agilent GC 7890 with mas-selective detector Agilent MD 5975 and Agilent DB-5ms (30 m × 0.25 mm × 0.25 μm) column. The following temperature regimen was used – initial temperature was 40°C and then increase to 300°C with 5°C min^{-1} (hold for 10 min); injector and detector temperatures – 250°C, helium was used as carrier gas at 1.0mL/min. The scanning range of mass-selective detector was m/z = 40-400 in splitless mode.

The individual compounds were identified comparing the retention times and the relative index (RI) with those of standard substances (linear n-alkanes (C_8–C_{40}) injected under the same conditions) and mas-spectral data from libraries of The Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) and NIST'08 (National Institute of Standards and Technology, USA).

2.2.3.Sensory analysis of aromatized wines

Sensory evaluation was performed according to ISO 13299:2016 with the following indicators: color intensity, aroma intensity, fruity nuances, flowery nuances, grassy nuances, taste intensity, acidity, and bitterness. Briefly, the bottles (15±0.5°C) were opened, poured in wineglasses and served coded to 21 (22-52 years old) untrained consumers. The degree of liking was based on an eleven-point scale (0: absence of the specified indicator, 10: extremely sensing the specified parameter). Organoleptic evaluation was done in three repetitions, and the values of individual attributes were averaged and added together.

2.2.4.Statistical analysis

The analyses were performed in triplicate and the data were given as mean values. Statistical significance was detected by analysis of variance (ANOVA, Tukey's HSD test; value of $p<0.05$ indicated statistical difference).

3.Results and discussions

3.1.Preparation and characterization of aromatized wines

3.1.1.Preparation and physico-chemical characteristics

Mavrud is among the highly valued local grape varieties. It is specific for the Western Thrace wine region of Bulgaria and traditionally is used for production of red wines. During the last years a tendency for making rosé wines on the basis of Mavrud was observed. The wines are distinctive with well-balanced fruity aroma bouquet and elegant taste of wild forest berries. Six variants with different amounts of added waste and control rosé wine were prepared based on preliminary experiments. At the end of fermentation flavor intensity was amplified in the variants with higher amounts of added rose waste and grassy and incomplete nuances were sensed. After removal of the precipitations and clarification the flavor was significantly improved and more harmonious nuances were detected while the grassy notes significantly decreased. The alcoholic content was in the 14.4-14.6±0.2 % (v) range and pH 3.43–3.62±0.1.

The total monomeric anthocyanins (Table 1) increased in W2 compared to control but then decreased in W4-W6 and the same trend was observed for the relative part of the red color due to flavilium cations of anthocyanins. In general the process of distillation of roses led to destruction of anthocyanins and the waste could not contribute to the final wine anthocyanin content. Slight differences in the color shades were observed: the lower the added rose waste, the more intensive peony color was obtained and this observation was confirmed with the increase of hue angle value – 46.21±0.84 for the control wine and 54.95±0.70 for the W6. From the other side the higher content of polyphenols in the

rose waste could probably contribute to stabilization of the anthocyanins in the beverage due to co-pigmentation (Shikov *et al.*, 2012). The total polyphenol content (TPP) increased significantly from 355.01±10.14 μmol GAE L^{-1} for the control wine to 576.08±12.08 μmol GAE L^{-1} for W6 which is due to extraction of polyphenols during maceration and co-fermentation. The increased TPP resulted in significant augmentation of antioxidant activity: for control wine 668.33±21.28 (by DPPH) and 918.33±15.64 mg TE L^{-1} (by FRAP) compared to W6 – 1991.67±23.95 (by DPPH) and 2850.00±24.85 mg TE L^{-1} (by FRAP).

Table 1. Physico-chemical characteristics of control and aromatized wines

	C	W1	W2	W3	W4	W5	W6
TMA, mg CG L^{-1}	2.62± 0.10^{a}	2.57± 0.12^{a}	2.94± 0.11^{b}	2.52± 0.10^{a}	2.38± 0.11^{a,c}	2.24± 0.12^{c}	2.06± 0.11^{c}
TPP, μmol GAE L^{-1}	355.01± 10.14^{a,b}	344.98± 15.21^{a,b}	355.27± 11.18^{a,b}	375.32± 12.08^{b,c}	395.44± 14.51^{c,d}	412.12± 10.14^{d}	576.08± 12.08^{e}
DPPH, mg TE L^{-1}	668.33± 21.28^{a}	558.33± 19.84^{b}	712.50± 18.30^{c}	885.00± 21.35^{d}	1016.67± 17.84^{e}	1423.33± 26.34^{f}	1991.67± 23.95^{g}
FRAP, mg TE L^{-1}	918.33± 15.64^{a}	941.67± 18.41^{a}	1085.00± 17.74^{b}	1288.33± 16.95^{c}	1488.33± 21.54^{d}	2091.67± 19.62^{e}	2850.00± 24.85^{f}
C (Chroma)	23.10± 0.68^{a,c}	22.81± 0.88^{a,c}	25.43± 0.72^{b,c}	22.81± 0.81^{a,c}	21.83± 0.95^{a}	23.80± 0.81^{a,c}	23.94± 0.72^{c}
h (Hue angle)	46.21± 0.84^{a}	45.34± 1.01^{a}	46.32± 0.74^{a}	44.33± 0.83^{a}	46.78± 0.84^{a}	49.56± 0.68^{b}	54.95± 0.70^{c}
L (Lightness)	82.10± 0.88^{a}	77.07± 1.15^{b,c}	79.96± 1.08^{b,a}	77.88± 1.17^{b,c}	76.06± 1.21^{c}	80.34± 1.35^{b,a}	79.86± 1.41^{b,a}
a	17.22± 0.45^{a}	17.11± 0.61^{a}	18.84± 0.48^{b}	17.25± 0.51^{a}	15.74± 0.49^{c}	16.42± 0.54^{a,c}	14.78± 0.57^{d}
b	15.51± 0.41^{a}	15.12± 0.61^{a}	17.17± 0.48^{b}	15.04± 0.74^{a}	15.12± 0.46^{a}	17.37± 0.50^{b}	18.92± 0.49^{c}
CI, *d*A %	62.53± 1.05^{a}	65.28± 1.16^{b}	53.32± 1.21^{c}	52.15± 1.30^{c}	56.21± 1.50^{d}	46.34± 1.17^{e}	41.02± 1.24^{f}

TMA – total monomeric anthocyanins; CG – cyanind-3-glucoside; TPP – total polyphenolic content; GAE – gallic acid equivalents; TE – Trolox equivalents; CI, *d*A% – relative part of the red color due to flavilium cations of anthocyanins; $^{a, b, c, d, e, f, g}$ Values with different letters in a raw are statistically different (Tukey's HSD test, $p < 0.05$)

3.1.2.HPLC determination of phenolic acids

Individual phenolic acids were determined by HPLC (Table 2). The highest increase was detected for gallic acid and 3,4-dihydroxy benzoic acid: from 1.2±0.9 mg L^{-1} and 5.4±0.2 mg L^{-1} in the control wine to 25.9±0.9 mg L^{-1} and 65.1±1.1 mg L^{-1} in W6, respectively. The increase of phenolic acids quantity in W3-W6 compared to control and W1-W2 could be explained with the addition of higher amounts of rose waste and subsequent extraction during fermentation, having in mind that the rose wastes are rich source of polyphenols (*Shikov et al.*, 2012). The higher amounts of phenolic acids determined was also related to increase in the antioxidant capacity of the aromatized wines from W1 to W6 (Table 1) and this could be explained with the higher amounts of total polyphenols extracted from the rose waste but not the anthocyanins (no significant difference in the TMA amounts in all wines).

Table 2. Phenolic acids in control and aromatized wines

Compound, mg L^{-1}	C	W1	W2	W3	W4	W5	W6
Gallic acid	1.2±0.9^{a}	3.3±0.9^{b}	4.1±0.8^{b}	8.8±0.7^{c}	17.9±0.5^{d}	19.2±0.7^{d}	25.9±0.9^{e}
3,4-dihydroxy benzoic acid	5.4±0.2^{a}	9.8±0.4^{b}	15.0±0.6^{c}	22.4±0.3^{d}	24.2±0.9^{d}	32.4±1.0^{e}	65.1±1.1^{f}
Chlorogenic acid	traces	0.1±0.0^{a}	traces	traces	10.8±0.5^{b}	11.7±0.6^{b}	10.2±0.8^{b}
Caffeic acid	0.7±0.2^{a}	0.7±0.2^{a}	0.7±0.2^{a}	1.8±0.1^{b}	1.9±0.1^{b}	2.3±0.2^{b}	3.4±0.2^{c}
Ferulic acid	traces	nd	traces	0.1±0.0^{a}	traces	traces	0.2±0.0^{a}
p-Coumaric acid	2.7±0.5^{a}	2.7±0.5^{a}	2.7±0.5^{a}	2.5±0.5^{a}	3.2±0.4^{a,b}	4.0±0.7^{b,c}	4.5±0.3^{c}
Sinapic acid	1.0±0.3^{a}	0.9±0.3^{a}	1.0±0.2^{a}	1.1±0.4^{a}	1.2±0.2^{a}	1.5±0.3^{a,b}	1.8±0.3^{b}
Rosmarinic acid	traces	traces	traces	traces	traces	traces	0.9±0.2
Cinnamic acid	traces	traces	traces	traces	traces	0.1±0.0^{a}	0.2±0.0^{a}

$^{a, b, c, d,e}$ Values with different letters in a raw are statistically different (Tukey's HSD test, $p < 0.05$)

Table 3. GC-FID analysis of control and aromatized wines

Compound, mg L^{-1}	C	W1	W2	W3	W4	W5	W6
Acetaldehyde	17.1± 1.2^{a}	19.9± 0.9^{a}	20.3± 1.3^{a,b}	22.2± 1.0^{b,c}	24.1± 0.9^{c}	25.2± 1.1^{c,d}	27.0± 0.9^{d}
Ethyl acetate	32.7± 2.1^{a}	33.8± 2.3^{a}	34.7± 2.0^{a}	33.3± 2.3^{a}	36.0± 1.9^{a,b}	37.6± 1.8^{b}	37.7± 1.9^{b}
Methanol	34.2± 2.1^{a}	35.1± 2.0^{a}	35.2± 2.3^{a}	36.4± 2.1^{a,b}	37.2± 2.5^{a,b}	38.7± 2.6^{b}	39.1± 1.8^{b}
2-butanol	29.4± 0.9^{a}	30.2± 1.2^{a,b}	32.8± 1.2^{a,b}	33.3± 1.1^{b}	29.0± 1.1^{a}	33.7± 1.2^{b}	34.0± 1.2^{b}
1-propanol	45.8± 0.9^{a}	45.9± 1.0^{a}	44.9± 1.1^{a}	46.4± 1.0^{a,b}	46.1± 1.1^{a,b}	47.7± 1.1^{a,b}	48.0± 1.0^{b}
i-butanol	27.3± 1.5^{a,b}	24.2± 1.4^{b,c}	28.1± 1.6^{a,b,d}	23.8± 1.5^{c}	25.6± 1.7^{a,b,c,d}	26.2± 1.7^{a,b,c,d}	28.3± 1.3^{d}
1-butanol	7.0± 0.3^{a}	7.2± 0.3^{a}	8.0± 0.2^{b}	8.6± 0.4^{b,c}	8.7± 0.2^{b,c}	9.0± 0.3^{c,d}	9.8± 0.3^{d}
i-amyl alcohols	7.9± 0.4^{a}	8.7± 0.5^{a}	8.7± 0.4^{a}	8.6± 0.3^{a}	8.6± 0.4^{a}	8.7± 0.5^{a}	8.9± 0.4^{a}
Sum of higher alcohols	117.4± 1.5^{a}	116.2± 1.4^{a}	122.5± 1.6^{b}	120.7± 1.5^{a,b}	118.0± 1.7^{a}	125.3± 1.7^{b}	129.0± 1.3^{c}

$^{a, b, c, d}$ Values with different letters in a raw are statistically different (Tukey's HSD test, $p < 0.05$)

3.1.3.GC-FID analyses

The GC-FID analysis (Table 3) revealed slight increase of acetaldehyde, ethyl acetate and methanol from W1 to W6 compared to control. The increased methanol content could be explained with the presence of pectic substance in the rose waste (Slavov *et al.*, 2017). Nevertheless, the amounts determined (even in W6) were within the permissible limits: for example the methanol limit is 250 mg L^{-1} for white and rosé wines and the higher amount observed in W6 was 39.1±1.8 mg L^{-1} (Compendium of international methods of analysis – OIV, 2018). The quantity of ethyl acetate determined was in the 32.7±2.1 – 37.7±1.9 mg L^{-1} range. An aroma similar to acetone is sensed if the concentration of ethyl acetate exceeds a threshold reported most often as being between 100-200 mg L^{-1} (Cliff and Pickering, 2006). Ethyl acetate concentrations below the threshold can contribute to the depth of body, richness and sweetness of wine and between 30-80 mg L^{-1} ethyl acetate can add to the wine character and be a part of the pleasant wine bouquet (Plata et al., 2003). The amounts of higher alcohols increased in the W1-W6 variants (except for i-amyl alcohols) compared to the control wine. Higher alcohols, also known as fusels, plays and important role in the formation of wine aroma and at concentrations below 300 mg L^{-1} positively influence aroma. The higher amounts negatively affect the proper bouquet of the wine. The aromatized wines and the control rosé had a total amounts of fusels in the 117.4-129.0±1.5 mg L^{-1} range and it could be concluded that addition of rose waste during fermentation did not affect negatively formation of higher alcohols.

3.1.4.GC-MS analyses – determination of polar volatile and non-volatile compounds

Furthermore the aromatized wines were subjected to GC-MS analysis. The preparation of rosé wines with Mavrud grape is a rare practice since this regional grape variety is mostly used for red wine preparation and to the best of our knowledge this is the first report for GC-MS profiling of Mavrud rosé. As a result of the analysis thirty nine polar non-volatile metabolites (amino acids, sugars, acids, sugar alcohols and sterols) were tentatively detected in the control and aromatized wines. In general increase in the content of most of the detected substances from control to W6 was observed (Table 4 and 5).

Table 4. Polar non-volatile metabolites in control and aromatized wines

Compound	RI	C	W1	W2	W3	W4	W5	W6
		% of TIC						
Lactic acid	1066	25.1± 0.7 $^{a, b}$	24.2± 0.6 ^{a}	24.8± 0.8 ^{a}	26.1± 0.5 ^{b}	24.3± 0.9^{a,b}	27.5± 0.4^{b,c}	29.2± 0.6 ^{c}
L-Valine	1228	10.6± 0.8 ^{a}	11.4± 0.9 $^{a, b}$	12.3± 0.8 $^{a, b, c}$	13.2± 0.7^{b,c,d}	13.9± 0.9 ^{c,d,e}	15.2± 0.7^{d,e}	15.9± 0.9 ^{e}
Glycerol	1266	426.3± 1.6 ^{a}	398.8± 1.4 ^{b}	431.7± 1.6 ^{c}	532.9± 2.1 ^{d}	586.8± 2.5 ^{e}	576.7± 2.0 ^{f}	592.4± 1.8^{e}
L-Leucine	1272	11.0± 0.7 ^{a}	11.9± 0.8 $^{a, b}$	12.7± 0.9 $^{a, b, c}$	13.8± 0.6^{b,c}	14.5± 0.7 ^{c}	12.9± 0.9^{a,b,c}	15.2± 0.8 ^{c}
Phosphoric acid	1278	88.0± 1.0 ^{a,b}	85.2± 0.9 ^{a}	90.3± 1.2 ^{b}	92.9± 1.1^{b,c}	94.2± 1.0 ^{c}	93.3± 1.5^{b,c}	95.4± 1.2 ^{c}
L-Isoleucine	1299	10.1± 0.5 ^{a}	10.8± 0.6 ^{a}	11.4± 0.5 $^{a, b}$	12.6± 0.6 ^{b}	14.3± 0.7 ^{c}	14.9± 0.5 ^{c}	15.6± 0.7 ^{c}
L-Proline	1307	42.0± 0.9 ^{a}	41.5± 0.7 ^{a}	49.3± 0.8 ^{b}	52.5± 1.0 ^{c}	58.4± 0.9 ^{d}	57.5± 0.8 ^{d}	59.9± 0.9 ^{d}

Succinic acid	1310	188.2± 1.9 [a]	187.2± 2.5 [a]	190.1± 2.0 [a, b]	191.2± 1.8[a,b]	195.4± 1.9 [b]	196.8± 1.6 [b]	195.1± 1.7 [b]
Glyceric acid	1339	55.7± 1.8 [a]	57.6± 1.2 [a]	62.4± 1.0 [b]	69.7± 1.4 [c]	72.9± 1.0 [d]	74.0± 1.1[d,e]	75.4± 1.2 [e]
Fumaric acid	1355	28.0± 1.1 [a]	27.9± 0.9 [a]	30.3± 0.8 [a]	35.0± 1.0 [b]	36.9± 0.8[b,c]	38.3± 0.9[c,d]	39.9± 1.0 [d]
Serine	1362	12.4± 0.6 [a]	13.5± 0.4 [a]	12.4± 0.8 [a]	15.5± 0.5[b,d]	16.8± 0.4 [c, d]	16.0± 0.5[d]	17.1± 0.6[c,d]
L-Threonine	1390	14.8± 0.8 [a]	16.0± 0.7 [a]	17.3± 0.5 [b]	18.4± 0.4 [c]	20.3± 0.7 [d]	21.4± 0.5[d,e]	22.5± 0.6 [e]
L-Malic acid	1488	297.0± 2.0 [a]	298.8± 1.1 [a]	299.9± 1.0 [a]	301.3± 1.9[a,b]	298.4± 1.8 [a]	302.3± 1.9[a,b]	305.6± 1.8 [b]
Pyroglutamic acid	1512	65.6± 0.9 [a]	69.8± 0.8 [b]	74.2± 0.7 [c]	82.0± 0.6 [d]	85.8± 0.8 [e]	86.7± 0.8[e,f]	88.3± 0.9 [f]
Salycilic acid	1516	24.0± 0.4 [a]	25.9± 0.8 [a,b]	26.3± 0.7 [b]	27.0± 0.8 [b]	25.2± 0.7[a,b]	26.9± 0.5 [b]	25.8± 0.4[a,b]
L-Aspartic acid	1531	11.0± 0.8 [a]	12.1± 0.9 [a]	12.8± 0.8 [a, b, c]	13.7± 0.9[b,c,d]	14.1± 0.7[c,d]	15.2± 0.8 [d]	15.9± 0.7 [d]
L-Threonic acid	1528	88.0± 1.2 [a]	95.2± 1.0 [b]	104.3± 1.1 [c]	110.0± 1.3 [d]	109.2± 1.1 [d]	111.4± 1.0 [d]	112.9± 1.1 [d]
L-(+)-Tartaric acid	1612	224.3± 1.6 [a]	228.4± 1.7 [b]	222.1± 1.4 [a]	230.8± 1.5 [b]	231.2± 1.1 [b]	229.9± 1.2 [b]	235.0± 1.3 [c]
L-Phenylalanine	1646	13.4± 0.9 [a]	14.4± 0.8 [a, b]	15.7± 0.7 [a, b, c]	16.8± 1.0[b,c,d]	17.3± 0.8[c,d]	17.8± 0.7 [d]	18.0± 0.5 [d]
Vanillic acid	1758	16.1± 1.0 [a]	16.9± 0.7 [a]	19.5± 0.5 [b]	20.2± 0.7[b,c]	21.1± 0.6[c,d]	22.0± 0.7 [d]	22.8± 0.9 [d]
Protocatechuic acid	1813	18.6± 0.6 [a]	19.5± 0.5 [a]	21.2± 0.8 [b]	23.3± 0.5 [c]	24.9± 0.8 [d]	25.3± 0.9 [d]	26.1± 0.5 [d]
Quinic acid	1843	22.5± 0.8 [a]	24.3± 0.9 [a]	27.8± 0.8 [b]	28.1± 0.7 [b]	29.8± 0.9[b,c,d]	30.5± 0.6[c,d]	30.4± 0.7 [d]
Fructose	1862	66.5± 1.4 [a]	70.1± 0.9 [b]	78.3± 1.0 [c]	83.1± 1.3 [d]	85.0± 1.0 [d]	88.2± 1.1 [e]	90.7± 0.9 [e]
Galactose	1884	62.3± 0.8 [a]	66.7± 0.9 [b]	69.2± 1.1 [c]	77.9± 0.9 [d]	79.3± 0.8[d,e]	80.4± 0.9[d,e]	80.8± 0.8 [e]
Syringic acid	1888	16.1± 0.6 [a]	17.2± 0.7 [a]	19.5± 0.8 [b]	20.2± 0.8 [b, c]	19.1± 0.7 [b]	20.5± 0.6[b,c]	21.4± 0.7 [c]
Glucose	1896	169.0± 1.9 [a]	181.5± 1.4 [b]	208.4± 1.5 [c]	211.3± 1.8 [c]	219.4± 1.9 [d]	215.8± 1.4 [e]	218.7± 1.5[d,e]
Glucitol	1930	60.9± 0.8 [a]	65.4± 0.7 [b]	66.9± 0.5 [c]	76.1± 0.9 [d]	77.8± 0.7 [d]	75.9± 0.9 [d]	78.3± 0.8 [d]
Gluconic acid	1991	36.8± 0.9 [a]	37.9± 0.8 [a]	41.2± 0.7 [b]	46.0± 0.7 [c]	45.8± 0.8 [c]	46.9± 1.0 [c]	47.8± 1.1 [c]
Palmitic acid	2039	54.7± 1.0 [a]	59.8± 1.1 [b]	63.5± 1.0 [c]	68.4± 0.9 [d]	66.3± 0.8 [d]	67.5± 0.9 [d]	67.6± 0.7 [d]
Glucaric acid	2013	27.7± 0.7 [a]	28.5± 0.8 [a, b]	30.8± 0.8 [b]	34.6± 0.9 [c]	35.7± 1.0 [c]	33.9± 1.1 [c]	36.8± 1.0 [c]

Myo-Inositol	2090	8.4± 0.6 [a]	8.9± 0.5 [a, b]	9.2± 0.6 [a, b, c]	10.5± 0.7[b,c]	10.9± 0.5 [c]	9.9± 0.8[a,b,c]	10.8± 0.6[b,c]
Stearic acid	2132	32.7± 1.1 [a]	31.5± 1.2 [a]	35.7± 1.3 [b]	40.9± 1.0 [c]	42.4± 0.8[c,d]	43.1± 0.9 [d]	43.8± 0.9 [d]
Caffeic acid	2140	14.5± 0.9 [a]	15.2± 0.7 [a]	16.9± 0.8 [a, b]	18.2± 0.8[b,c]	18.9± 0.5 [c]	18.5± 0.7 [c]	19.3± 0.6 [c]
Linoleic acid	2209	48.3± 1.0 [a]	51.9± 0.8 [b]	55.5± 0.9 [c]	60.3± 1.1 [d]	62.5± 1.0[d,e]	61.9± 1.1[d,e]	64.1± 1.0 [e]
α-Linolenic acid	2217	23.9± 0.8 [a]	24.8± 0.7 [a]	28.1± 0.4 [b]	29.8± 0.9 [c]	31.2± 0.8[c,d]	32.4± 0.7 [d]	30.3± 0.8[c,d]
Sucrose	2649	63.4± 1.1 [a]	66.8± 1.0 [b]	68.9± 1.1 [b]	79.2± 1.0 [c]	77.8± 1.2 [c]	78.9± 1.1 [c]	79.9± 1.0 [c]
Turanose	2742	35.1± 1.3 [a]	39.8± 1.0 [b]	42.7± 1.2 [c]	43.9± 1.1 [c]	42.3± 1.2 [c]	41.9± 1.0[b,c]	40.8± 1.1[b,c]
Stigmasterol	3315	12.3± 0.7 [a]	14.1± 0.8 [a, b]	13.3± 0.9 [a, b]	15.4± 0.8[b,c]	16.0± 0.7 [c]	15.8± 0.9 [c]	16.7± 0.8 [c]
β-Sitosterol	3355	11.7± 0.8 [a]	12.9± 0.7 [a, b]	12.7± 0.5 [a]	14.6± 0.7[b,c]	15.7± 0.8 [c]	15.4± 0.7 [c]	15.8± 0.8 [c]

RI: relative index (Kovats retention index)
TIC: total ion current
The results are presented as mean ± SD (n=3)
[a, b, c, d, e, f, g] Values with different letters in a raw are statistically different (Tukey's HSD test, $p < 0.05$)

Table 5. Polar volatile (aroma) substances in control and aromatized wines

Compound	**RI**	**C**	**W1**	**W2**	**W3**	**W4**	**W5**	**W6**
		% of TIC						
Alcohols								
Propan-1-ol	599	0.36± 0.08 [a]	0.35± 0.05[a]	0.36± 0.06[a]	0.37± 0.07[a]	0.38± 0.05[a]	0.38± 0.04[a]	0.39± 0.08[a]
Butan-1-ol	660	0.30± 0.08 [a]	0.30± 0.07[a]	0.31± 0.04[a]	0.31± 0.05[a]	0.30± 0.06[a]	0.30± 0.06[a]	0.30± 0.05[a]
Pentan-1-ol	768	0.40± 0.04 [a]	0.41± 0.02[a]	0.40± 0.04[a]	0.40± 0.05[a]	0.41± 0.03[a]	0.42± 0.04[a]	0.42± 0.02[a]
Hexan-1-ol	867	1.80± 0.10 [a]	1.79± 0.10[a]	1.84± 0.09[a]	1.85± 0.09[a]	1.86± 0.07[a]	1.86± 0.08[a]	1.87± 0.06[a]
Heptan-1-ol	912	0.48± 0.07 [a]	0.49± 0.06[a]	0.49± 0.05[a]	0.50± 0.05[a]	0.50± 0.07[a]	0.50± 0.06[a]	0.50± 0.05[a]
Octan-1-ol	993	0.60± 0.06 [a]	0.59± 0.06[a]	0.61± 0.07[a]	0.61± 0.06[a]	0.61± 0.06[a]	0.61± 0.07[a]	0.62± 0.06[a]
Nonan-1-ol	1170	0.21± 0.01 [a]	0.21± 0.01[a]	0.21± 0.01[a]	0.21± 0.02[a]	0.21± 0.03[a]	0.22± 0.02[a]	0.23± 0.03[a]
Decan-1-ol	1272	0.25± 0.04 [a]	0.26± 0.03[a]	0.27± 0.04[a]	0.26± 0.03[a]	0.27± 0.04[a]	0.27± 0.04[a]	0.28± 0.06[a]
Acids								
Acetic acid	640	0.41± 0.06 [a]	0.41± 0.05[a]	0.42± 0.06[a]	0.42± 0.07[a]	0.41± 0.05[a]	0.42± 0.06[a]	0.43± 0.04[a]

Butanoic acid	785	0.32± 0.07 [a]	0.31± 0.06[a]	0.31± 0.05[a]	0.33± 0.04[a]	0.33± 0.04[a]	0.33± 0.05[a]	0.33± 0.06[a]
Octanoic acid	1192	5.88± 0.09 [a]	5.89± 0.08[a]	5.95± 0.06[a]	6.07± 0.09[a]	6.08± 0.08[a]	6.11± 0.05[a]	6.13± 0.04[a]
Nonanoic acid	1281	0.76± 0.10 [a]	0.77± 0.08[a]	0.78± 0.06[a]	0.78± 0.09[a]	0.79± 0.07[a]	0.78± 0.08[a]	0.79± 0.07[a]
Decanoic acid	1388	6.90± 0.11 [a]	6.95± 0.08[a]	7.03± 0.09[a]	7.12± 0.12[a]	7.09± 0.09[a]	7.13± 0.06[a]	7.15± 0.05[a]
Dodecanoic acid	1573	1.48± 0.08 [a]	1.49± 0.05[a]	1.50± 0.06[a]	1.53± 0.09[a]	1.54± 0.08[a]	1.55± 0.07[a]	1.56± 0.05[a]
Tetradecanoic acid	1774	0.30± 0.06 [a]	0.30± 0.04[a]	0.31± 0.03[a]	0.31± 0.04[a]	0.32± 0.05[a]	0.32± 0.04[a]	0.33± 0.03[a]
Aldehydes								
Acetaldehyde	400	0.27± 0.06 [a]	0.30± 0.05[a]	0.30± 0.05[a]	0.28± 0.05[a]	0.34± 0.04[b]	0.38± 0.06[c]	0.42± 0.05[d]
Hexanal	800	1.72± 0.09 [a]	1.73± 0.05[a]	1.74± 0.08[a]	1.77± 0.10[a]	1.78± 0.09[a]	1.79± 0.07[a]	1.78± 0.08[a]
Decanal	1205	0.87± 0.08 [a]	0.87± 0.07[a]	0.88± 0.06[a]	0.90± 0.09[a]	0.90± 0.07[a]	0.91± 0.06[a]	0.92± 0.07[a]
Hydrocarbons								
Hexadecane	1600	2.81± 0.10 [a]	2.83± 0.09[a]	2.86± 0.08[a]	2.91± 0.11[a]	2.92± 0.08[a]	2.93± 0.09[a]	2.95± 0.10[a]
Octadecane	1800	3.34± 0.11 [a]	3.36± 0.08[a]	3.41± 0.09[a]	3.45± 0.12[a]	3.44± 0.09[a]	3.46± 0.10[a]	3.48± 0.09[a]
Nonadecane	1900	2.68± 0.09 [a]	2.69± 0.07[a]	2.71± 0.08[a]	2.76± 0.14[a]	2.77± 0.08[a]	2.75± 0.10[a]	2.79± 0.08[a]
Eicosane	2000	2.01± 0.12 [a]	2.02± 0.08[a]	2.05± 0.06[a]	2.08± 0.09[a]	2.08± 0.09[a]	2.10± 0.08[a]	2.12± 0.07[a]
Heneicosane	2100	2.10± 0.10 [a]	2.11± 0.08[a]	2.15± 0.07[a]	2.17± 0.09[a]	2.18± 0.08[a]	2.19± 0.09[a]	2.22± 0.10[a]
Docosane	2200	1.76± 0.08 [a]	1.78± 0.06[a]	1.77± 0.09[a]	1.82± 0.11[a]	1.85± 0.10[a]	1.87± 0.08[a]	1.89± 0.11[a]
Terpenes								
Linalool	1097	1.53± 0.10 [a]	1.54± 0.09[a]	1.57± 0.08[a]	1.58± 0.14[a]	1.63± 0.09[a]	1.69± 0.09[a]	1.75± 0.08[a]
Phenethyl alcohol	1110	nd	1.48± 0.11[a]	1.95± 0.08[b]	2.18± 0.14[b]	2.68± 0.11[c]	2.99± 0.10[d]	3.44± 0.12[e]
Cis-Rose oxide	1112	0.08± 0.05 [a]	0.49± 0.11[b]	0.57± 0.10[b]	0.69± 0.13[b,c]	0.86± 0.14[c]	1.24± 0.09[d]	1.68± 0.08[e]
Trans-Rose oxide	1127	0.10± 0.06 [a]	0.57± 0.05[b]	0.61± 0.08[b]	0.59± 0.07[b]	0.89± 0.11[b]	1.11± 0.09[c]	1.79± 0.08[d]
β-Citronellol	1228	nd	0.12± 0.02[a]	0.15± 0.03[a,b]	0.16± 0.01[a,b]	0.18± 0.02[b]	0.19± 0.01[b]	0.25± 0.02[c]
Geraniol	1255	nd	0.63± 0.11[a]	0.69± 0.10[a]	0.78± 0.09[a]	1.25± 0.11[b]	1.68± 0.12[c]	2.19± 0.14[d]
Eugenol	1356	2.10± 0.09 [a]	2.15± 0.10[a]	2.18± 0.07[a]	2.17± 0.08[a]	2.19± 0.09[a]	2.22± 0.08[a]	2.28± 0.08[a]
***β*-Bourbonene**	1383	1.26± 0.11 [a]	1.27± 0.09[a]	1.29± 0.08[a]	1.30± 0.10[a]	1.33± 0.07[a]	1.34± 0.06[a]	1.38± 0.05[a]

***β*-Elemene**	1390	2.54± 0.12 [a]	2.57± 0.09[a]	2.63± 0.10[a]	2.62± 0.08[a]	2.64± 0.06[a]	2.66± 0.05[a]	2.69± 0.04[a]
***β*-Caryophyllene**	1419	2.93± 0.06 [a]	2.95± 0.07[a,b]	2.98± 0.08[a,b]	3.02± 0.09[a,b]	3.03± 0.08[a,b]	3.08± 0.06[a,b]	3.10± 0.07[b]
***β*-Cubebene**	1389	0.76± 0.05 [a]	0.76± 0.07[a]	0.77± 0.08[a]	0.78± 0.10[a]	0.80± 0.05[a]	0.85± 0.04[a]	0.89± 0.03[a]
***α*-Guaiene**	1438	1.08± 0.11 [a]	1.08± 0.10[a]	1.09± 0.06[a]	1.11± 0.10[a]	1.15± 0.12[a]	1.18± 0.07[a]	1.22± 0.06[a]
***α*-Humulene**	1454	1.51± 0.09 [a]	1.53± 0.08[a]	1.55± 0.08[a]	1.56± 0.08[a]	1.55± 0.07[a]	1.59± 0.08[a]	1.67± 0.07[a]
(*Z*)-*β*-Farnesene	1459	1.81± 0.08 [a]	1.82± 0.09[a]	1.86± 0.07[a]	1.87± 0.09[a]	1.96± 0.08[a,b]	2.05± 0.06[b]	2.15± 0.05[b]
Germacrene D	1479	3.18± 0.14 [a]	3.20± 0.08[a]	3.24± 0.09[a]	3.28± 0.11[a]	3.29± 0.10[a]	3.33± 0.09[a]	3.39± 0.08[a]
***δ*-Guaiene**	1508	1.88± 0.10 [a]	1.89± 0.09[a]	1.92± 0.07[a]	1.94± 0.12[a]	1.95± 0.11[a]	1.99± 0.08[a]	2.12± 0.09[a]
***δ*-Cadinene**	1524	2.42± 0.10 [a]	2.45± 0.08[a]	2.49± 0.06[a]	2.50± 0.09[a]	2.54± 0.08[a]	2.59± 0.08[a]	2.66± 0.07[a]

RI: relative index (Kovats retention index); TIC: total ion current; nd – not determined;The results are presented as mean ± SD (n=3)

[a, b, c, d, e] Values with different letters in a raw are statistically different (Tukey's HSD test, $p < 0.05$)

The major primary acids in wine grapes and subsequently in wines are tartaric, malic and depending from the grape variety but usually in minor amounts, citric acid *(*Bellman and Gallander, 1979). During the winemaking process and mainly fermentation, lactic, succinic, acetic and other acids could be formed and they play significant role in the final wine quality. The amounts of tentatively determined tartaric and malic acid are comparable for all variants of aromatized vines with control rosé with slight significant increase in W5 and W6. Malic acid is an important precursor of lactic acid through malolactic fermentation and the lactic acid formed is giving milder acidic taste *(*Bellman and Gallander, 1979). The amount of lactic acid found in all the variants is comparable which suggested that the added rose wastes of grape must did not influenced substantially the fermentation process.

A total of 41 volatile substances were tentatively detected (alcohols, acids, aldehydes, hydrocarbons and terpenes). Significant effect on the aroma substances formation/extraction in the aromatized wines and control were observed (Table 5) for β-citronellol, phenethyl alcohol, rose oxides, and geraniol. These substances were absent or present in the control in low amounts and appeared in the aromatized wines due to addition of rose waste. Phenethyl alcohol is among the compounds which contribute significantly to the favorable aroma of white and rosé wines (Cabaroglu and Canbas, 2002). β-caryophyllene, β-citronellol, phenethyl alcohol, rose oxides, and geraniol increased significantly and distinctive rose aroma in W2-W6 variants was sensed. The amounts of alcohols increased in the aromatized wines compared to control but this increase was insignificant. The amount of acetaldehyde increased significantly for W4-W6 variants which confirm the GC-FID analysis results.

3.2.Sensory analysis of aromatized wines

An important attribute of every new or modified food system is the consumers' opinion and for this reason in the subsequent experiments sensory analysis of aromatized wines was conducted (Figure 1). The results of the sensory tests revealed most of the panelists preferred W1 and W2 variants as wines with characteristics closer to the control rosé. This could be explained with the more traditionally oriented taste of the Bulgarian consumers

concerning wines. The variants W3 to W6 were characterized with more pronounced rose aftertaste, the grassy nuances became more intense, the bitterness increased (along with the astringency, although astringency was not included in the indicators of the sensory analysis but most of the panelists expressed such sensations), as well as the flowery nuances increased. The W5 and W6 were disliked by most of the panelists (Overall acceptability 2.1±0.8 and 2.0±0.8, respectively, compared with 6.1±1.2 for the control).

In general the panelists divided wines in three groups: 1). Control, W1 and W2; 2). W3 and W4; and 3). W5 and W6. The group one was preferred mostly by the traditionally oriented consumers. It is interesting to note the opinion of some of the consumers towards group 3: they gave highest marks to these variants based on their personal preferences for aromatized (especially with rose notes) low-alcoholic beverages.

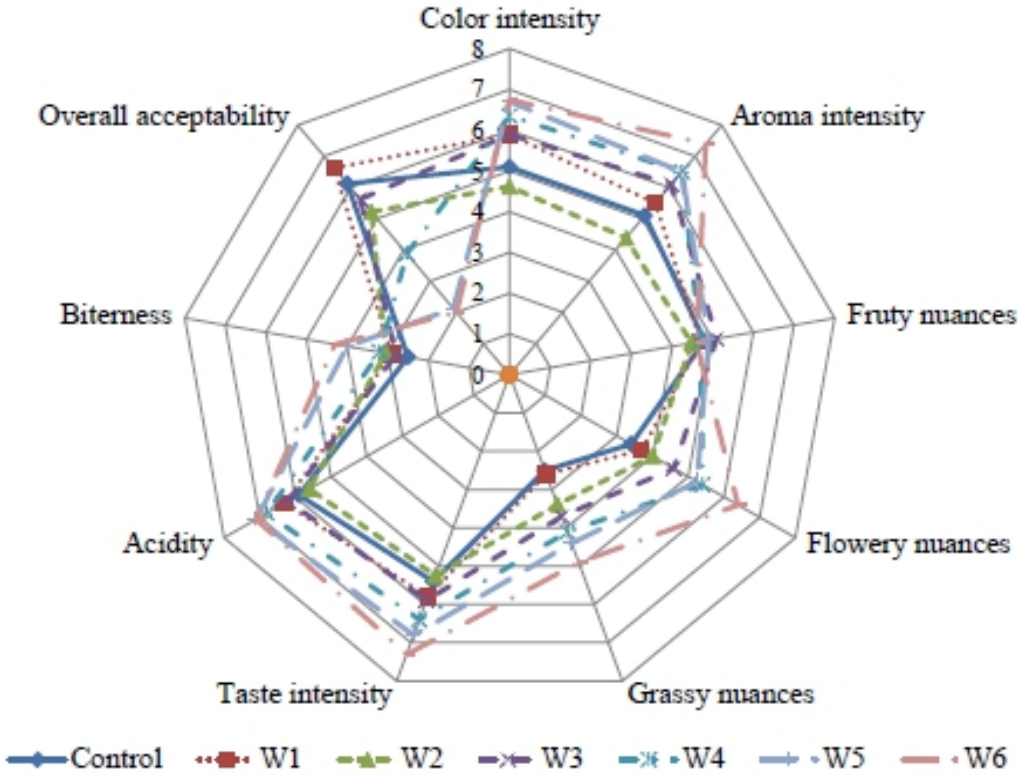

Figure 1. Sensory profile of wines

4. Conclusions

The present work explored the possibility for preparation and managing the quality of aromatized wines with addition of essential rose oil industry waste during grape must fermentation. To the best of our knowledge this is the first attempt for manufacturing of aromatized wines with addition of essential rose oil industry waste and the first experimental data for GC-MS profiling of Mavrud rosé. Control rosé and six variants with addition of different amounts of dry rose wastes (from 0.05% to 2%) were obtained. The aromatized wines and the control had alcoholic content in the 14.4-14.6±0.2 % range and pH 3.43÷3.59±0.1. The polyphenol content increased significantly from 355.01±10.14 to 576.08±12.08 μmol GAE L^{-1} for the control and W6, respectively. The higher amounts of phenolic acids (mainly gallic acid and 3,4-dihydroxy benzoic acid: from 1.2±0.9 mg L^{-1} and 5.4±0.2 mg L^{-1} in the control to 25.9±0.9 and 65.1±1.1 mg L^{-1} in W6, respectively) is also related to increase in the antioxidant capacity of aromatized wines from

W1 to W6 and this could be explained with the higher amounts of total polyphenols extracted from the rose waste but not the anthocyanins (no significant difference in the TMA amounts). The aromatized wines and the control rosé had a total amounts of fusels in the 117.4-129.0±1.5 mg L^{-1} range and it could be concluded that addition of rose waste during fermentation did not affect negatively formation of higher alcohols. β-caryophyllene, β-citronellol, phenethyl alcohol, rose oxides, and geraniol increased significantly and rose aroma in W1-W6 was achieved. The sensory analysis revealed W1 and W2 (overall acceptability 6.6±1.0 and 5.9±0.9, respectively) were considered with closer characteristics to control wine (overall acceptability 6.1±1.2) and more appropriate for consumption by the consumers. The overall interpretation of experimental data suggested that added rose wastes in the grape must during its fermentation, did not influenced substantially the fermentation process. The results of the present study confirmed the main hypothesis that rose oil industry by-products successfully could be utilized for preparation of aromatized wine and contributed for augmentation of total polyphenol content, antioxidant capacity, and new aroma profile of the final product was obtained.

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ASSESSMENT OF *MORING OLEIFERA* LEAVES AS NATURAL SOURCE TO PRODUCE HYPOLIPIDEMIC AND ANTIOXIDATIVE FUNCTIONAL FOOD

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ABSTRACT

The objectives of this work is to investigate the antioxidative and hypolipidemic effect of Moringa oleifera leaf powder(MOLP). For this purpose 36 Male rats were assigned into six groups, each of six rats; negative control group feeding with basal diet, positive control group feeding with high cholesterol diet (HCD) and three tested groups fed on HCD and different ratios of MOLP; 25, 50 or 75g/kg diet, in addition to initial group, the feeding period was 45 days.

At the end of feeding period, compared to the positive control group, all tested groups demonstrated decrease in body weight. Also , as compared to the positive control, significant decreases in serum biological parameters were found, including; glucose, total lipids, triglycerides , total cholesterol, and LDL- cholesterol. Moreover increase was found in HDL-cholesterol and decrease in cholesterol and triglycerides in liver, compared to positive control group. Changes in all above parameters were according to the ratio of MOLP, each of the above three ratios with wheat flour was used to produce crackers . The dough of each product was submitted to rheological measurements using farinograph and extensograph . The best results for the rheological parameters, including; water absorption, stability time, dough development time, degree of softening and elasticity were found in dough containing 25% MOLP.The best dough was used to manufacture hypolipidemic and antioxidant crackers as functional food.

1.Introduction

Diet plays on important role in decreasing of atherosclerosis and coronary heart diseases, which considered the major health problem in all the world. Dietary composition plays a role in disordered lipid and lipoprotein metabolism which led to hyperlipidemia and hyperlipoproteinemia, that considered important factors in developing of coronary heart diseases. Herbal medicine contains natural substances which are important to promote health and decrease illness, it helps in decreasing lipid and cholesterol in blood (Alattar, 2006). Moringa oleifera lam is an Indian tree belongs to genus family moringacease, it was used by Greeks , Roman and anciente Egyptians (Paikra*et al.,* 2017). *M .oleifera* contains;carotenoids, minerals, vitamins, amino acids , sterols, glycosides and phenols, also the leaves are source of protein, vitamin C, B. carotene iron and potassium (Joshi and Mehta, 2010;

Rajanandh *et al.,*2012). Moreover the plant contains different phytochemicals including ; saponins, steroids, tannins, flavonoids and alkaloids (Shousha *et al.,* 2019).,The plant can be used as fresh, cooked, or dried and powdered. The leaves are used as hypoglycemic and hypocholesterolemic agent. (Ghasi *et al.,* 2000 and Leone *et al*., 2015). The administration of M. oleifera's crude extract of leaves reduced cholesterol levels. In serum, liver, and kidney in the rats feeding with a high-fat diet (Ghasi *et al.,* 2000; Vagelas and Sugar 2020). The leaves extract of *M.oleifera* inhibit high antioxidant activity, total phenolic and flavonoids. Using synthetic antioxidants in food products is not popular anymore because of their instability and also due to suspended action as promoters of carcinogenesis, therefore, there is interest in the studies of natural, nontoxic and healthy antioxidants (Tomaino *et al.,* 2005). In the light of the above data, the objectives of the present study was to evaluate the effect of the leaves powder of *M. oleifera* as hypolipidemic and antioxidant agent ,in prevention of lipid elevation in rats fed on high cholesterolemic diet, also helps as natural antioxidant in decreasing the chance of disease progression and using the leaves powder in producing type of functional bakery product.

2. Materials and methods

2.1.Materials

Moringaoleifera leaves were harvested from the Aschrafsaad Farm, Elbuhera Governerate **.**Commercial wheat flour was obtained from the local market. All chemicals were of analytical grade and purchased from Sigma Chem. Co., (St. Louis, MO, USA), Merck (Germany), and Bipdigonostic lChems Co. (Egypt).

2.2. Methods

Moringaoleifera leaves were dried in an oven at 50°C for 8 hours ,the dried leaves were ground and sieved using sieve (20 mesh) and kept in the refrigerator until needed.

2.2.1.Proximate composition

On dry powder, the chemical composition of dry components such as protein, fat, crude fiber, and ash was measured. according to association of Official Analytical Chemists (A.O.A.C, 2016), total carbohydrates were calculated by difference (Valdez-Solana *et al*., 2015)**.**The atomic absorption method was used to determine minerals including calcium, phosphorus, and iron. (A. Analysit 400.perkineler), (Mouminah, 2015).

2.2.1.1.Determination of antioxidants

10 gm of MOLP were dissolved in 100 ml boiling water with swirling for 30 minutes, then filtered using filter paper NO (1), and centrifuged for 30 min at 2000×g, then stored for analyses at −20º C (Sarkar *et al.*,2017).

2.2.1.2.Determination of ascorbic acid:-

The ascorbic acid content was measured by titration method using 2,4-Dichloro-phenol-Indophenol dye A.O.A.C. (2016).

2.2.1.3.Determination of total polyphenols and flavonoids

Total polyphenols content of leaves extract of *M. oleifera* was measured as gallic acid calculated using the method of ciocalteu's. Total flavonoids in leaves extract was assayed with colorimetric method using aluminium chloride and expressed as quercetin equivalent (Akter *et al.,* 2015).

2.2.2.4.Evaluation of free radical scavenging activity

The free radical scavenging activity of the *M* Moringaoleifera leaves extract-picryl-hydrazyl (DPPH) was determined according to (Akter *et al.,* 2015) by determining the absorbance decreasing of the DPPH solution at 517 nm in methanolic extract.

% Inhibition = $[(A_c - A_s) / A_c] \times 100$

$\mathbf{A_c}$: Absorbance of control

$\mathbf{A_s}$: absorbance of sample

2.3.Experimental animals

Thirty six male albino rats with a body weight ranging 130 ±10g were obtained from animal house of National Organization for Drug Control and Research(NODCAR) Giza, Egypt.

The animals were kept in cages for a week to acclimatize under standard conditions included 12/12 hour dark/light cycle, relative humidity, and free access to water and food. The investigation complies with the guide for the care and use laboratory animals (NODCAR/II/28/20).Six rats(initial group) were randomly chosen, then weighed ,blood samples were obtained from retrobulbar venous plexus of each rat (Shermer and Jones,1967). The serum was separated and its biochemical parameters were determined, then rats were sacrificed and liver was excised and subjected to its tissue biochemical analysis.

The reminder rats were divided into five groups each of 6 rats as follows:

control: Rats fed on basal diet control+: Rats fed on high cholesterol diet (HCD) containing cholesterol (1.25%) and bile acids(0.5%) (Pandu*et al.*, 2018)

Gr I : Rats fed on HCD and *M. oleifera leaves* powder*(MOLP),*250mg /Kg

Gr II : Rats fed on HCD and *MOLP*500 mg/Kg

Gr III: Rats fed on HCD and *MOLP*750mg/Kg

Feeding duration was 45 days, changes in body weight were recorded and blood samples were taken at time intervals 15, 30 and 45 days, then serum was separated and subjected to the biochemical analysis. Rats were sacrificed at the end of the experiment, and the liver was excised and subjected to tissue biochemical analysis.

2.3.1.Serumbiochemical analysis

Serum total lipids were determined according to (Zoliner and Kirsch,1962), triglycerides(TG) were determined according to(Fossati, 1982) totalcholesterol (TC) was determined according to(Allain *et al.,* 1974),HDL-Cholesterol(HDL-C) was determined according to the method of (Lopez-Virella,1977) and LDL –cholesterol(LDL-C) was calculated (Salamatullah *et al.*,1983)

2.3.2.Tissue biochemical parameters

One gram of liver was homogenized in 9 mL of ice cold saline and centrifuged at 6000 xg for 10 minutes, then TC and TG were determined in the supernatant
(Zhanga *et al*., 2013).

2.4. Preparation of crackers

The flour formulations which used to make cracker samples were produced in various ratio as follow: :

control: 100g wheat flour .

Sample A: 75g wheat flour + 25g MOLP

B: 50g wheat flour + 50g MOLP

C: 25g wheat flour + 75g MOLP

The dough of products was prepared by mixing each above mixture with corn oil, active yeast ,salt and spices mixture(Abd-El Rahim, 2005)**,** conditions of baking were adjusted as**:** - Temperature for fermentation 30°C for 30 minutes, then bake at 200°C /15 minutes(Saba, 1993).

2.4.1.Rheological measurements

Rheological measurements for flour mixtures were carried out using farinograph and extensograph tests(A.A.C.C, 2007). The parameters including ; water absorption, dough development time, stability time, degree of softening and elasticity were determined using farinograph Brabender OHG Duisurg kulturstrasse 51- 55. extensibility P.N and energy of the dough were determined using Brabender extensograph device .

2.4.2.Sensory evaluation

The staff members were chosen as twenty panelists to test the crackers' sensory properties. Indication of preference was madeon a scale of evaluation to 10 for each of sensory characteristic namely ; appearance, color, flavor, Oder, taste and acceptability(Abd-El Rahim, 2005).

2.5. Statistical analysis

The data can be displayed as mean ± standard error and analyzed using one-way ANOVA, followed by Tukey post-hoc test, using Graph Pad Prism data analysis program (Graph Pad software, Inc., San Diego, CA, USA). A value of $p \leq 0.05$ was considered statistically significant.

3.Results and discussions

3.1.Nutritional composition of Moringaoleifera leaves powder

The proximate chemical composition of Moringa oleifra leaves powder(MOLP) on dry basis was shown in tab(1),Protein content was 30.29 %.protein values ranged from 22.60 to 38.25 % according to(Lesten and Emmanuel, 2018),M. leaves contain a high-quality protein that is easy to absorb and is determined by the amino acids quality.(Mune *et al.,* 2016).Fat content value was 5.4 %as seen in Table (1), ether extract for air dried M.leaves has a fat content of 6.5%.(Busani *et al.*, 2011).The results also showed that ash content of leaves powder was 8.96%, ash content ranging from 4.60 to 11.24 %(Lesten and Emmanuel, 2018),the high ash content of M. leaves indicates that they are a good source of minerals(Shokry, 2017).

On the other hand ,the percentage of fiber content was 9.2% as seen in table (1).Fibers content of M. leaves was 8.96%(Aye and Adegun, 2013). Fibers are important part in a healthy balanced diet; prevent weight gain, improve digestive , diabetes, heart disease, and some cancer diseases (Shokry, 2017),also the ability of fiber to absorpt water led to positive effect against constipation(Gatade *et al.*,2013). However the percentage of carbohydrates of dried leaves was 46.15% calculated by difference which confirmed with Rajput *et al.*, (2017).

Table 1. Nutritional composition of Moringaoleifera leaves powder on dry basis (g/100gm)

Parameters	
Protein	30.29±1.04
Fat	5.4±0.09
Ash	8.96 ± 0.08
Crude Fiber	9.2±0.34
Total Carbohydrate	46.15±0.11
Mineral :	
Calcium	3.60 ±0.08
Phosphorus	0.198 ±0.02
Iron	0.021±0.07

Mean ±SE, n = 3

The leaves of *M. oleifera* contain high amount of calcium in value of 3.60g/100g and very high amount of Phosphorus and iron in values of 0.198and 0.021g/100g powder respectively as seen in table (1)calcium are required for a range of activities in the body, including the bones and teeth. Iron, as the nucleus of hemoglobin, is required for the production of red blood cells in the body.(Mulyaningsih and Yusuf, 2018).

3.2.Antoxidants content of Moringaoleifera leaves powder:

Ascorbic acid content was 0.51 mg/g as seen in table (2).Content of ascorbic acid ranged from56 to 129mg/100g (Hanson *et al.,* 2006).There was a significant difference in ascorbic acid content in plant parts(Asghari *et al.*, 2015).Ascorbic acid is good source for cardiovascular health and the suppression of free radicals in cells.(Sohaimy *et al*.,2015).

Phenolic compounds and flavonoids are very important constituents due to its antioxidative activity. Phytochemical compounds including ; phenols and flavnoids, are shown in table (2) in values of 71.65mg gallic acid /g for phenols and 8.43 mg querctin / g for flavonoids. Querctin content in M. oleifera leaves was found in range from 0.46 to16.64 mg/g (Amaglo *et al.,* 2010).

Table 2. Phytochemicals content of Moringaoleifera leaves powder (mg/g).

Parameters	
Ascorbic acid	0.51±0.11
Total phenols (equivalent galic acid)	71.65± 0.89
Total flavonoieds (querctin equivalent)	8.43±0.17

Mean ±SE, n = 3

The total phenolic content ranged between 37.7 to 137.53 mg gallic acid /g and the total flavonoids content ranged between 8.22 to 33.40 (mg QE/g) in Moringa peregrine and *M.*oleifera extracts, respectively (El-awady*et al.*, 2016). Antioxidant from natural source helps in scavenging free radicals and improve the

antioxidant system in body. An increasing in the interest for antioxidant from natural source faster than synthetic sources, *M.oleifera*is rich in phenolic compound which can reduced many diseases as antioxidant compound(Andréa *et al.*,2012).

3.3.Antioxidant activity of Moringa oleifera leaves powder:

Antioxidant activity at various concentrations of MOLP20,40,80 and 100µg/ml were evaluated and the results are presented in fig (1). The results showed inhibition of 2, 2-diphenyl-1-piccrlhydrazyl hydrate (DPPH) radicals in percentage values of; 42.44,46.64,55.49,60.49 and 67.11 in the above concentrations respectively. The antioxidants activity of M. leaves was found between 59.8 to 40.4(Pakade *et al.,* 2013).DPPH radical scavenging assay for MOLP was used as one of indicated that It can be used in food products as an antioxidant source**.**

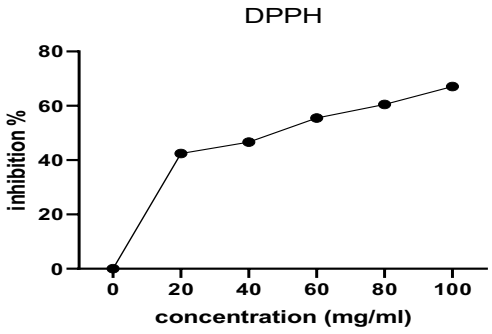


Figure 1.DPPH radical scavenging activity of Moringaoleiferaleaves extract

3.4.Biological evalution

3.4.1.Effect on body weight

The results showed the effect of administration the three doses of MOLP; 25 , 500 and 750 g/kg diet on rats fed on high cholesterol diet for 45 days. The changes in body weight in the five groups during the experimental period were found in table (3). There was non significant differences in body weight among all groups at the beginning of the experiment, gradually increasing in body weight was found during feeding period till the end of experiment in positive control group which was fed on high cholesterol diet compared to the negative control group in values of 106.20 and 40.84g in the weight gain of the two groups respectively. The obese status of rats was maintained when they were fed a high fat diet for two weeks. Also there was a significant difference in the body weight between positive control group which was fed on HCD and tested groups I, II and III which were fed on HCD and different ratios of MOLP, the increasing in all tested groups ranged between27.39to 60.74g.From the aforementioned data, it can be seen that the least values in body weight gain was obtained in rats fed on HCD and 75g / kg diet of MOLP(III) followed by II , then group I which were fed on MOLP in values of 50 and 25 g/kg diet respectively. While compared to rats fed a high-fat diet, MOLP supplementation at 200 and 400 mg/kg b.w. reduced body weight gain and resulted in a significant reduction in body weight(Bais *et al.,* 2014), which led to inhibition in HMG COA reductase activity, that is the key enzyme in pathway of cholesterol biosynthetic, or because of inhibition in body tissues cholesterol deposition. (Atsukwei1*et al.,*2014)

Table 3.Changes in body weight (g) of male rats fed on high cholesterol diet supplemented with Moringa oleifera leaves powder.

Animal Group	Initial	Feeding period (days)			Body weight gain (g)
	0	15	30	45	
Control$^-$	$123.96^{a} \pm 0.42$	$133.71^{a} \pm 0.55$	$153.46^{c} \pm 0.44$	$164.80^{a} \pm 0.42$	40.84
control$^+$	$121.03^{a} \pm 0.38$	$152.80^{c} \pm 0.64$	$194.77^{e} \pm 0.42$	$227.23^{d} \pm 5.30$	106.20
Group I	$124.13^{a} \pm 0.53$	$143.74^{d} \pm 0.57$	$163.17^{d} \pm 0.95$	$184.77^{c} \pm 0.42$	60.74
II	$125.98^{a} \pm 0.31$	$137.42^{b} \pm 0.44$	$150.15^{b} \pm 0.59$	$156.64^{ab} \pm 0.56$	30.66
III	$124.12^{a} \pm 0.36$	$136.09^{b} \pm 1.37$	$143.78^{a} \pm 0.51$	$151.51^{a} \pm 0.71$	27.39

n=6 rats

control$^-$: rats fed on basal diet, control$^+$ rats fed on high cholesterol diet(HCD).

Group I : rats fed on HCD + MOLP(25g/kg diet), Group II : rats fed on HCD + MOLP(50 g/kg diet).

Group III: rats fed on HCD +MOLP(75 g/kg/diet), Mean ± SE, a, b, c, d e: Significantly different tasted groups ; I , II and III from control$^-$ or control$^+$ for at P<0.05 using one way ANOVA followed by Tukey as post-hoc test

3.4.2.Effect on glucose

Data in fig(2) showed a significant (*p<0.05*) increase in glucose level in the positive control group which was fed on high cholesterol diet compared to negative control group which was fed on standard diet at the end of experimental period in values of 229.55 and 90.82 mg/dl in the two groups, respectively. On the other hand, Blood glucose levels decreased significantly (*p<0.05*) in all studied groups but the highest effective was observed in group III which was fed on HCD and MOLP,75g/kg diet followed by group II then group I as seen in fig(2) in values of 94.64,141.46 and 160.74 mg/dl, Also, non significant differences between group III and negative control group was found in percentage values of 94.64 and 90.82mg/dl after the end of experiment, respectively. Moringa leaves caused a significant reduction in blood glucose levels in waster rats with type 2 diabetes.(Ndong *et al.,*2007), also, another study reported as a mechanistic model for anti-diabetic activity, that the extract from M. leaves is effective for reducing sugar within 3 hours of ingestion(Mittall *et al.,* 2007).

On the other hand, Moringa leaves were shown to have beneficial effects on carbohydrate metabolism with different mechanisms including; improving glucose uptake and utilization , increasing insulin activity, restoring and preventing the integrity and function of β-cells (Vergara-Jimenez *et al.,* 2017), the dried leaves or aqueous extract of M. oleifera were more effective and may be it have some effect by increasing the glucose utilization in tissue by inhibiting absorption of glucose or hepatic gluconeogenesis into the muscles and adipose tissue(Gray *et al.,*2000)

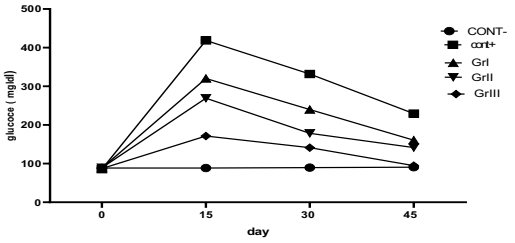


Figure 2. Serum glucose (mg/dl) in rats fed on high cholesterol diet and different ratios of *Moringa oleifera* leaves powder for 45 days. (n=6 rats). Groups abbreviations as seen in table(3)

3.4.3.Effect on total lipids

The results in fig (3) showed highly significant increasing in serum total lipids in positive control feeding with diet containing 0.5% cholesterol compared to negative control group which was fed on basal diet in values of 628.96 and 337.37mg/dl in the two groups respectively .the results were in agreement with Muniz *et al.,* (2019)The tested groups I, II and III which were fed on HCD and different concentrations of MOLP, showed gradually significant decreases in total lipids compared to positive group, the highest decrease was found in group III then group II then group I which were fed on HCD supplemented with MOLP in concentration of 75,50 and 25 g/kg diet in the three groups respectively, the decreases were in values of 313.83, 467.50 and 501.50 mg/dl in the three groups, respectively.

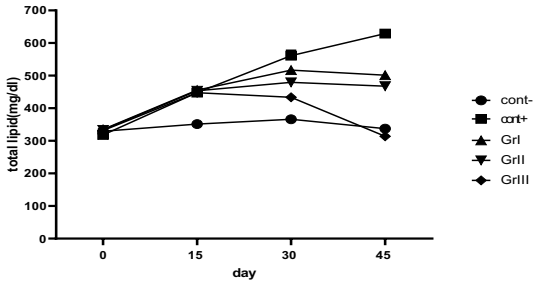


Figure 3. Serum total lipids (mg/dl) in rats fed on high cholesterol diet and different ratios of *Moringa oleifera* leaves powder for 45 days. (n=6 rats).Groups abbreviations as seen in table(3)

3.4.4. Effect on triglycerides

The results in fig(4) showed significant increases in triglycerides in positive control group compared to negative control group in values of 276.43 and 87.02 mg/dl, respectively. On the other hand, tested groups showed also significant gradually decreases in triglycerides during feeding period, the highest decreases was observed in group III followed by group II , then group I in values of 99.88, 105.31 and 135.57, in the three groups respectively compared to positive and negative control rats in values of 276.43and 87.02mg/dl respectively, the content of flavonoids and vitamin C in *M oleifera* can lower TG and LDL-C levels as a result for the inhabitation of 3-Hydroxy-3-methyglutary Coenzyme A (HMG-CoA) reductase this decomposition of cholesterol, LDL-C, TG become slow which results in the formation of cholesterol, LDL-C and TG decreased ,also, triacylglycerols are storage form of fatty acids(Affan*et al*., 2018).the improved serum values of TG, TC, LDL-C, and VLDL-C that were observed simultaneously in the study, the lowering in total lipids in treated animals is logical.(Atsukwei1 *et al.,* 2014;Gheith and El-Mahmoudy, 2019) .

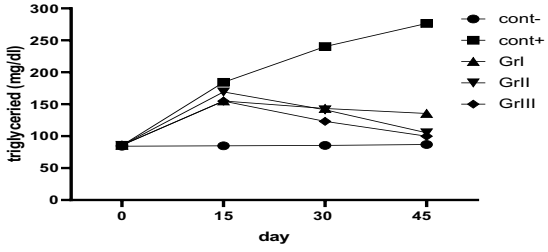


Figure 4.Serum triglycerides (mg/dl)) in rats fed on high cholesterol diet and different ratios of *Moringa oleifera* leaves powder for 45 days. (n=6 rats). Groups abbreviations as seen in table(3).

3.4.5.Effect on Total-cholesterol, LDL-cholesterol and HDL-cholesterol

As shown in fig (5) , positive control group which was fed on basal diet containing 1.25% cholesterol for 45days showed significantly increases in serum parameters including ;TC , LDL- C and decrease in HDL- C in values of 205.24, 126.25 and 19.04 mg/dl compared to negative control group in values of 82.71, 28.40, and53.93mg/dl at the end of experiment in the aforementioned parameters, respectively.

Total cholesterol and LDL-C were decreased significantly (P<0.05) in all tested groups ; I, II and III which were given different concentrations from MOLP ; 25,50 and 75g/kg diet at the end of experiment in values of 115.79, 98.51and 90.97 mg/dl and 32.30, 30.25 and 27.45 mg/dl in the two parameters respectively compared to positive control rats in values of 205.24 and 126.25 mg/dl as seen in figure (5) A and B. Moringa oleifera leaves containing β-sitosterol inhibit the cholesterol absorption in the intestines, which limit the ability of the cholesterol entrance to the body, and helps in reducing cholesterol limit (Frawley, 2009), similarity of B-sitosterol and cholesterol led to it takes place of micelles cholesterol in the intestinal lumen, which caused reduction in cholesterol absorption, and will be absorbed in stead of cholesterol. Reduced cholesterol caused reduction in LDL-C, which carries cholesterol to the blood serum from liver**(**Rana *et al.*,2007).

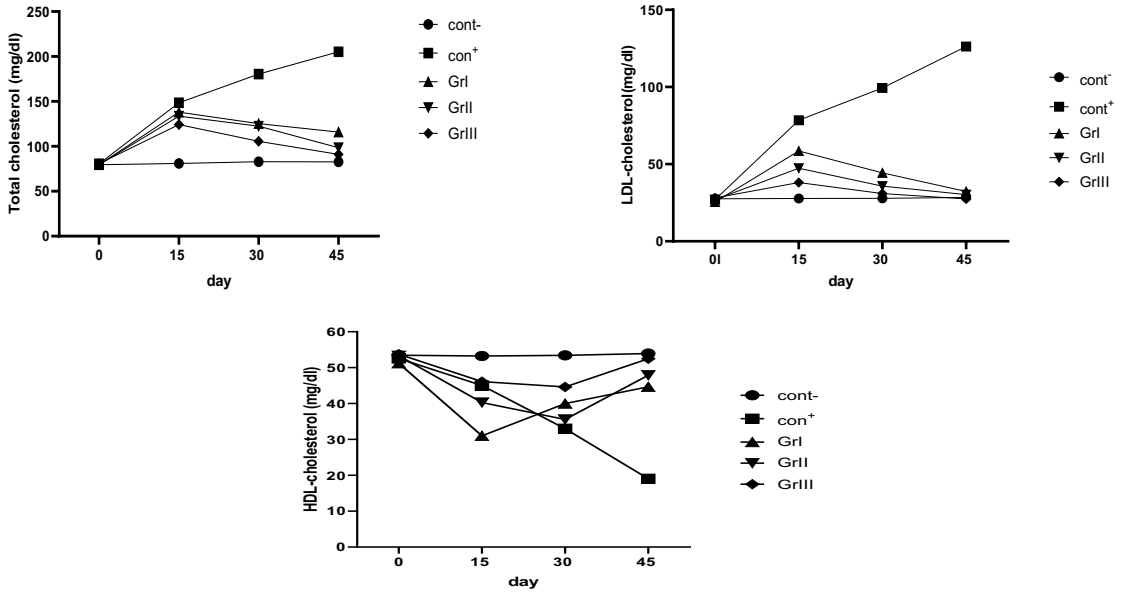


Figure 5(A-C). Serum total cholesterol, HDL and LDL cholesterol (mg/dl) in rats fed on high cholesterol diet and different ratios of *Moringa oleifera* leaves powder for 45 days. (n=6 rats).Groups abbreviations as seen in table(3)

Acetyl-CoA is important as a key substrate in cholesterol biosynthesis, extract of Moringa oleifera at 600 mg/kg body weight lead to a decrease in acetyl CoA concentration, leading to low -oxidation of fatty acid.(Atsukwei, 2014)**.**Also, HDL-cholesterol was increased significantly (P<0.05) in all the tested groups which were given different ratios of MOLP for 45 days as seen in figure (5C). The highest increase was found in group III , followed by group II then group I which were fed on supplemented diet with MOLP in 75,50and 25g/kg diet respectively, the increase was in values of 52.48 , 47.89and 44.67 mg/dl, respectively.

Oral administration of M leaves extract to rats at dose of 100 and 200 mg/kg b.w for 28 days improved HDL-C levels.(Ma *et al.,* **2018**).

Also, the treated group which was given 4.6 g of leaves powder in tablet form had a 1.6% decrease in cholesterol and 6.3 % rise in HDL compared to the control group(Stohs and Hartman, 2015), *M*. leaves extracts at doses of 300 and 600mg/kg b.w, significantly decreased TG, TC, LDL-C, VLDL-C, atherogenic index, and increased HDL-C in rats fed on high-fat diet (Jain *et al.,* 2016).

3.4.6.Effect on liver enzymes

The data in fig 6 (A-B) showed a significant increase in the activity of all liver enzymes Alanin aminotransferase (ALT) and Aspartate amino transferase (AST) in control positive group fed on HCD in values of 60.97 and 47.60mg/dl. compared to the normal control group in values of 42.89and 40.73mg/dl ($P < 0.05$) respectively, when compared to other tested groups that had a significant decline in liver enzyme activities, after administration of different concentrations of MOLP.

Also group ;I, II and III fed on HCD and MOLP showed significant improvement in serum ALT and AST activity at the end of experiment in the values of 44.71 and 44.94 for group I,41.56 mg/dl and 41.08 for group II , 41.78and 39.64mg/dl for group III for the two parameters compared to positive control rats in values of 60.97 and 47.60mg/dl respectively ($P < 0.05$). In mice fed a high fat diet, hepatoprotective properties of MOLP were found to protect against liver damage by lowering (AST) and (ALT)(Nilanjan Das *et al.*, 2012)**,** ALT and AST measurement is of clinical and toxicological importance because their activity indicates tissue damage by toxicants or in disease condition liver (Rania *et al.,* 2016)**.** Feeding on high fat diet with *Moringa oliefera* at 3% & 5% caused decrease in serum AST and ALT as compared to positive control group(Etab and Mona, 2017).Also *Moringa oleifera* fortified diet improves liver functions, in addition it can protect the liver from chemically induced (Buraimoh, 2011)**.**

3.4.5.Effect on Liver lipid

Total cholesterol and triglycerides in liver were shown in fig 7(A-B) with higher level in positive high fat group in values of 315.07 and 200.59mg/dl than that of negative group in values of 95.72 and 123.59mg/dl for the two parameters respectively. Tested groups; I, II and III showed significant decrease in both parameters with the highest decrease level in group III in values of 132.49 and 108.96mg/dl followed by group II in values of 77.69 and 96.49mg/dl then group I in values of 112.49 and 108.96mg/dl compared to positive group with values of 315.07 and 200.59mg/dl respectively.

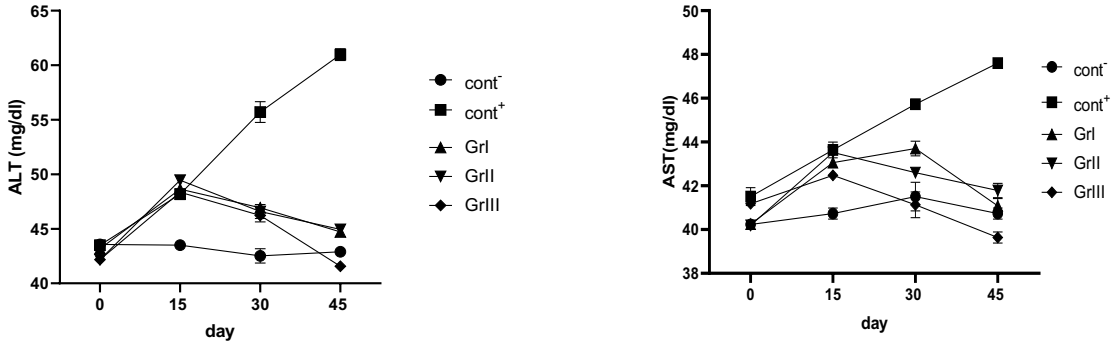


Figure 6(A-B). Serum ALT and AST (mg/dl) in rats fed on high cholesterol diet and different ratios of *Moringa oleifera* leaves powder for 45 days. (n=6 rats)
Groups abbreviations as seen in table(3).

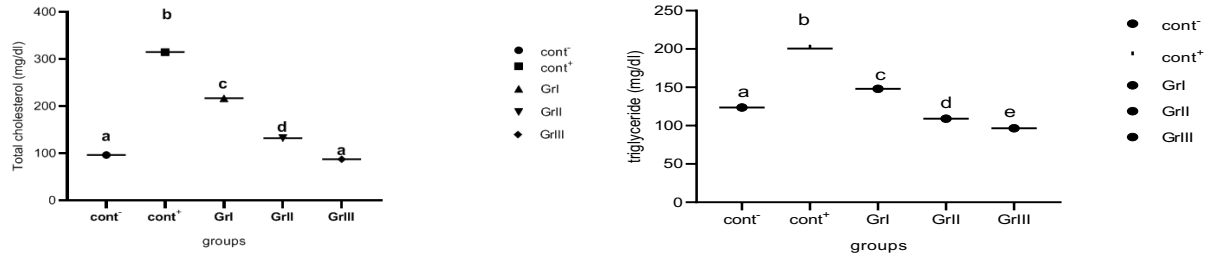


Figure 7(A-B). liver total cholesterol and triglyceride (mg/dl) of rats fed on high cholesterol diet and different ratios of *Moringa oleifera* leaves powder for 45 days. n=6 rats. Groups abbreviations as seen in table(3).

The changes in high fat group were recovered in both tested groups and the values were less than that in negative group, the best results were found in groups III followed by group II. Moringa oleifeara leaves extract improved the status of liver disease in mice feeding high fat diet and can protect the liver from the hepatotoxic drugs and toxins(Nilanjan*et al.,*2012)

3.5.Rheological properties

The dough's rheological properties were determined using farinograph and Extensograph, which showed non response and could not obtained any results for the two dough samples contain 50 and 75%MOLP,only sample contains 25%showed clear results.

3.5.1.Farinograph parameters

Rheological properties of dough ,which are important for dough quality were determined using pharinograph for dough prepared from wheat flour supplemented with MOLP(25%) and control sample contain wheat flour 100% ,The result is presented in table (4.A-B)).

The results in this study showed increasing in water absorption in tested sample compared to control sample in percentage values of 75.0 and 61.5 %respectively. Damage starch content and soluble protein may influence water absorption(Rakszegi *et al.,* 2014).Results in table (4A) showed also equal arrival time in value of one minute for both tested and control samples. On the other hand increasing in dough development time in tested sample was observed which is the need time to mix flour and water to form dough with the best consistency compared to control sample in values of 3.5 and 1.5min in the two groups, respectively. Development time of dough which was determined with pharinograph ranged from 1.8 to 4min(Pelin and Incilay, 2020).

The stability value was decreased in strength of the tested dough compared to control in values of 4.5 and 5.5 min as seen in table (4A). Normal stability by pharinograph ranged from 2.3 to 10 min, also dough stability for MOLP- wheat flour tested sample was around 5 min(Pelin and Incilay, 2020) .

3.5.2.Extensograph parameters:

Extensograph was used to determine resistance and extension ability of the dough, data in table (4B) and figure(7B)showed the results of extensograph analysis of parameters for tested sample supplemented with MOLP(25%) and control sample contains wheat flour (100 %).Elasticity and extensibility were decreased in tested sample compared to control sample in values of 180 and 320(B.U)in the two samples respectively ,the same trend was in agreement with Rania *et al.,*(2016)**.**proportional number which calculated from elasticity and extensibility was increased in tested sample contains MOLP(25%),compared to control group in values of 5.14 and 1.94 P.N., respectively. Finally dough energy had the highest value in control in value of 105cm^3, these results are in agreement with those obtained by Rania *et al.,*(2016**)** and El-Karamany, (2015

Table 4.Farinograph and Extensograph parameters for control and tested dough supplemented with Moringa oleifera leaves powder(25%)

(A)

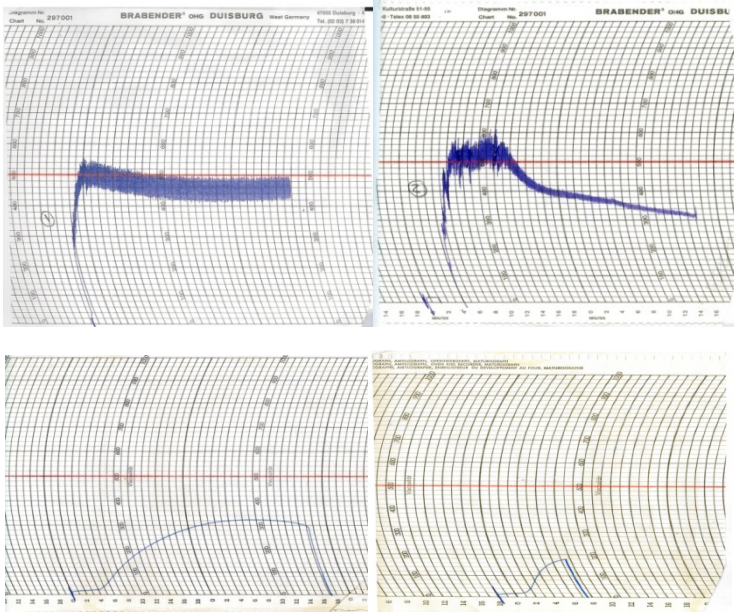
Parametres / Sample	Water absorption %	Arrival time (min)	Dough development (min)	Degree of softening (B.U)
Control	61.5	1.0	1.5	50
Sample A	75.0	1.0	3.5	180

(B)

parameters / sample	Elasticity (B.U)	Extensibility (mm)	Proportional number P.N	Energy ($cm^{2)}$
Control	320	165	1.94	104
Sample A	180	35	5.14	12

Control: 100% wheat flour Sample

Sample A: 75% wheat flour + 25% *Moringa oleifera* leaves powder



Control Sample (A)

Figure 8 (A-B). Farinograph and Extensograph parameters for control and tested dough supplemented with *Moringa oleifera* leaves powder(25%)

3.6.Sensory evaluation of crackers :

Data in fig. (9) show sensory evaluation of crackers manufactured with MOLP25%.

Significant differences ($P<0.05$) in all sensory evaluation compared to control (A) were found.

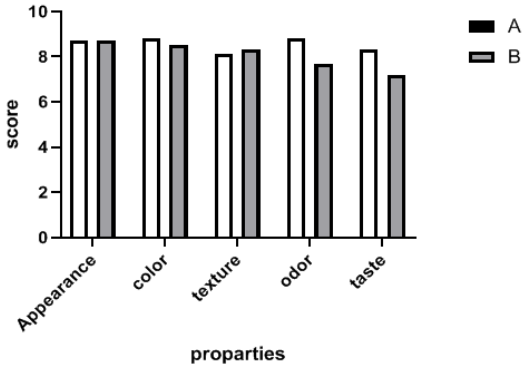


Figure 9. Sensory evaluation of crackers prepared from dough supplemented with (25%) *Moringa oleifera* leaves powder.

4. Conclusions

Hypolipidemic and antioxidative effect of different ratios of MOLP were studied; therefore its chemical composition was determined and the biological evaluation on male rats were estimated. Supplemented dough containing different ratios of MOLP were prepared, and dough which showed the best rheological parameters(25%) was used to manufacture crackers , that can be used as hypolipidemic and antioxidative functional food with acceptable sensory characteristics. This study indicated that MOLP can be used as natural source in production of hypolipidemic and antioxidative functional food.

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CHEMICAL AND MICROBIOLOGICAL QUALITY DURING STORAGE: HALF-DRIED SALTED ROUND SCAD (*DECAPTERUS MARUADSI*)

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ABSTRACT

Half-dried salted round scad can be stored for only a short time due to high-moisture content. The objective of this study was to evaluate the quality changes during storage under 3 packaging; air (T1), vacuum (T2), and air with oxygen absorber (T3). Moisture content, pH, TVB value, TMA value, TBA value, total viable count as well as yeast and mold of half-dried salted round scad significantly increased along with the longer storage time ($p<0.05$). The vacuum packaging had the lowest effect on the quality changes of half-dried salted round scad. When stored at room temperature)(30±2 ^{0}C) half-dried salted round scad gained higher amounts of yeast and mold than the standard of TISI. On day 15 of storage, yeast and mold of the sample were 6.4×10^2 CFU/g When stored under chilling condition (4±1 ^{0}C)for 30 days, half-dried salted round scad gained higher TMA value as well as yeast and mold values than the standard of TISI at 11.48 mg/100 g and 5.2×10^2 CFU/g, respectively. Therefore, the shelf life of half-dried salted round scad packed under vacuum condition were 10 and 25 days for samples stored at room temperature and cold storage, respectively

1. Introduction

Half-dried salted fish is one of the most popular traditional products. It has high nutrition, soft texture, unique taste and flavor. It is also a great source of high protein which contains all essential amino acid. Half-dried salted fish is different from normal salted fish in term of the moisture and salt content in their final products. For salted fish, the a_w is usually under 0.85 and the salt content is over 10% (Thai industrial standards institute; TISI. 312/2006). For half-dried salted fish, it is a high-moisture content product and the salt content is less than 10% (light cure). Moreover, the main characteristics of salted fish affecting consumer buying decision are light salty taste, special flavor without stinky smell and absence of fish bone (Sukjuntra, 2014). Due to the lower salt content in half-dried salted fish, food spoilage bacteria and food-born pathogenic bacteria can easily grow in this product which leads to the product's shorter shelf life. Nowadays, snake-head fish and sepat siam, freshwater fish, are commonly used as half-dried salted fish products.

Among the sea water fish species, the round scad (*Decapterus maruadsi*), a fatty fish, has been chosen as the raw material for half-dried salted fish production because of its appearance and taste which are better than those of lean fish. Half-dried salted round scad also has soft texture during to the low salt added which leads to no denaturation of fish protein (Ana and Rui, 2010; Magnus and Turid, 2012). Moreover, the half-dried salted round scad is healthy food for

consumer, due to its low salt content (Nuwanthi *et al*., 2016). For the above reasons, half-dried salted round scad is widely consumed and available in local markets especially in the lower southern part of Thailand. However, the supply of half-dried salted round scad are still low because of its traditional sun drying process with the lack of proper technology to improve the production. Furthermore, during the rainy season, the half-dried salted round scad is not available in the market because of the sun drying process. The factors determining the half-dried salted round scad product's quality are raw material quality, salt quantity and quality, good and hygienic processing, packaging and suitable storage condition (Koral, 2013; Lorentzen *et al*., 2016).

In order to improve the quality and extend the shelf life of half-dried salted round scad, appropriate packaging is crucial. Nowadays, there are many studies on various packaging for shelf life extension of fish and fishery products including vacuum packaging, oxygen absorber, moisture absorber and modified atmosphere packaging (Mohan *et al*., 2009). The vacuum packaging is a widely used method in the market as its efficiency and low cost. This packaging can prevent oxidative rancidity, and inhibition of the growth of aerobic bacteria and spoilage bacteria, especially *Macrococcus* and *Aeromonas* (Masniyom, 2011; Renato, 2012; Pinar, 2013; Kumar and Ganguly, 2014).

Appropriate packaging is able to increase the shelf life of half-dried salted round scad, increase the marketing competition capacity and also enhance reputation of this product. Thus, the objectives of this study were to select the practical packaging methods conforming to entrepreneur in the lower southern part of Thailand for increasing the shelf life of half-dried salted round scad. Samples were stored at room temperature (30±2 ^{0}C) and at cold storage (4±1 ^{0}C) for 15 and 30 days, respectively. The changes of physical, chemical and microbiological properties of sample were determined at 5 days interval.

2. Materials and methods

2.1. Round Scad Preparation

The A grade to low B grade of round scad (*Decapterus maruadsi*) (8-12 fish/kg) according to EU grade was beheaded, gutted, butterflied and deboned. After that, the butterflied fish was soaked in 6% (w/v) brine solution for 20 min at a ratio of 1:1 (fish: brine solution). Then the soaked fish was dried in a solar dryer (Electricity Generating Authority of Thailand; SD-050 model) until a_w of fish fresh ranged from 0.85 to 0.90. Then, the fish was stored in low density polyethylene bags (LDPE) under 3 types of packaging; air (T1), vacuum (T2) (VAC-STAR S220, Switzerland), and air with an iron-based oxygen absorbent (T3) which utilized in sachet form and meant to be used in products with maximum water activity (a_w) of 0.85 and minimum oxygen absorption of 300 ml. Afterwards, half-dried salted round scad was stored at room temperature (30±2 ^{0}C) for 15 days, chilled in condition (4±1 ^{0}C) for 30 days, and sampled every 5 days for evaluation of the shelf life.

2.2. Quality Analysis

2.2.1.Chemical Analysis

The pH measurement was determined by homogenizing samples with distilled water at ratio of 1:5 (w/v), as described by Manthey et al. (1988). First, pH values of homogenate were measured by using a pH meter (Schott, model G 0840). Also, moisture content was determined using an oven method (Association of official Analytical Chemists, 2000). Then, salt content was analyzed using AOAC method. Moreover, the Total volatile base nitrogen (TVB) and Trimethylamine (TMA) value were analyzed using conway unit (Siang and Kim, 1992) by grinding 2 g of grounded sample with 8 ml of 4% (w/v) Trichloroacetic acid (TCA) in a mortar and then filtrating the texture through filter paper (Whatman No. 41). The volume of filtrate was adjusted to 10 ml using 4% (w/v) TCA. After that, 1 ml of sample extract was pipetted into outer ring of conway unit. Then 1 ml of inner ring solution (boric acid in ethanol) was pipetted into inner ring of conway unit.

Subsequently, 1 ml of saturated K_2CO_3 was pipetted into another side outer ring of conway unit. Moreover, The Trimethylamine value was carried out according to the methodology of TVB with a slightly different. After the sample extraction, the sample extract, inner ring solution and saturated K_2CO_3 were added into conway unit. Then, 1 ml of 10% (v/v) Formaldehyde solution was pipetted into the sample extract portion. After conway unit was closed, both of the TVB and TMA portion were gently mixed and incubated at 37 ^{0}C for 45 - 60 min, respectively. After incubation, the inner ring solution was titrated with 0.02 N Hydrochloric acid (HCl) using micro-burette until its green color turned pink color. The blank test was done using 1 ml of 4% (w/v) TCA instead of sample extract. TVB and TMA value was calculated as follows.

$$\text{TVB (mg/100mg)} = \frac{\text{N} \times 14 \times (\text{A} - \text{C}) \times 10 \times 100}{\text{W}}$$

$$\text{TMA (mg/100mg)} = \frac{\text{N} \times 14 \times (\text{B} - \text{C}) \times 10 \times 100}{\text{W}}$$

Where; N = Normality of HCl

A = Titration volume (ml) of 0.02N HCl for sample extract (TVB)

B = Titration volume (ml) of 0.02N HCl for blank

C = Titration volume (ml) of 0.02N HCl for sample extract (TMA)

W = Weight of sample (g)

Besides, the Thiobarbituric acid (TBA) value was determined by a distillation method (Egan *et al*., 1981). First, 10 g of sample was homogenized with 50 ml of distilled water, then adding 4 N HCl. After that, the mixture was heated with steam distillation until 50 ml of distillate was collected. A 5 ml of distillate was mixed with 5 ml of TBA reagent and incubated in boiling water for 35 min. After cooling, the absorbance of the solution was read at 538 nm. The blank test was done using distilled water instead of sample. TBA value was calculated as follows.

$$\text{TBA(mg MAD/kg)} = 7.8 \times \text{A}$$

Where A; Absorbance of the solution was read at 538 nm

2.2.2.Microbiological analysis

A microbiological analysis was carried out by examining total plate count (TPC) as well as yeast and mold according to the technique recommended by Bacteriological Analytical Manual (2001). *Staphylococcus aureus* and *Escherichia coli* MPN (3 tubes method) were analyzed using the technique recommended by Bacteriological Analytical Manual (2002).

2.3. Statistical analysis

The experiment was carried out with a completely randomized design (CRD). Data obtained from chemical parameters were analyzed by analysis of variance (ANOVA) using computer software. Duncan's New Multiple Range Test (DMRT) was used to compare the means with a significance level of 95%.

3.Results and discussions

3.1. Chemical quality changes

3.1.1.pH value

The result of this study indicated that the storage at room temperature (30±2 ^{0}C) had affected the pH value of half-dried salted round scad. pH values of the samples significantly increased with the increasing storage time. All 3 samples packed in air (T1), vacuum (T2) and air with oxygen absorber (T3) had the pH values of 5.98 on day 0 and their pH values reached to 6.73, 6.63 and 6.74 at the end of storage (day 15), respectively ($p>0.05$) (data is not shown). Corresponding to the result of Farid *et al.* (2014) who found that the pH values of 2 dried salted snake-head fish products stored at room temperature (27-30 ^{0}C) increased from 6.3 and 6.5 (day 0) to 8.1 and 7.9 (day 150). For half-dried salted round scad stored under chilling condition) 4±1 ^{0}C (in the present study, the pH value used for indicating fish quality of all 3

samples slightly increased with the longer storage time ($p>0.05$) (data is not shown). During the post-mortem period, the decomposition of nitrogenous compounds occured by microorganisms and formed volatile amines, ammonia, amine and trimetylamineoxide, which are basic alkalinity compounds affecting on the increasing pH value of fish (Farid *et al.,* 2014; Frangos *et al*., 2010; Lorentzen *et al*., 2015). Thus, the storage of dried salted round scad in chilling temperature is able to retard microorganism activities leading to the minor changes in pH.

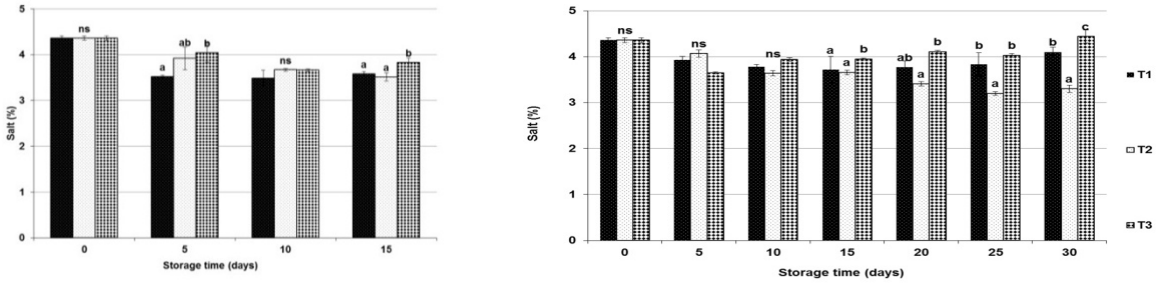
3.1.2.Moisture content

Moisture content of half-dried salted round scad stored at room temperature significantly increased with the longer storage time ($p<0.05$). The moisture content of samples packed in air (T1), vacuum (T2) and air with oxygen absorber (T3) were 54.93% on day 0 and increased to 61.27, 57.43 and 58.39% on day 15 of storage, respectively (data not shown). From these results, the moisture content of the sample stored in non-vacuum packaging (T1) was higher than other packaging. ($p<0.05$). The increase in moisture content of half-dried salted round scad was due to the moisture absorption of products an environment with from higher humidity (Farid *et al*., 2014). This is in agreement with the study of Adenike (2014), Ikutegbe & Sikoki (2014) and Reza *et al*. (2015) who found that moisture content of smoked catfish, dried chela *(Laubuka dadiburjori*) and smoked long-neck croaker (*Pseudotolithus typus*) increased with increasing the storage time ($p<0.05$). The moisture content of smoked catfish increased from 62.52% to 69.13% (on week 6), the moisture content of dried chela increased from 5.88% to 10.83% (on day 30) and the moisture content of smoked long-neck croaker increased from 10.90% to 13.10% (on week 4).

The result of half-dried salted round scad stored in chilling condition indicates that the changes of moisture content in the final products were lower than those stored at room temperature. The moisture content of sample stored in air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging was 54.93% on day 0 and increased to 59.87, 58.68 and 58.77% on day 30 of storage, respectively (data not shown). This was because dried fish slowly re-absorbed moisture from the relatively humidity condition like a chilling storage. However, moisture content of half-dried salted round scad is a factor of shelf life because it affects microbial growth and chemical changes including oxidation reaction and browning reaction. Normally, high moisture food is highly perishable.

3.1.3.Salt content

The salt content analysis of half-dried salted round scad stored at room temperature showed that the salt contents of samples packed in air (T1), vacuum (T2) packaging significantly decreased on day 5 of storage with a constant value on day 5-15 of storage. Whereas, the salt content of samples packed in air with oxygen absorber (T3) was stable during 15 days of storage ($p>0.05$). On day 15, salt content of 3 half-dried salted round scad were 3.38, 3.51 and 3.83% respectively (Figure 1(a)). In refrigerator storage ($4\pm1\ ^0C$) condition, salt contents of all 3 samples varied with increasing storage time and showed the difference of salt content on day 15 of storage (Figure 1(b)). It can be seen that a decrease in salt content of products corresponded to the increased moisture content similar to Oyarekua (2014) who found that the salt content of smoked catfish slice decreased with an increase in storage time ($p<0.05$). The salt content decrease from 16.66% to 12.05% on week 6 of storage. In order to increase the shelf life of salted fish with 55-58% moisture content, Loannis (2014) demonstrated that salt content of salted fish should be in the range of 18-21%. From this study, high moisture content with 0.85 -0.90 of a_w and low salt content (less than 5%) in half-dried salted round scad promoted the growth of microorganism and has effects the on short shelf life of this product.

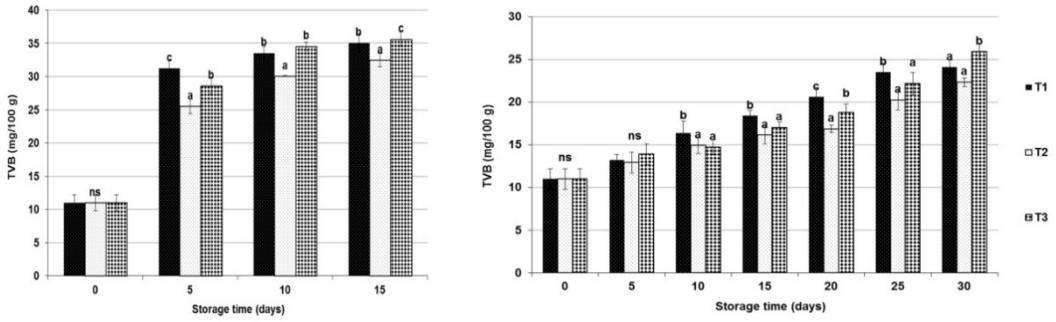


(a) **(b)**

Figure 1. Salt content of half-dried salted round scad stored at (a) room temperature and (b) chilling condition under air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging

3.1.4.Total volatile base nitrogen (TVB)

The TVB value in half-dried salted round scad stored at room temperature significantly increased along with the longer storage time ($p<0.05$). After 15 days of storage, TVB value in sample packed by vacuum packaging (T2) was lowest at 32.47 mg/100 g as shown in Figure 2(a). Moreover, the TVB value in half-dried salted round scad stored in refrigerator significantly increased with the longer storage time ($p<0.05$). The results showed that TVB value increased from an initial value of 10.99 mg/100 g on the day 0 to 24.24, 23.33 and 25.92 mg/100 g on the day 30 of storage in T1, T2 and T3, respectively (Figure 2(b)). Similar results were reported by Latifa et al. (2014) about refrigerated storage (4 ^{0}C) of smoke-dried chapila (*Gudusia chapra*) that TVB value increased from 8.84 to 20.04 mg/100 g after 9 months of storage. In addition, Farid et al. (2014) reported that TVB value in sun-dried salted shoal fish at room temperature (27-30 ^{0}C) storage increased from 4.89 to 30.86 mg/100 g after 5 months of storage. TVB is a group of biogenic amine produced by bacteria including ammonia, dimethylamine and trimethylamine. This increasing values correspond to the increasing fish spoilage; therefore, TVB is used for indicating the spoilage of fish and the consumer acceptance. The acceptability level of TVB in salted fish is 35 - 40 mg/100g. Above that level, fish products are considered unsuitable for human consumption (Bilgin and Degirmenci, 2019).



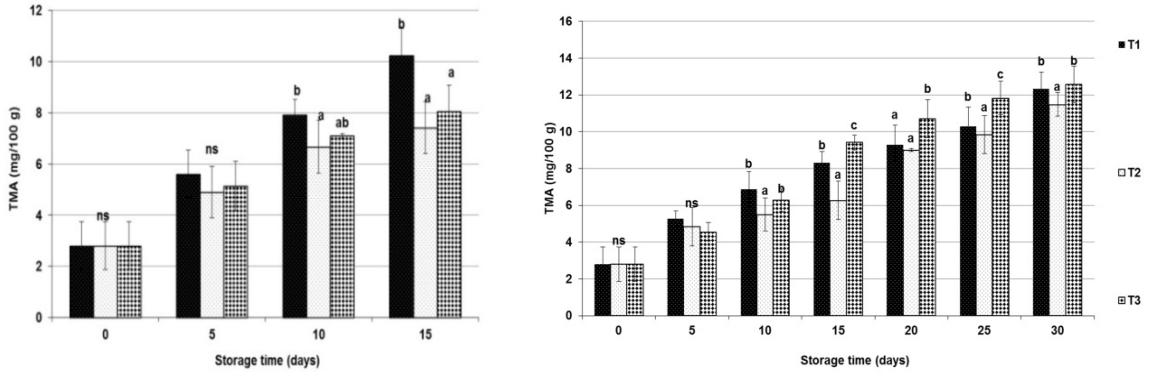
(a) **(b)**

Figure 2. Total volatile base (TVB) values of half-dried salted round scad stored at (a) room temperature and (b) chilling temperature under air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging

The results of this study also showed that the different packaging effects on the growth of spoilage microorganism in fish products. Sample stored in air packaging had the highest spoilage. Whereas, sample stored in vacuum condition was not able to inhibit the growth of aerobic bacteria (Fuentes *et al*., 2011). In addition, the storage under chilling condition was able to prevent the growth of microorganism and enhance the shelf life of product. This study suggested that samples packed in air (T1), and air with oxygen absorber (T3) conditions and stored at room temperature had TVB value over the limitation of 35 mg/100 g. on day 15 of storage at 35.09 and 35.61 mg/100 g, respectively. On the contrary, TVB values of all 3 samples stored at chilling condition were within the standard of 35 mg/100 g TVB during the storage time. These results indicated that the quality of half-dried salted round scad stored at chilled condition was acceptable.

3.1.5.Trimethylamine (TMA)

The TMA value of half-dried salted round scad stored at room temperature significantly increased along with the longer storage time ($p<0.05$). After 15 days of storage, TMA value of sample packed by vacuum packaging (T2) was lowest at 7.42 mg/100 g (Figure 3(a)). Likewise, the values of half-dried salted round scad stored in refrigerator significantly increased along with the longer storage time ($p<0.05$). The results showed that TMA value increased from an initial value of 2.81 mg/100 g on the day 0 to 12.23, 11.48 and 12.57 mg/100 g on the day 30 of storage in T1, T2 and T3, respectively (Figure 3(b)). Similar results have been report by Bilgin and Degirmenci (2019) during refrigerated storage (4±1 ^{0}C) of hot-smoked meager (*Argyrosomus regius*). TMA value increased from 1.35 to 5.57 mg/100 g after 56 days of storage. TMA was derived from non-protein nitrogen compounds (trimethylamine oxide; TMAO) in fish by trimethylamine oxide reductase and degraded to TMA, dimethylamine (DMA), formaldehyde (FA) and ammonia (Benjakul *et al*., 1997). Thus, the increasing TMA value stimulates the increase of TVB value. The fresh fish normally has TMA value of 10-15 mg/100 g (Venugopal, 2006). TMA value of sample packed in air (T1) condition and stored at room temperature was >10 mg/100 g on day 15 of storage. Whereas, TMA value of samples stored under chilling condition was >10 mg/100 g on day 25, 30 and 20 of storage for samples packed in air (T1), vacuum (T2) and air with oxygen absorber (T3) conditions, respectively. Therefore, the vacuum packaging of half-dried salted round scad is the best packaging in slowing down the growth of aerobic bacteria.

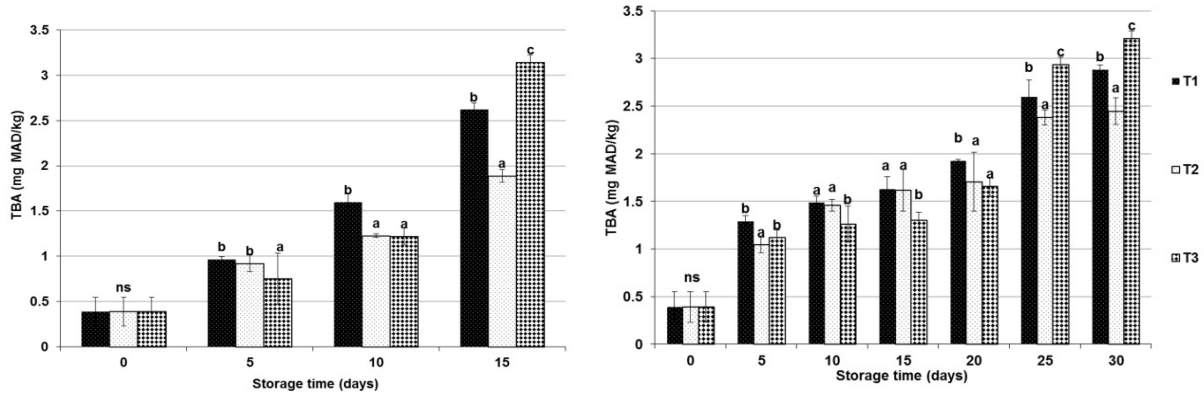


(a) **(b)**

Figure 3. Trimethylamine values (TMA) of half-dried salted round scad stored at (a) room temperature and (b) chilling condition under air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging

3.1.6. Thiobarbituric acid (TBA)

The TBA value of half-dried salted round scad stored at room temperature significantly increased along with the longer storage time ($p<0.05$). The sample packed under vacuum condition (T2) had the lowest increasing TBA value with an initial of 0.39 mg MAD/kg and increased to 1.89 mg MAD /kg on day 15 of storage. The TBA value of samples stored under chilling condition significantly increased with an increased storage time ($p<0.05$) with 2.45 mg MAD /kg on day 30 of storage. The increase in TBA led to the oxidation reaction of products and degraded to malonaldehyde. The increased TBA of half-dried salted round scad also has effects on food spoilage, nutrition and consumer health safety. Comparing to striped snaked-head fish, catfish and snapper queen fish, fatty fish with highly unsaturated fat, such as round scad, tend to have more oxidation reaction (Oyarekua, 2014; Lohalaksanadech and Sujarit, 2016). Moreover, Oxygen was a factor leading to oxidation reaction. So half-dried salted round scad packed in vacuum condition had the lowest TBA value according to the result of Antonios and Michael (2007) who found that the packaging of mackerel *(Scomber japonicas*) under vacuum condition resulted in the lowest increase of TBA when comparing to modified atmosphere packaging and air packaging. However, the limitation of TBA in fish was lower than 8 mg MAD /kg and fresh fish has <3 mg MAD /kg. Among fishery products, the TBA of 3-4 mg MAD /kg indicates the low quality product. When considering the TBA in dried salted round scad, the results indicated that half-dried salted fish still had acceptable levels of TBA even at the end of storage time.



(a) **(b)**

Figure 4. Thiobarbituric acid values (TBA) of half-dried d salted round scad stored at (a) room temperature and (b) chilling condition of air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging

3.2. Microbiological quality changes

3.2.1. Total plate count (TPC)

An initial number of TPC in half-dried salted round scad was 1.6×10^2 CFU /g in room temperature storage, and reached 2.9×10^6, 2.2×10^5 and 3.0×10^5 CFU/g on day 15 of storage for 3 samples packaging, respectively. Whereas, The TPC in samples stored in refrigerator reached 3.3×10^6, 2.0×10^5 and 3.5×10^5 CFU/g on day 30 of storage for samples packed in air (T1), vacuum (T2) and air with oxygen absorber (T3) packaging respectively (Table 1-2). The highest numbers of TPC were observed in dried salted round scad packed in air packaging and stored at room temperature and refrigerator. Meanwhile, the lowest number of TPC was observed in samples packed in vacuum packaging and stored at room temperature and refrigerator. Even though, the Thai Community Products

Standards does not define the TPC in sun-dried fish (Thai industrial standards institute; TISI. 298/2006), the TPC is still necessary for indicating the spoilage of products, especially the high moisture and low salt content products. If salt content in salted products is above 10% and their moisture content is below 40%, toxigenic-halophilic amine which forms bacteria will be inhibited (Koral *et al*., 2013).

3.2.2.Escherichia coli and *Staphylococcus aureus*

Escherichia coli (E. coli) and *Staphylococcus aureus* (*S. aureus)* of all 3 half-dried salted round scad packaging stored at room temperature and refrigerator were less than Thai Community Products Standards for sun-dried fish. The standard limitation of *E. coli* is less than 50 MPN/g and *S. aureus* is less than 200 CFU/g (data not shown). *E. coli* is used as a hygienic indicator for food processing plants, indicating the fecal contamination from animals and humans. *S. aureus* is a toxigenic microorganism which is harmful to consumers.

Table 1. The microbial count of half-dried salted round scad stored at room temperature (30±2 ºC)

Storage time (days)	Total plate count (CFU/g)			Yeast and mold (CFU/g)		
	Air (T1)	Vacuum (T2)	Absorber (T3)	Air (T1)	vacuum (T2)	absorber (T3)
0	1.6×10^2	1.6×10^2	1.6×10^2	2.0×10^2	2.0×10^2	2.0×10^2
5	1.9×10^3	1.8×10^3	1.9×10^3	$1.6\text{x}10^2$	1.7×10^2	2.6×10^2
10	2.0×10^6	1.3×10^4	2.2×10^4	2.6×10^2	1.5×10^2	1.6×10^2
15	2.9×10^5	2.2×10^5	3.0×15^5	2.7×10^3	6.4×10^2	5.4×10^2

Table 2. The microbial count of half-dried salted round scad stored in chill condition (4±1ºC)

Storage time (days)	Total plate count (CFU/g)			Yeast and mold (CFU/g)		
	Air (T1)	Vacuum (T2)	absorber (T3)	Air (T1)	vacuum (T2)	absorber (T3)
0	1.6×10^2	1.6×10^2	$1.6\text{x}\times10^2$	2.0×10^2	2.0×10^2	2.0×10^2
5	1.3×10^3	1.4×10^3	1.8×10^3	1.9×10^2	1.8×10^2	2.5×10^2
10	1.1×10^4	1.6×10^4	2.3×10^4	1.7×10^2	2.0×10^2	3.0×10^2
15	2.4×10^4	1.9×10^4	2.8×10^4	2.9×10^2	3.4×10^2	2.1×10^2
20	2.9×10^5	2.2×10^4	3.0×10^4	2.7×10^2	3.7×10^2	2.3×10^2
25	3.1×10^6	2.7×10^4	3.2×10^4	6.9×10^2	3.5×10^2	5.5×10^2
30	$3.3\text{x}10^6$	$2.0\text{x}10^5$	$3.5\text{x}10^5$	$7.2\text{x}10^2$	$5.2\text{x}10^2$	$5.1\text{x}10^2$

4. Conclusions

Half-dried salted round scad stored in vacuum packaging showed the lowest quality changes comparing to samples stored in air and air with oxygen absorbers packaging. The chilling storage was able to increase the shelf life of half-dried salted round scad. It can be concluded that, the shelf life of half-dried salted round scad packed in air condition was 5 and 10 days length when stored at room temperature and chilled condition, respectively. On the other hand, the shelf life of half-dried salted round scad stored in vacuum package was 10 and 25 days under room temperature and chilled condition, respectively. For the greater amount of half-dried salted round scad as an industrial product, food additives are recommended to extend the product's shelf life.

5.References

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HPLC QUANTIFICATION OF THE CHEMICAL CONSTITUENTS FROM INDIGENOUS FRUITS AND VEGETABLES OF INDIAN HIMALAYAN REGION

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ABSTRACT

The purpose of the present work was to determine the phytochemical profiles by HPLC of the indigenous fruits and vegetables. The phenolic contents showed diverse variation in the selected fruits and vegetables. Development of genuine and dependable analytical methods with profile marker phytoconstituents in an extract containing a mixture of several components is a challenging task. A simple, rapid, precise and reliable HPLC method was developed for the quantification of phytochemicals from the extracts of selected minor fruits and vegetables. The *Taraxacum officinale* genus comprised a mixture of different bioactive compounds belonging to different chemical types, such as flavonoids, sesquiterpenes, triterpenes, phenolic acids, sterols. *Malva neglecta* contains different compounds including several phenolic acids, flavonoids and some non-phenolic compounds. Caffeoylquinic acids (3-, 4-, and 5-O-caffeoylquinic acids and 3,5-dicaffeoylquinic acid) are mainly present in *Cydonia oblonga* pulps. Three different hydroxycinnamic acid derivatives (neochlorogenic acid, p-coumaroylquinic acid and chlorogenic acid) were detected and quantified in *Prunus avium*.

1.Introduction

Indigenous fruits and vegetables are widely used by various traditions and cultures to fulfil energy source. They are potent sources of phytochemical components like polyphenols, antioxidants, minerals, vitamins, etc. Due to the special agro-climatic conditions in the temperate Himalayas of India, Kashmir has a variety of fruits and vegetables. The indigenous crops of the area are used by people to meet their basic needs. The indigenous fruits selected were Cherry (*Prunus avium)* and Quince (*Cydonia oblonga*) and the selected vegetables were Handh (*Taraxacum officinale*) & Sustchal (*Malva neglecta*).

Handh has potential health benefits due to the presence of phenolics, flavonoids, coumarins, terpenoids, sesquiterpene lactones, carotenoids, chlorophylls, dietary fibre and alkaloids (Colle *et al*., 2012; Gonzalez-Castejon, Visioli, & Rodriguez-Casado, 2012). Among flavonoids, apigenin-7-Oglycoside, luteolin-7-O-glycoside and naringenin-7-O-glycoside were identified and furthered analysed for their antimicrobial and antioxidant activities (Kenny *et al*., 2015).

Malva neglecta is referred to as Khebaiz or Khobb eiza in Arabic and belongs to Malvaceae family and is wildly grown in the Northern Border Province, Saudi Arabia. It has been traditionally used for insect bites, bladder infection, burns, inflammation, ulcers and wounds, as astringent, demulcent, diuretic, expectorant and laxative. Some of the

phytoconstituents reported in the literature are Quinic acid, Aconitic acid, Chlorogenic acid, Caffeic acid, Coumaric acid, Rutin, Hyperoside, Myricetin, Fisetin, Coumarin, Quercetin, Naringenin, Luteolin, Kaempferol, Apigenin, Rhamnetin and Chysin (Nesrin *et al*., 2017; Guder *et al*., 2012; Karak 2019; Essafi Benkhadir *et al*., 2012; Fattouch *et al*., 2007).

A large number of polyphenolic constituents were identified in quince fruit; in particular, flavan-3-ols such as Epicatechin, Procyanidin B2, eight Hydroxycinnamates, derivatives of Caffeoylquinic acid (CQA) and Coumaroylquinic acid, Kaempferol and Quercetin derivatives (Nagahora *et al*., 2013; Wojdylo *et al*., 2013; Hamauzu *et al*., 2005). The analysis of the phenolic profile indicated that derivatives of Quercetin and Kaempferol were minor components of quince fruit, while Procyanidins and Chlorogenic acid (CGA) derivatives constituted the majority of the polyphenolic fraction (Bystricka *et al*., 2017; Crozier *et al*., 2009; Strek *et al*., 2007; Joana *et al*., 2019). The other main component of quince are proanthocyanidins, compounds widely known for their antioxidant and protective effects on cardiovascular and cancer-related diseases, in part due to their ability to modulate pro-inflammatory and carcinogenic signals. (Joana *et al*., 2019; Serra *et al*., 2011). Procyanidins from quince fruit have been studied for their chemoprevention activity, and preliminary investigation indicated an effect against enzymes and receptors, suggesting a role in cancer prevention (Serra *et al*., 2011).

Sweet cherries are an excellent source of phytochemicals, namely phenolic compounds (Bastos *et al*., 2015). The concentration of polyphenols can vary between different sweet cherry cultivars and in different parts thereof (Tural and Koca 2008). Studies have shown that the antioxidant capacity in the stem is greater than the antioxidant capacity in the pulp, leading people to believe that this is due to the higher concentration of phenols found here as well. (Wang *et al*., 2017).The levels of phenolic compounds can be affected by several factors, and this is associated to both climatic and agronomic conditions (Toydemir *et al*., 2013; Liu 2013). Another study in acid cherry (*Prunus cerasus* L.) also allowed the phenolic composition to be determined using a high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD) (Monteiro Egydio *et al*., 2013). A study of several sweet cherry cultivars using high-performance liquid chromatography equipment coupled to ultraviolet (HPLC–UV) and diode array (HPLC–DAD) (Toydemir *et al*., 2013) is also reported.

Currently, some studies support the idea that a healthy diet can prevent the development of certain diseases. (Liu 2013). In addition, research works have shown the high relationship between the consumption of fruits in prevention of various chronic health problems (Monteiro Egydio *et al*., 2013). Not only fruit pulp, but also peels and seeds from dietary plants and traditional medicinal herbs, play an important role in health because of their nutritional, antioxidant properties and wealth due to bioactive compounds. It is also interesting to know the presence and concentration of polar compounds (such as organic acids), because these compounds affect sensory and sensory properties. In addition, organic acids can be used as a chemical indicator of maturity, bacterial activity or storage conditions, because these compounds have good stability during processing and storage. (Sandin-Espana *et al*., 2016; Vagelas and Sugar, 2020).

Currently, there are few studies on phenolic compounds and organic acids of the selected minor fruits and vegetables. Due to the few available research studies about the characterization of bioactive compounds in selected minor fruits and vegetables, the aim of the present work was to comprehensive and tentative identify phenolic compounds and organic acids in the edible part of selected minor fruits and vegetables. This study presents the selected minor fruits and vegetables as potential sources of bioactive compounds with applications in the food, pharmaceutical and cosmetic industries.

2. Materials and Methods

2.1. Chemicals and Reagents

HPLC grade formic acid, Methanol, Water & Acetonitrile were purchased from Himedia (LBS, Marg, Mumbai, India).

Solvents were filtered using a Solvent Filtration Apparatus 58,061 (Supelco, Bellefonte, PA, USA). Double- deionised water was obtained with a Milli-Q-system from Millipore (Bangalore India). The standard compounds Quinic acid, Malic acid, tr-Aconitic acid, Gallic acid, Chlorogenic acid, Protocatechuic acid, Tannic acid, tr-Caffeic acid, Vanillin, P- Coumaric acid, Rosmarinic acid, Rutin, Hesperidin, Hyperoside, 4-OH-Benzoic acid, Salicylic acid, Myricetin, Fisetin, Coumarin, Quercetin, Naringenin, Hesperidin, Luteolin, Kaempferol, Apigenin, Rhamnetin, Chyrsin, 3-O-Caffeoyl quinic acid, 4-O-Caffeoyl quinic acid, 5-O-Caffeoyl quinic acid, 3,5-di Caffeoyl quinic acid, Quercetin 3- galactoside, Kaempferol glycoside, Kaempferol 3-glycoside, Kaempferol 3-Rutinoside, NeoChlorogenic acid, P- Coumaryl quinic acid, Cyanidin 3-glucoside, Cyanidin 3- Rutinoside, Peonidin 3-glucoside, Pelargonidin 3- Rutinoside, Peonidin 3- Rutinoside, Catechin, Epicatechin, 9- Cis Violaxanthin, Neochrome, All- trans-Neoxanthin, All-trans-Violaxanthin, 9-cis-Neoxanthin, Luteoxanthin, cis-Violaxanthin, Antheraxanthin, 9-cis-Violaxanthin, 13- cis-Lutein, All trans Lutein, All trans Zeaxanthin, 9 cis- Lutein, were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Extraction

A solid- liquid extraction was used to extract the polar fraction. Briefly, 0.5 g of freeze-dried sample powder was dissolved in 15 mL of a solution of methanol/ water (80: 20 v/v). The mixture was placed in a water bath for 15 min at room temperature and then it was centrifuged for 15 min at 5000 rpm, the supernatant was removed, and extraction was repeated once more. The supernatants were collected, evaporated and reconstituted in 2 mL of methanol/ water (80: 20, v/v). The final extracts were filtered with regenerated cellulose filters 0.2 µm, (Millipore, Bedford, MA, USA) and stored at -18 °C until the analyses.

2.3. UPLC DAD Analyses

The extracts obtained by solid-liquid extraction were analysed by using UPLC coupled to DAD in order to identify phenolic compounds. An Agilent 1290 Infinity II- LC system (Agilent Technologies, Waldbronn Germany) equipped with a vacuum degasser, autosampler, quaternary pump, and DAD was used for the chromatographic determination. All the methods were validated and optimized.

For *Taraxacum officinale*, the separation was done using an Agilent Poroshell Eclipse plus C30 column (4.6 × 250 mm, 5 µm) from Agilent Technologies operating at a flow rate of 1 mL/min and 40 ° C, thorough out the gradient. The mobile phases used were water with MeOH/ACN in the ratio of 79:14:7 (Phase A) and Methylene chloride (Phase B), and the solvent gradient changed according to the following conditions: 95% A in the beginning and maintained for 9 min, decreased to 85% A in 23 min, 83% A in 33 min, 71% A in 35 min, 70% A in 45 min, 66% A in 66 min and returned to original ratio in 71 min. The injection volume was 20 µL and chromatograms were recorded from 190-600 nm with 450 nm as maximum wavelength.

For *Malva neglecta*, the chromatographic separation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3µm) analytical column. The column temperature was fixed at 40°C. The elution gradient consisted of mobile phase A (water, 5mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5mM ammonium formate and 0.1% formic acid). Use the gradient program t (min) of solvent B in the following proportions,% B: (0.40), (20.90), (23.99.90), (24.40), (29.40). The solvent flow rate was maintained at 0.5 ml / min and the injection volume was stable at 4 µl.

For *Cydonia oblonga*, chromatographic separation was carried out on a LiChroCART column (250 × 4 mm, RP-18, 5-µm particle size, Merck, Darmstadt, Germany) using two solvents: water/formic acid (19:1) (A) and methanol (B); starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min, and 80% B at 60 min. The flow rate was 0.9 mL/min, and the injection volume was 80 µL. Detection was carried out at 270, 320, and 350 nm.

In case of *Prunus avium*, the HPLC equipment was used with a diode array detector (DAD). The system consists of a binary pump, a degasser and an autosampler. The chromatographic column used is Beckman Ultrasphere ODS (Roissy CDG, France): 4.6 mm x 250 mm, 5 µm, equipped with a guard column of 4.6 mm x 10 mm. The mobile phase consists of two solvents: solvent A, water/formic acid (95:5; v/v) and solvent B, acetonitrile/solvent A (60:40; v/v). Phenolic compounds were eluted under the following conditions: 1 mL/min flow rate and the temperature was set at 25 °C, isocratic conditions from 0 to 10 min with 0% B, gradient conditions from 0% to 5% B in 30 min, from 5% to 15% B in 18 min, from 15% to 25% B in 14 min, from 25% to 50% B in 31 min, from 50% to 100% B in 3 min, followed by washing and reconditioning the column.

3. Results and discussions

The separation of all the compounds in complex extract is one of the challenging tasks in analytical HPLC.

3.1. Taraxacum Officinale (Handh)

According to current knowledge, the genus Taraxacum includes a mixture of different biologically active compounds belonging to different chemical types, such as flavonoids, sesquiterpenes, triterpenes, phenolic acids, sterols, etc. Among them, flavonoids and phenolic acids were the more abundant metabolites, which always had similar structure skeleton. It has been well established that a C30 column can provide a much better resolution than a C18 column in separating carotenoids and their geometrical isomers (Chen et al., 2004; Dugo et al., 2008). Initially a gradient solvent system of methanol/acetonitrile/water (84:14:2, v/v/v) and methylene chloride developed by Inbaraj et al., (2008) was used to separate the various carotenoids in T. formosanum by a YMC C30 column (250 mm × 4.6 mm I.D., particle size 5 µm). However, several carotenoid peaks overlapped, which may be due to the high concentration of solvent in the mobile phase. Therefore, it is necessary to reduce the strength of the solvent by increasing the polarity of the mobile phase. After various studies, a gradient mobile phase of methanol/acetonitrile/water (79:14:7, v/v/v) (A) and methylene chloride (B) was developed to separate 25 carotenoids and their geometrical isomers within 66 min (Fig. 1), with the retention times ranging from 11.54 to 64.22 min, retention factor from 2.51 to 18.57, separation factor from 1.01 to 1.44 and peak purity from 88.3 to 99.8% (Table 1). Most carotenoid peaks were adequately resolved (Fig. 1), implying both solvent strength and selectivity of mobile phase to sample components were properly controlled.

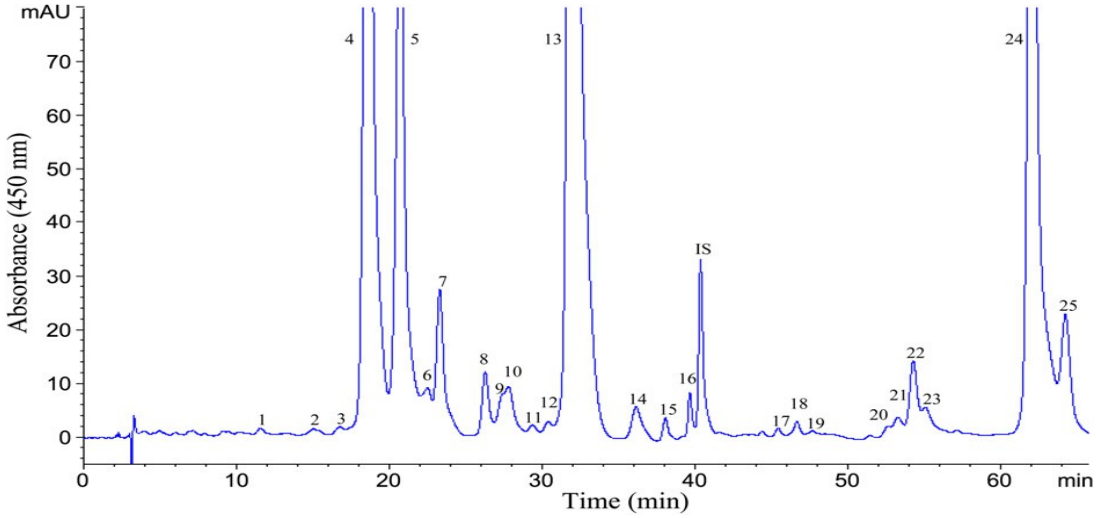

Figure 1. HPLC chromatogram of Standards of *T. officinale* (column: C30, mobile phase: (A) methanol–acetonitrile–water (79:14:7, v/v/v), (B) methylene chloride, flow rate: 1 mL/min, detection wavelength: 450 nm).

In Fig. 1, peak 1 was identified as 9- or 9'-cis- violaxanthin. Peak 2 was identified as neochrome. Both peaks of 3 and 4 were identified as all –trans forms of neoxanthin and violaxanthin, respectively. Peak 5 was identified as 9 or 9'- cis-neoxanthin. Both peaks 6 and 10 were identified as luteoxanthin based on absorption spectra and mass spectra characteristic and compared with that reported by Chen et al., (2004) and Dugo et al., (2008). Following the same approach, peaks 7 and 9 were identified as cis-violaxanthin and 9 or 9'-cis violaxanthin, respectively (Melendez-Martinez et al., 2008; Chen et al., 2004). Peak 8 was identified as antheraxanthin. Peaks 13, 14, 17 and 24 were positively identified as all-trans forms of lutein, zeaxanthin, β-cryptoxanthin and β-carotene, respectively. In several similar studies the major carotenoids, including neoxanthin, violaxanthin, β-carotene, zeaxanthin, β- cryptoxanthin and antherxanthin were also detected in Taraxacum officinale.

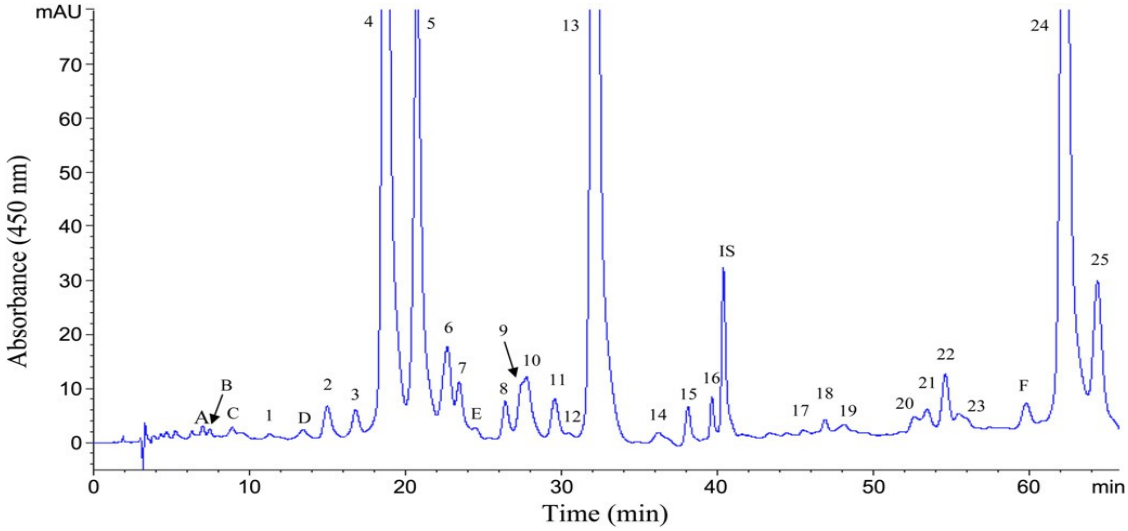

Figure 2. HPLC chromatogram of extract of *T. officinale*. Peak numbers with alphabetical letters (A-F) indicate additional compounds identified in extract fraction isolated by column chromatography, while 1-25 denote the same standard compound.

Fig. 2 shows the HPLC chromatogram of various carotenoids in carotenoid fraction isolated from Taraxacum extract. Following the same identification and quantitation criteria, a total of 31 carotenoids were separated and identified (Faria et al., 2009; Zepka et al., 2009). The peaks marked as A-F were identified as Auroxanthin, 13- cis-neoxanthin, Violaxanthin and 9-cis-βcarotene.

3.2. Malva Neglecta (Sustchal)

In the quantitative analysis of phenolic compounds various studies exist in literature reporting the use of HPLC (Mousavi et al., 2015; Fahimi et al., 2016). Figure 3 depicts the chromatogram of the standards of *Malva neglecta*.

Therefore, an accurate quantitative method was developed on HPLC for the analyses of twenty-seven compounds. The methanol extracts of M. neglecta were screened to quantify their phenolic composition by using an Agilent 1290 Infinity II- LC system.

This accurate method allowed us to qualify and quantify 27 different compounds including several phenolic acids, flavonoids and some non-phenolic compounds. To summarize, this is the first study to screen 27 compounds in M. neglecta. It is rich in terms of phenolic acids.

The detected compounds were Quinic acid, Malic acid, tr- Aconitic acid, Gallic acid, Chlorogenic acid, Protocatechuic acid, Tannic acid, tr- caffeic acid, Vanillin, p- Coumaric acid, Rosmarinic acid, Rutin, Hesperidin, Hyperoside, 4- OH Benzoic acid, Salicylic acid, Myricetin, Fisetin, Coumarin, Quercetin, Naringenin, Hesperedin, Luteolin, Kampferol, Apigenin, Rhamnetin and Chysin (Fig. 4).

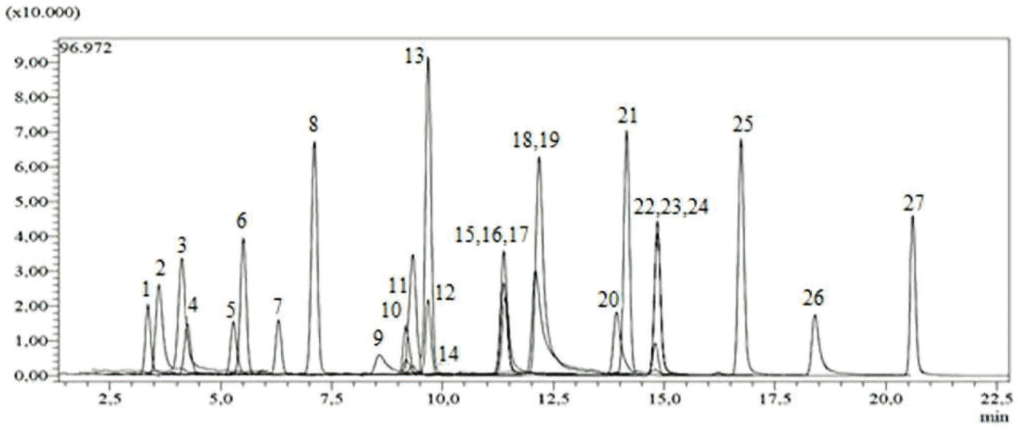

Figure 3. HPLC chromatogram of Standards of *Malva neglecta*.

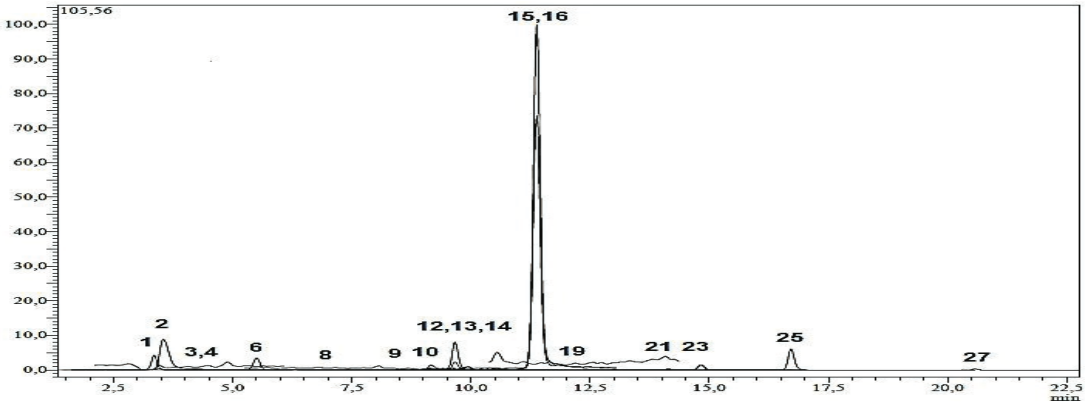

Figure 4. HPLC profile of *Malva Neglecta* methanol Extract. 1: Quinic acid, 2: Malic acid, 3: tr-Aconitic acid, 4: Gallic acid, 5: Chlorogenic acid, 6: Protocatechuic acid, 7: Tannic acid, 8: tr- caffeic acid, 9: Vanillin, 10: p-Coumaric acid, 11: Rosmarinic acid, 12: Rutin, 13: Hesperidin, 14: Hyperoside, 15: 4-OH Benzoic acid, 16: Salicylic acid, 17: Myricetin, 18: Fisetin,19: Coumarin, 20: Quercetin, 21: Naringenin, 22: Hesperetin, 23: Luteolin, 24: Kaempferol, 25: Apigenin, 26: Rhamnetin, 27: Chrysin.

3.3. Cydonia oblonga (Quince)

All quince pulps presented the same chemical profile, composed by at least five identified phenolic compounds: 3-, 4-, and 5-O-caffeoylquinic acids, 3, 5-dicaffeoylquinic acid, and rutin. Fig. 5 depicts the chromatogram of the standards of *Cydonia oblonga*.

The first group (39-46 min) was formed by five major peaks (peaks 5 to 9) and the second group (51-56 min) was formed by three peaks (Fig. 6).

All of the peaks showed identical spectral profile, with two maxima at 257 and 353-355 nm, which indicated that they could be flavonols or flavonol derivatives. HPLC analyses provided interesting information on the two mentioned groups of flavonoids. Quince fruit composition was characterised by the presence of 4- caffeoylshikimic acid, 4-caffeoyl quinic acid, quercetin-3, 7-diglucoside, kaempferol-3-O- rhamnoside and kaempferol-7-O-glucoside.

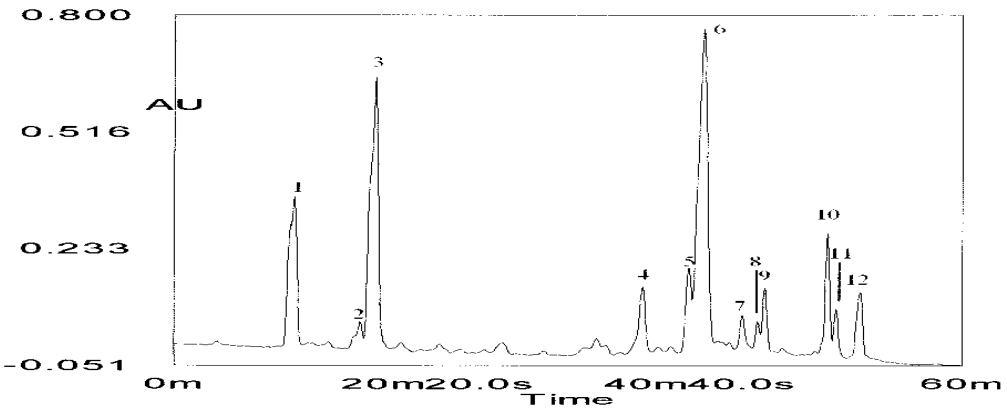

Figure 5. HPLC profile of a *Cydonia oblonga* Standards. Detection at 350 nm. Peaks: (1) 3-*O*-caffeoylquinic acid; (2) 4-*O*-caffeoylquinic acid*;* (3) 5-*O*caffeoylquinic acids; (4) 3,5-dicaffeoylquinic acid; (5) Quercetin 3-galactoside; (6) Rutin; (7) Kaempferol glycoside; (8) Kaempferol 3-glucoside; (9) Kaempferol 3-rutinoside; (10) and (11) Quercetin glycosides acylated with *p*-coumaric acid, and (12) Kaempferol glycoside acylated with *p*-coumaric acid.

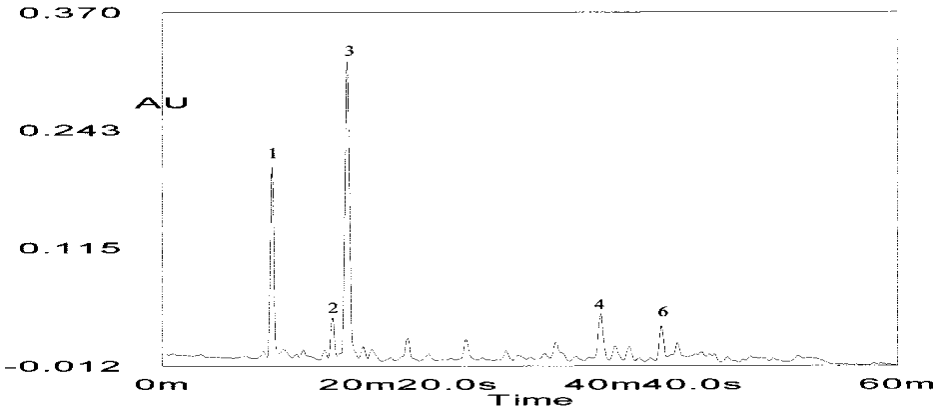

Figure 6. HPLC profile of *Cydonia oblonga* Extract. Detection at 350 nm. Peaks: (1) 3-*O*-caffeoylquinic acid; (2) 4-*O*-caffeoylquinic acid*;* (3) 5-*O*caffeoylquinic acids; (4) 3, 5-dicaffeoylquinic acid, and (6) Rutin.

3.4. Prunus avium (Cherry)

Figure 7 depicts the chromatogram of the standards of *Prunus avium.*

A total of 11 phenolic compounds, hydroxycinnamic acid derivatives (3), anthocyanins (5), flavan-3-ols (2) and flavonol (1), were identified and quantified in sweet cherry cultivars. Three different hydroxycinnamic acid derivatives (neochlorogenic acid, p-coumaroylquinic acid and chlorogenic acid) were detected and quantified in the cherry (Fig. 8). Among the hydroxycinnamic acids, neochlorogenic acid showed the highest concentration of the total hydroxycinnamic acid content. Goncalves et al., (2004) reported that neochlorogenic acid was the major hydroxycinnamic acid, varying from 22 to 190 mg ⁄ 100 g of FW and represented 19% to 71% of phenolics (Goncalves et al., 2004). Chlorogenic acid was the least abundant hydroxycinnamic acid. When we compare the total hydroxycinnamic acid derivative content of sweet cherries analysed with the previous reports, we see that our results were higher than the values obtained by Usenik et al., (2008) while lower than the values obtained by Goncalves et al., (2004). It was noted that cyanidin 3-rutinoside was the most dominant anthocyanin and it accounted for the largest proportion of the total anthocyanin contents.

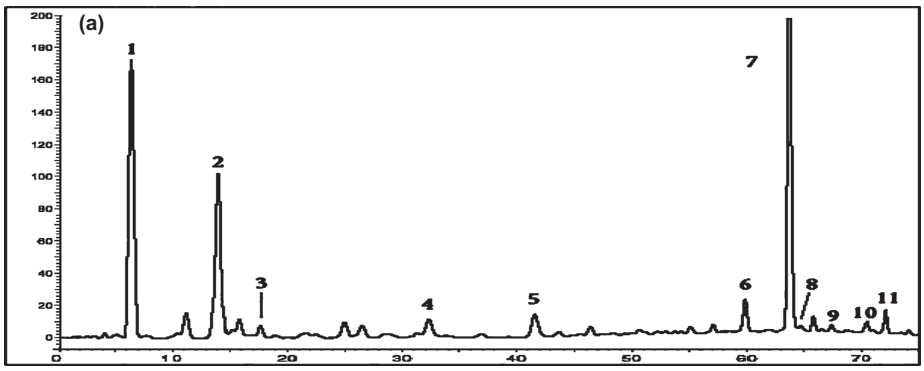

Figure 7. HPLC profile of Standards of *Prunus avium*. (1) Neochlorogenic acid; (2) *p*-coumaroylquinic acid; (3) Chlorogenic acid; (4) Cyanidin 3-glucoside; (5) Cyanidin 3-rutinoside; (6) Peonidin 3-glucoside; (7) Pelargonidin 3-rutinoside; (8) Peonidin 3-rutinoside; (9) Catechin; (10) Epicatechin; (11) Rutin

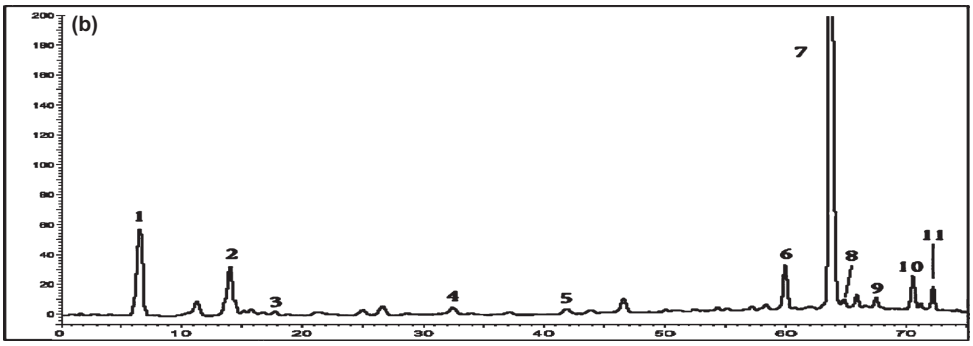

Figure 8. HPLC chromatograms of *Prunus avium* Extract recorded at 280 nm.

4. Conclusions

The HPLC gradient mobile phase was developed to separate different types of phytochemicals in the selected indigenous fruits and vegetables. The choice of mobile phase and buffer (organic matter and pH) composition plays an important role in the selectivity of separation. The final optimization can be done by changing the temperature, gradient slope and flow rate, and the type and concentration of the mobile phase modifier.

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Conflicts of Interest:

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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DIVERSITY OF MICROORGANISMS CAUSING SOFT ROT DISEASE OF FRUITS AND VEGETABLES MARKETED IN TAMANGHASSET (ALGERIA)

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ABSTRACT

The nutritional richness of fruit and vegetables makes them an ideal target for microorganisms, causing in particular soft rot. The aim of this work is therefore to isolate, characterize and identify these microorganisms, based on random sampling of rotten fruits and vegetables marketed in the city of Tamanghasset (Algerian Sahara). The study was carried out on 76 samples of fruits and vegetables showing symptoms of soft rot (from 2 to 14 units each). The strains were characterized phenotypically, enzymatically and pathogenically. In addition, the antimicrobial activity of sodium bicarbonates, white vinegar and bleach, was investigated *in-vitro* on the isolates. The results showed a high diversity of microorganisms involved. The dominance of yeasts was reported, especially of the *Candida* genus, followed by the Fungi *Cladosporium* and *Botrytis* and finally the bacteria which the most important ones are *Staphylococcus xylosus* and *Neisseria cinerae*. In addition, these microorganisms are capable to produce a wide range of PCWDE (Plant Cell Wall Degrading Enzymes), represented according to the experimental case by: gelatinase (30.26%), caseinase (31.57%), amylase (4.47%), lipase (35.52%), lipoproteinase (63.15%) and haemolysin (30.26%). However, the production of pectinase in 27.63% of the isolated microorganisms which is the most phytopathogenic enzyme for the deterioration of fruits and vegetables. This was confirmed by the pathogenicity test on three types of fruits and vegetables (zucchini, mandarin, tomato). Furthermore, the *in-vitro* evaluation of the sensitivity of these pectinolytic microorganisms to the three usual disinfectants, showed a remarkable efficiency of white vinegar and sodium bicarbonate, but none with bleach. Finally, it is clear from this work that the microorganisms involved in soft rot present a significant taxonomic diversity. Their capacity to resist disinfectants and their enzymatic background are the main factors of pathogenicity.

1. Introduction

Worldwide, concerns about plant diseases that can affect agriculture are becoming increasingly serious due to severe crop failures, and economic losses (Aouar, 2012). Approximately 30% of fresh vegetables are lost due to spoilage, primarily due to

phytopathogenic microorganisms colonization (Ife Fitz and Bas, 2003; Lee *et al.,* 2013). Chemical reactions that cause unpleasant sensory changes in food are the result of microbes' presence that use food as a source of carbon and energy (Gram *et al.,* 2002). Generally, deterioration leads to undesirable changes at organoleptic level, nutritional and healthy quality of food. This type of deterioration known as decay, some of which appear soft, reducing the shelf life of fruits and vegetables (Rosset 1990; Hozbor *et al.,* 2006). However, the main cause of soft rot in fruits and vegetables is the proliferation of bacteria, Fungi and sometimes yeast (Wallen, 1983). These microorganisms use the nutrients (sugar, protein, fat and vitamins) found in the plant product to survive (Ife Fitz and Bas, 2003; Hozbor *et al.,* 2006). However, some opportunistic germs can infect fruits and vegetables already damaged by other phytopathogenic microorganisms that have penetrated the protective wall of vegetables (Lee *et al.,* 2013).

The pathogenicity of these microorganisms is mainly related to the production of a wide range of enzymes called PCWDE (Plant Cell Wall Degrading Enzymes), which have the ability to degrade plant structures composed of: pectins, cellulose, hemicellulose and others, causing cell necrosis and tissue maceration (Lee *et al.,* 2013). Bacterial deterioration first results in tissue softening as pectin degrades (Rawat, 2015). Several bacterial species can penetrate the protective shell of vegetables and damage these products, while others can only penetrate when the product has been damaged (Alfano and Collmer, 1996). Deteriorating bacteria are found on the plant surface and soil and may enter the host from damaged areas or at natural openings during field crop growth, harvest and post-harvest handling, or during storage and distribution (Perombelon, 2002).

To our knowledge, no studies have been carried out on the microorganisms causing soft rot of fruits and vegetables marketed in the arid region of Tamanghasset. This has encouraged us to work in this direction, first know the diversity of the microorganisms involved, in order to test their phytopathogenicity and the production of the main PCWDE related to vegetable deterioration on the one hand, and the usual disinfecting agents on the other hand, to control this disease.

2. Materials and methods

2.1. Materials

2.2.1. Sample site and collection

Seventy-six (76) units of soft rot fruits and vegetables were sampled in February 2020 at various stores (Figure 1) in the city of Tamanghasset (2000 km south of the Algerian coast: Latitude: 22.7869, Longitude: 5.52722 22° 47 13 North, 5° 31 38 East). The samples are presented unpacked in the sampling sites according to their type (each type of crop being in a different stand from the other), and placed in an insulated plastic bag suitable for food use, hermetically sealed and labelled to identify the samples (number, location and date of sampling).

2.2. Methods

2.2.1. Isolation and Purification of Microorganisms from rotten fruits and vegetables

The damaged plant tissue (object of microbial infection) or the resulting liquid has been spread on the nutrient agar (Laboratory of Conda S.A, Madrid, Spain), as shown in Figure 2. After incubation, the isolates were purified according to the type of microorganism observed under an optical microscope, which led us to use two culture media: (1) PCA (Plate Count Agar) medium (Laboratory of Conda S.A, Madrid, Spain): for the isolation of bacteria and (2) OGA Milieu (Oxytetracycline Gelose Agar) (Institut Pasteur, Algiers, Algeria): for the isolation of yeasts and fungi.

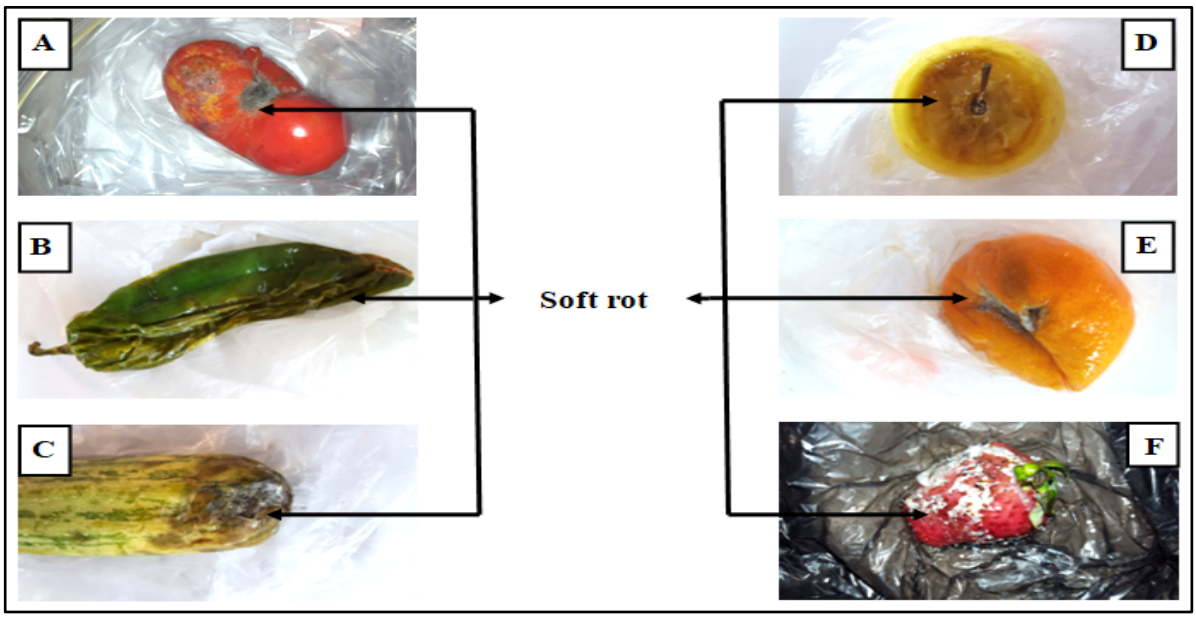

Figure 1. Photograph of some rotten fruits and vegetables sampled in the shops of Tamanghasset city (A- Tomato with white and viscous rot, B- Pepper with brown and viscous rot, C- Zucchini with brown rot with hypha, D-E- Apple and Mandarin with wound in the stem, F- Strawberry with white rot on the surface).

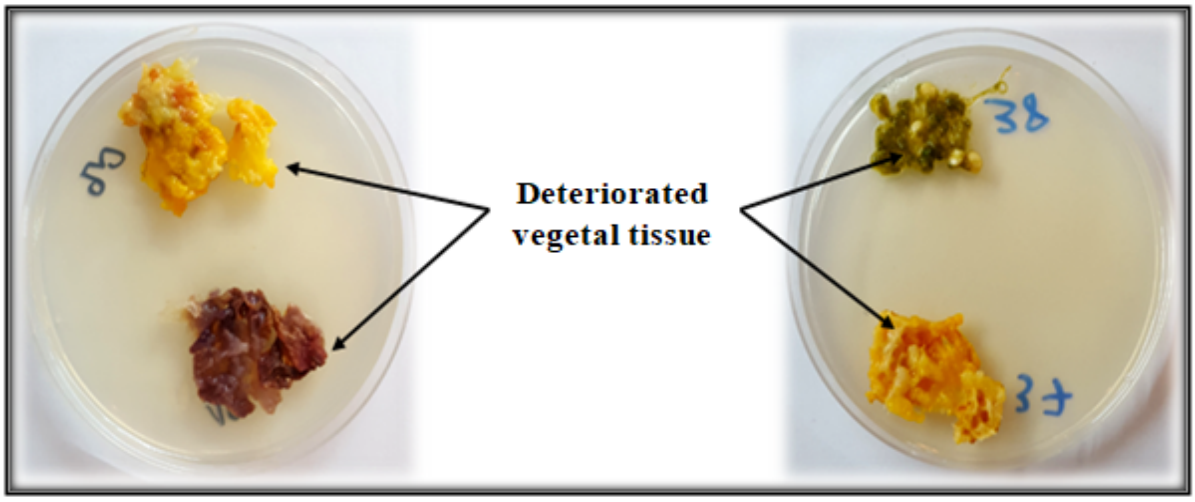

Figure 2. Photograph the isolation of the microorganisms responsible for the soft rot from the damaged tissues before incubation (part of the tissues from the infection site and a few drops of the resulting liquid are spread on the agar).

2.2.2. Screening for enzymatic activity (PCWDE)

The enzymatic activity of soft rot microorganisms is related to the presence of a set of enzymes. The hydrolysis test of gelatin was performed as described by Egamberdiyeva (2004) modified, using a nutrient broth supplemented with 50 g/L gelatin powder as a solidifying agent. Casein hydrolysis is tested on Mueller Hinton agar (MH) supplemented with 10% skimmed milk (CastroEscarpulli *et al.,* 2003) and amylolytic activity was detected on

Tryptic Soy Agar (TSA 1/10) with 1% starch added (Delarras 2014). Lecithinase was revealed on an ordinary nutrient agar supplemented by an emulsion of egg yolk and distilled water (2 mL / 20 mL) (Delarras, 2007). Pectinase is sought in a medium consisting of: 15 g of agar in 519 mL of distilled water, 1 g of yeast extract in 20 mL $(NH_4)_2SO_4$ 20%, 5 mL of 87% aqueous glycerol solution, 250 mL of 2% polygalacturonic acid aqueous solution, 0.2 M phosphate buffer at pH = 8, 100 mL distilled water, 1 mL $MgSO_47H_2O$ to 1 M (Snaiki *et al.,* 2006). Haemolytic activity of strains has been studied on blood agar (oxoid) and recorded as "positive" when a clear, colorless area around the colonies appeared or "weak" when incomplete hemolysis was observed (Hoeffler, 1977).

2.2.3. Phenotypical characterization of isolates

The strains were phenotypically characterized using standard procedures of Gram ©, catalase and oxidase tests supplemented by several biochemical tests as nitrate reductases, sugar assimilation (glucose, fructose, lactose and sucrose), growth at 4° C and 42° C and growth in salinity condition (7.5% NaCl) . All strains were stored in a nutrient broth containing 20% glycerol at -80° C. The morphology of isolated microorganisms is studied according to two (02) types of observation: macroscopic and microscopic. However, microscopic observation includes two types of examination: (1) examination in the fresh state and (2) examination after coloring. In addition to the examination in the fresh yeast observation was carried out by adding a few drops of iodine solution (Lugol) (Laboratory of Conda S.A., Madrid, Spain) on the slide in the fresh state. After 2-3 minutes, brown inclusions appear, representing the glycogen seeds present in the yeast cell (Dolisi, 2007). Methylene blue is used to visualize the structure of yeast and Fungi. Pure isolated fungi have been identified in accordance with the recommendations of Dufresne and Guy (2018).

In addition, the identification of bacterial strains was carried out using different types of API galleries: 20 Staph, 20 Strep, 20 NE (BioMérieux, Lyon, France), and their reading is done according to the procedure by the Excel Taxon 2007 software for gallery 20 Strep and Api Web^{TM} for gallery 20 NE and 20 Staph, in order to have reliable identification results.

2.2.4. Pathogenicity test

In order to study the relationship between the pectinolytic strains and soft rot, a test is conducted on intact fruit and vegetables. In fact, we have chosen to apply a procedure below to the intact samples spread at this time of year; namely: zucchini, mandarin and tomato. A disinfection of the surface of the whole fruit and vegetables in alcohol at 70°, and leave for 1 minute, in order to eliminate the saprophytic flora, then rinses the samples for 3 minutes with distilled water. After that, samples are dried in the oven at 30°C. On the other hand, the suspensions of the pectinolytic strains were sown by swabbing on the fruit and vegetables (three samples for each strain). The results were interpreted with the naked eye, assessing the degree of deterioration (soft rot) by each strain.

2.2.5. Evaluation of the antimicrobial activity of common liquid disinfectants on pectinolytic microorganisms

To control the soft rot disease of fruit and vegetable consumption, we used the descriptions provided by the FDA (Sanchez, 2018): [1 tablespoon (20 g) sodium bicarbonate in 1 L distilled water; 240 mL white vinegar in 1 L distilled water] and FAO (Lopez Camelo, 2007): [1 L domestic bleach diluted in 400 L distilled water]. For this, the agar direct diffusion technique (Miyadoh, 1993) was used to assess the effect of some common disinfectants on pectinolytic microorganisms, which are considered among the most dangerous plant pathogens.

All values are the mean ± SE (standard error) of three replicates of a single sample. The obtained data have been submitted to ANOVA

using the Statistical Analysis System (XLSTAT) version 2016. 02.

3. Results and discussions

This work is conducted to study the microorganisms responsible for soft rot disease of fruits and vegetables marketed in the city of Tamanghasset. This study is considered as the first report on the state of this plant disease in the Algerian Sahara. The purpose consists first of isolating these microorganisms and then characterizing them at the morphological, physiological and biochemical levels, then identify and preserve them, in order to launch research to combat them in a future study.

3.1. Isolated microorganisms responsible for soft rot

Out of seventy-six (76) isolated strains: thirteen (13) bacteria, forty-nine (49) yeasts and fourteen (14) fungi were found. Figure 3 represents the percentage of each phylum relative to the total number of isolates.

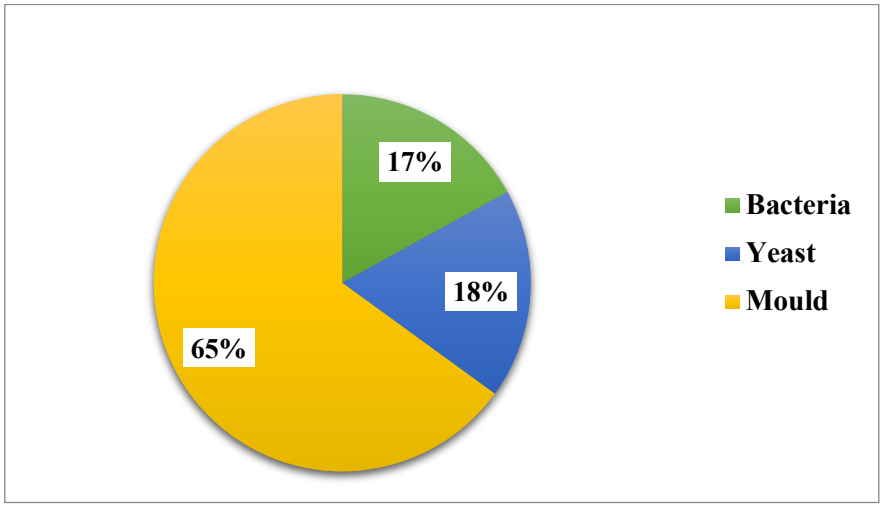

Figure 3. Percentage of each phylum of microorganisms involved in the soft rot of fruits and vegetables marketed in the city of Tamanghasset (southern Algeria).

3.1.1. Phenotypical characterization of isolated microorganisms

Characterization of the seventy-six (76) isolated microorganisms (bacteria, yeasts and fungi) was made according to the characteristics described above, the results of which are presented in the following paragraphs.

3.1.1.1. Bacteria

The results of staining (Table 1) showed that among the thirteen (13) bacterial strains found: nine (09) strains are cocci (5 Gram positive and 4 Gram negative) and four (4) strains (one 1 Gram positive and three 3 Grams negative) are rod.

3.1.1.2. Yeast and Fungi

Of the sixty-three (63) isolated yeasts and Fungi, and based on the macroscopic aspect of the colonies, we were able to distinguish two (02) groups: I. Thirty-nine (39) colonies have a smooth appearance, with a creamy or mucous or dry consistency and II. Twenty-three (23) colonies have a rough appearance, with a creamy or dry consistency. In addition, sixteen (16) colonies have aerial hyphae and four (04) colonies have spread green pigment on the agar (Table 2).

The results of the microscopic observation allowed us to visualize two (02) types: yeast and Fungi, where the differentiation between

them was carried out using an iodine solution, which colors the glycogen seeds existing in the yeast cells with a brown color under the microscope, also the appearance of the cells after staining with methylene blue. We noted that the most yeasts have a pseudo or true mycelium, with spores of the arthrospore or chlamydospore type (Figure 4). Unlike Fungi, observation of the yeasts under a photonic microscope allowed us to visualize the type of budding (bipolar or multilateral) for a few that were probably in the growth stage.

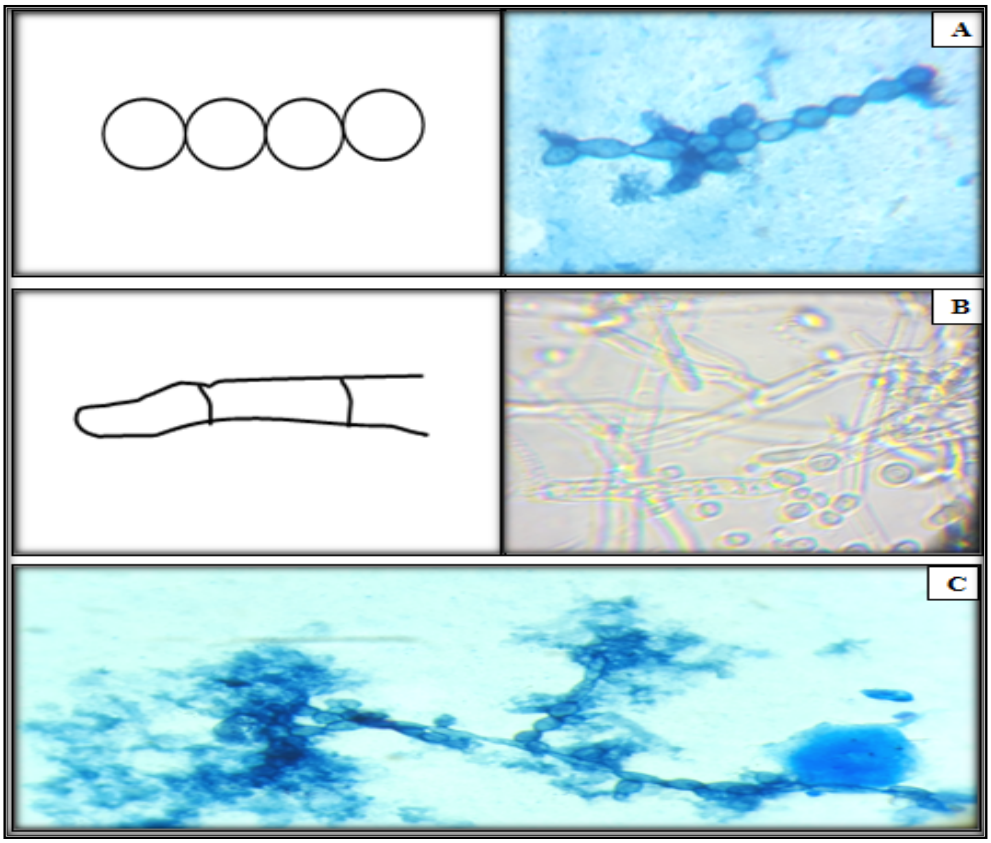

Figure 4. Microscopic observation photograph of yeast and Fungi involved in the soft rot of fruits and vegetables marketed in the city of Tamanghasset (Southern Algeria) under an optical microscope with X40 magnification (B- Yeast with true mycelium, chlamydospore) and X100 magnification (A- Yeast with pseudomycelium, arthrospore; C- Fungi with true mycelium, chlamydospore).

3.1.2. Physiological characterization

We have found that of the seventy-six (76) strains isolated, seventy (70) strains possess catalase; most of them are bacteria and yeasts. In addition, we found that among the 76 strains isolated, only thirty-four (34) strains possess cytochrome oxidase, and these strains are mostly yeasts. From the results obtained and presented in tables 1 and 2, we observed that most strains do not possess the enzyme nitrate reductase.

A relative neutral effect of abiotic stress referred to as salinity (7.5% NaCl growth) and temperature (4°C and 42°C growth) on microbial isolates showed their ability to survive under these conditions (Table 1 and 2). This finding concludes the virulence of our strains, which are principally inhibited by high salinity and low storage temperature (Selmaoui et *al.,* 2016; Benidire et *al*., 2015).

Table 1. Phenotypic characterization of isolated bacteria

Sample number	Microscopic aspect				Physiological characters						Genus and species	Vegetal host
	Form	Regroupement mode	Gram	Mobility	Catalase	Oxydase	NR	Salinity 7.5 % NaCl	Temperature			
									4 °C	42 °C		
05	**Cocci**	**Chain**	+	+	+	+	-	-	+	+	*Staphylococcus xylosus*	Tomato
06	**Cocci**	**Clusters**	-	-	+	-	+	-	+	+	*Neisseria cinerea*	Orange
21	**Cocci**	**Chain**	+	-	+	+	+	-	+	+		Strawberry
24	**Cocci**	**Diplo**	+	-	+	-	-	+	+	+	*Staphylococcus xylosus*	Strawberry
37	**Cocci**	**Chain**	+	+	+	+	-	-	+	+	*Staphylococcus xylosus*	Strawberry
43	**Cocci**	**Chain , isolated**	+	+	+	-	+	-	+	+	*Neisseria mucosa*	Mandarin
44	**Cocci**	**Chain**	-	-	+	+	+	-	+	+	*Neisseria mucosa*	Mandarin
51	**Cocci**	**Chain**	-	+	+	+	-	+	+	+	*Neisseria cinerea*	Mandarin
56	**Bacilli**	**Diplo, isolated**	-	+	+	+	-	+	+	+	*Vibrio vulnificus*	Apple
61	**Bacilli**	**Diplo**	+	+	+	+	+	+	+	+	*Bacillus coagulans*	Apple
65	**Cocci**	**Diplo, isolated**	-	+	+	+	+	+	+	+	*Haemophilus paraphraphilus*	Onion
69	**Bacilli**	**Diplo, isolated**	-	+	+	+	+	+	+	+	*Photobacterium damselae*	Onion

Gram type: Gram positive (+), Gram négative (-) ;
Mobility: absence (-), presence (+).
Catalase : absence (-), presence (+) ;
Growth at 4 °C / 42 °C: absence (-), presence.
Oxydase : absence (-), presence (+) ;
NR (Nitrate réductase) : absence (-), présence (+) ;
Salinity 7.5 % NaCl : absence (-), presence (+) ;

Table 2. Phenotypic characterization of isolated fungi

Sample number	The thallus aspect			Microscopic aspect (hypha)		Nature of the strands	Physiological characters						Genus and species	Vegetal host
	Aerial mycelium	Color	Diffusible pigment	Type of mycelium	spores		Catalase	Oxydase	NR	Salinity 7.5 % NaCl	Temperature			
											4 °C	4 °C		
1	**-**	**Yellow**	**-**	**TM**	**C**	Fungus	+	-	-	-	+	+	*Candida kefyr*	Mandarin
2	**-**	**Yellow**	**-**	**TM**	**A**	Fungus	+	-	-	+	+	+	*Geotrichum capitatum*	Mandarin
3	**-**	**White**	**-**	**PM**	**A**	Fungus	+	+	-	+	+	+	*Pichia anomala*	Mandarin
4	**-**	**White**	**-**	**TM**	**A**	Fungus	+	+	-	-	+	+	*Trichosporon sp*	Zucchini
7	**-**	**White**	**-**	**TM**	**A**	Fungus	+	-	-	-	+		*Candida guilliermondii*	Tomato
8	**+**	**Yellow**	**-**	**TM**	**C**	Fungus	-	+	-	-	+	+	*Candida kefyr*	Tomato
9	**-**	**Jaune**	**-**	**PM**	**C**	Fungus	+	-	-	+	+	+	*Candida kefyr*	Apple
10	**-**	**White**	**-**	**TM, septate**	**C**	Fungus	+	-	+	+	+	+	*Candida tropicalis*	Tomato
11	**+**	**White**	**-**	**PM**	**A**	Fungus	+	-	-	+	+	+	*Candida dubliensis*	Tomato
12	**+**	**White**	**-**	**TM**	**A**	Fungus	+	-	-	+	+	+	*Trichosporon sp*	Tomato
13	**+**	**White**	**-**	**TM**	**A**	Fungus	+	+	+	+	+	+	*Candida kefyr*	Tomato
14	**-**	**White**	**-**	**TM**	**A**	Fungus	+	+	+	-	+	+	*Candida kefyr*	Pepper
15	**-**	**White**	**-**	**TM, no septate**	**A**	Fungus	+	-	-	+	+	+	*Candida dubliensis*	Pepper
16	**+**	**White**	**-**	**TM**	**A**	Fungus	+	+	-	-	+	+	*Candida tropicalis*	Zucchini
17	**+**	**Yellow**	**-**	**TM**	**A**	Fungus	+	-	-	-	+	+	*Trichosporon sp*	Onion
18	**-**	**Yellow**	**+**	**TM**	**C**	Fungus	+	+	-	-	+	+	*Botrytis sp*	Onion
19	**-**	**White**	**+**	**TM**	**A**	Fungus	+	+	-	-	+	+	*Trichosporon sp*	Onion
20	**-**	**White**	**-**	**PM**	**A**	Fungus	+	+	-	+	+	+	*Candida guilliermondi*	Mandarin
22	**-**	**White**	**-**	**TM**	**A**	Fungus	-	+	+	+	+	+	*Botrytis sp*	Orange
23	**-**	**White**	**-**	**TM**	**A**	Fungus	+	+	-	-	+	+	*Botrytis sp*	Orange
25	**-**	**Yellow**	**-**	**TM**	**C**	Fungus	+	+	-	-	+	+	*Trichosporon sp*	Tomato
26	**-**	**White**	**-**	**PM**	**C**	Fungus	+	-	-	+	+	+	*Candida kefyr*	Pepper
27	**-**	**White**	**+**	**TM, septate**	**C**	Fungus	+	+	+	-	+	+	*Trichosporon sp*	Tomato
28	**-**	**Yellow**	**-**	**PM**	**A**	Fungus	+	-	-	-	+	+	*Botrytis cinerae*	Zucchini
29	**-**	**White**	**-**	**TM**	**A**	Fungus	+	+	-	+	+	+	*Botrytis sp*	Onion
30	**-**	**White**	**+**	**TM**	**A**	Fungus	+	-	-	+	+	+	*Trichosporon sp*	Pepper

31	-	**White**	-	**TM**	**A**	Fungus	+	+	-	+	+	+	*Trichosporon sp*	Pepper
32	+	**White**	-	**TM, no septate**	**A**	Fungus	+	-	-	-	+	+	*Trichosporon sp*	Pepper
34	-	**White**	-	**TM**	**A**	Fungus	+	+	-	+	+	+	*Candida dubliensis*	Pepper
35	+	**White**	-	**TM**	**A**	Fungus	+	-	-	+	+	+	*Trichosporon sp*	Apple
36	-	**Yellow**	-	**TM**	**A**	Fungus	+	+	-	+	+	+	*Trichosporon sp*	Apple
38	-	**White**	-	**TM**	**A**	Fungus	-	-	+	-	-	+	*Candida dubliensis*	Pepper
39	-	**White**	-	**PM**	**A**	Fungus	+	+	-	+	+	+	*Pichia anomala*	Pepper
40	-	**White**	-	**PM**	**C**	Fungus	+	-	+	+	+	+	*Candida kefyr*	Mandarin
41	-	**White**	-	**PM**	**C**	Fungus	+	-	-	+	+	+	*Candida parapsilosis*	Eggplant
42	-	**White**	-	**PM**	**C**	Fungus	+	-	+	+	+	+	*Pichia anomala*	Apple
45	-	**White**	-	**PM**	**A**	Fungus	+	-	-	+	+	+	*Candida guilliermondii*	Mandarin
46	+	**White**	-	**TM**	**A**	Fungus	+	-	-	-	+	+	*Cladosporium sp*	Mandarin
47	-	**White**	-	**PM**	**A**	Fungus	+	-	+	-	+	+	*Candida kefyr*	Mandarin
48	-	**White**	-	**TM**	**A**	Fungus	+	-	+	-	+	+	*Trichosporon sp*	Mandarin
49	+	**White**	-	**TM**	**C**	Fungus	+	-	-	-	+	+	*Trichospron sp*	Mandarin
50	+	**White**	-	**PM**	**C**	Fungus	+	-	-	+	+	+	*Penicillium sp*	Mandarin
52	-	**White**	-	**PM**	**A**	Fungus	+	+	-	+	+	+	*Candida guilliermondii*	Apple
53	+	**White**	-	**TM**	**A**	Fungus	+	-	-	+	+	+	*Geotrichum capitatum*	Apple
54	-	**White**	-	**PM**	**C**	Fungus	+	-	-	+	+	+	*Candida kefyr*	Apple
55	-	**White**	-	**PM**	**A**	Fungus	+	-	-	+	+	+	*Cladosporium sp*	Apple
57	-	**White**	-	**PM**	**C**	Fungus	+	+	+	+	+	+	*Geotrichum capitatum*	Apple
58	+	**White**	-	**TM**	**A**	Fungus	+	-	-	+	+	+	*Candida guilliermondii*	Apple
59	-	**White**	-	**TM**	**A**	Fungus	+	-	-	+	+	+	*Cladosporium sp*	Apple
60	-	**Yellow**	-	**TM**	**C**	Fungus	+	+	+	+	+	+	*Fusarium sp*	Apple
62	-	**White**	-	**TM**	**C**	Fungus	+	-	-	+	+	+	*Trichosporon sp*	Tomato
63	-	**White**	-	**TM**	**A**	Fungus	+	+	-	+	+	+	*Penicillium digitatum*	Tomato
64	-	**White**	-	**TM**	**A**	Fungus	+	+	-	+	+	+	*Cladosporium sp*	Mandarin
71	-	**White**	-	**PM**	**C**	Fungus	+	-	+	+	+	+	*Candida kefyr*	Tomato
72	-	**White**	-	**PM**	**A**	Fungus	+	+	+	+	+	+	*Candida kefyr*	Zucchini
73	-	**Yellow**	-	**TM**	**A**	Fungus	+	+	-	+	+	+	*Botrytis sp*	Onion
74	-	**White**	-	**TM**	**A**	Fungus	+	-	+	+	+	+	*Tricosporon sp*	Zucchini
75	-	**White**	-	**PM**	**A**	Fungus	-	-	+	-	+	+	*Candida kefyr*	Spinach
76	-	**White**	-	**TM**	**C**	Fungus	-	-	-	+	+	+	*Cladosporium sp*	Spinach
77	-	**White**	-	**TM**	**C**	Fungus	+	-	+	+	+	+	*Trichosporon sp*	Wild truffle
78	+	**White**	-	**PM**	**A**	Fungus	-	-	-	-	+	+	*Penicillium sp*	Wild truffle

79	+	**White**	-	**PM**	**A**	Fungus	+	-	+	+	+	+	*Candida lypolytica*	Wild truffle
80	+	**White**	-	**TM**	**A**	Fungus	+	-	+	+	+	+	*Geotrichum capitatum*	Eggplant

Aerial mycelium : absence (-), presence (+) ;
Diffusible pigment : absence (-), presence (+) ;
Type of spores: Arthrospore (A), Chlamydospore (C).
Type of mycelium : Pseudomycelium (PM), True mycelium (TM) ;
Catalase : absence (-), presence (+) ;
Oxydase : absence (-), presence (+) ;
NR (Nitrate réductase) : absence (-), présence (+) ;
Growth at 4 °C / 42 °C: absence (-), presence (+).

Table 3. Assessment of the Degree of Soft Rot Caused by Pectinolytic Strains in Three (03) Plant Varieties

Strand		**01**	**04**	**05**	**06**	**11**	**16**	**19**	**20**	**21**	**24**	**25**	**26**	**27**	**28**	**32**	**39**	**40**	**41**	**57**	**62**	**63**
Code		**PS1**	**PS2**	**PS3**	**PS4**	**PS5**	**PS6**	**PS7**	**PS8**	**PS9**	**PS10**	**PS11**	**PS12**	**PS13**	**PS14**	**PS15**	**PS16**	**PS17**	**PS18**	**PS19**	**PS20**	**PS21**
Zucchini	**After 24h**	+++	+++	+++	+++	-	+	+++	-	+	+	+	+	+++	+	-	-	-	-	+++	-	-
	After 6 days	+++	+++	+++	+++	+	+	+++	-	++	+	++	+++	+++	+	-	-	-	+	+++	-	-
Mandarin	**After 24h**	-	+	+	-	-	+	-	-	+	+	++	‡	-	-	-	‡	‡	++	+	-	+
	After 6 days	-	++	‡	+	‡	+	-	-	‡	+	+++	+++	+	+	-	+++	+++	+++	+	+	‡
Tomato	**After 24h**	-	+	+	-	-	-	+	-	+	-	‡	-	+++	-	+	-	‡	-	‡	+	-
	After 6 days	‡	‡	‡	‡	+	-	+++	+	+	‡	+++	+++	+++	+	+	‡	‡	‡	+++	‡	-

(-) : No rot ; **(+) :** Weak rot ; **(++) :** Medium rot ; **(+++) :** Strong rot ; **(++++) :** Very strong rot (appearance of mashed potatoes) ; **PS :** pectinolytic strand; **PS1 :** *Candida kefyr* ; **PS2 :** *Trichosporon sp* ; **PS3 :** *Staphylococcus xylosus* ; **PS4 :** *Neisseria cinerea* ; **PS5 :** *Candida dubliniensis*; **PS6 :** *Candida tropicalis* **; PS7 :** *Trichosporon sp* ; **PS8 :** *Candida guilliermondii* ; **PS9 :** *Staphylococcus xylosus* ; **PS10 :** *Staphylococcus xylosus* ; **PS11 :** *Trichosporon sp* ; **PS12 :** *Candida kefyr* ; **PS13 :** *Trichosporon sp* ; **PS14 :** *Botrytis cinerea* ; **PS15 :** *Trichosporon sp* ; **PS16 :** *Pichia anomala* ; **SP17 :** *Candida kefyr* ; **PS18 :** *Candida parapsilosis* ; **PS19 :** *Geotrichum capitatum* ; **PS20 :** *Trichosporon sp* ; **PS21 :** *Penicillium digitatum.*

Table 4. Antimicrobial activity of common disinfectants on isolated pectinolytic strains expressed by diameter inhibition zones (mm)

Strains	01	04	05	06	11	16	19	20	21	24	25	26	27	28	32	39	40	41	57	62	63
Code	**PS1**	**PS2**	**PS3**	**PS4**	**PS5**	**PS6**	**PS7**	**PS8**	**PS9**	**PS10**	**PS11**	**PS12**	**PS13**	**PS14**	**PS15**	**PS16**	**PS17**	**PS18**	**PS19**	**PS20**	**PS21**
Sodium bicarbonate	7 ± 1.08b	8.66 ± 1.08b	7.66 ± 1.08b	8.66 ± 1.08b	8 ± 1.08b	6.33 ± 1.08b	8 ± 1.08b	16 ± 1.08a	8 ± 1.08b	0 ± 1.08c	7 ± 1.08b	6 ± 1.08b	7.33 ± 1.08b	10 ± 1.08b	6.66 ± 1.08b	8.66 ± 1.08b	6.66 ± 1.08b	8.33 ± 1.08b	9.33 ± 1.08b	7 ± 1.08b	6.66 ± 1.08b
Bleach	7.66 ± 0.81a	0 ± 0.81b	9.33 ± 0.81a	7.33 ± 0.81a	7 ± 0.81a	9.66 ± 0.81a	8.33 ± 0.81a	0 ± 0.81b	9.66 ± 0.81a	8 ± 0.81a	6.66 ± 0.81a	6.66 ± 0.81a	6.33 ± 0.81a	9.66 ± 0.81a	9.33 ± 0.81a	6 ± 0.81a	6.66 ± 0.81a	9.33 ± 0.81a	9.66 ± 0.81a	6.66 ± 0.81a	7 ± 0.81a
White vinegar	10.66 ± 0.83c	10.33 ± 0.83c	12.66 ± 0.83abc	9.66 ± 0.83	7.33 ± 0.83	12.33 ± 0.83abc	11.33 ± 0.83bc	7.33 ± 0.83	12 ± 0.83abc	13.66 ± 0.83abc	11.33 ± 0.83bc	0 ± 0.83	14.66 ± 0.83abc	11 ± 0.83b	16 ± 0.83a	13 ± 0.83abc	0 ± 0.83	13.66 ± 0.83abc	10 ± 0.83	15.66 ± 0.83ab	15.66 ± 0.83ab

PS1 : *Candida kefyr* ; **PS2 :** *Trichosporon sp* ; **PS3 :** *Staphylococcus xylosus* ; **PS4 :** *Neisseria cinerea* ; **PS5 :** *Candida dubliniensis*; **PS6 :** *Candida tropicalis* **;** **PS7 :** *Trichosporon sp* ; **PS8 :** *Candida guilliermondii* ; **PS9 :** *Staphylococcus xylosus* ; **PS10 :** *Staphylococcus xylosus* ; **PS11 :** *Trichosporon sp* ; **PS12 :** *Candida kefyr* ; **PS13 :** *Trichosporon sp* ; **PS14 :** *Botrytis cinerea* ; **PS15 :** *Trichosporon sp* ; **PS16 :** *Pichia anomala* ; **SP17 :** *Candida kefyr* ; **PS18 :** *Candida parapsilosis* ; **PS19 :** *Geotrichum capitatum* ; **PS20 :** *Trichosporon sp* ; **PS21 :** *Penicillium digitatum.*

We can explain the survival of these pathogens by their adaptations or their ability to sporulate, which allow them on the one hand, sporulant bacteria *(Bacillus*) to resist adverse conditions, and on the other hand fungi to propagate and colonize other niches (Pozzi, 2014). Moreover, the city of Tamanghasset in terms of agricultural self-sufficiency is less developed, as it is considered an arid zone, which requires the import of fruits and vegetables from other areas of the country. This transport can probably play a major role in the spread of spores as agents of resistance and propagation of the microorganisms causing soft rot.

3.1.3. Biochemical Characterization

3.1.3.1. Sugar assimilation

As part of the characterization of our strains, several phenotypic tests were performed including the assimilation of sugars. We noted a strong assimilation for the three sugars: sucrose, fructose and lactose, compared to glucose (Figure 5). Gupta *et al.* (2015) explained this latter as the fact that plant pathogens primarily target complex compounds in the early phase of infection, which explains the low metabolization of glucose compared to other sugars. In addition, the assimilation of lactose, which is not part of the carbohydrate composition of fruits and vegetables (Rémésy, 2008), according to Rosset (1995), indicates that these microorganisms are capable to alter other foods.

3.1.3.2. Enzymatic activity

In this same perspective, we studied the enzymatic potential of isolated strains that seem to be a very important player in the alteration of fruits and vegetables. Phytopathogenic microorganisms are capable to produce one or more enzymes, which enable them to break down fruit and vegetable tissues. These enzymes are called PCWDE (Plant Cell Wall Degrading Enzymes) (Lee et *al.,* 2013). We observed that all isolated strains possess this hydrolytic activity, but in different proportions for the selected substrates, the most important of which is lipoprotein-bound, followed by casein and gelatin. Based on the results obtained and presented in Figure 6, all isolated strains have a low capacity for protein hydrolysis (casein and gelatin). However, their hydrolytic properties are notable for lipids, particularly lipoproteins, and for polysaccharides, specifically starch. On the other hand, most strains are not able to hydrolyze hemoglobin (hemolytic activity), and those that have this activity are mainly of the β type. This can be explained first, that the majority of our isolates are fungi, whose infections are characterized by the secretion of the protease enzyme in the early stage of infection (Movahedi and Heale, 1990; Zalewsky-Sobcazak, 1985). In addition, our isolates are capable to hydrolyze lipids and polysaccharides (starch), suggesting that they use both substrates as sources of carbon and energy, according to Boiron (1996) and Nicklin *et al.* (2000). Moreover, some of our phytopathogens have an enzymatic characteristic and not the least, namely the hydrolysis of pectin. The latter is the most important polysaccharide in the cell wall of fruits and vegetables (Esquerré-Tugayé et *al*., 2000). Noted that, the pectinolytic activity of these microorganisms is the most studied among PCWDE, because it induced degradation of this protective wall, and softening of tissues (Selmaoui *et al.,* 2017), which, led opportunistic plant pathogens that possess PCWDE enzymes to attack fruits and vegetables (the remaining 55 strains), and do not possess pectinase (Willats *et al.,* 2001). Therefore, pectinase is the most important virulence factor of most pathogenic isolated microorganisms.

On this basis, these microorganisms can use a wide range of substrate to grow, making them more competitive and good colonizers. In

addition to these enzymes, we also looked at their hemolytic activity. It is true that these germs contaminate the plant in the first place (no blood) but can pass to the human being, and cause diseases, most often serious after eating the infected fruits and vegetables (Linares *et al.,* 2007) hence searching for hemolytic activity.

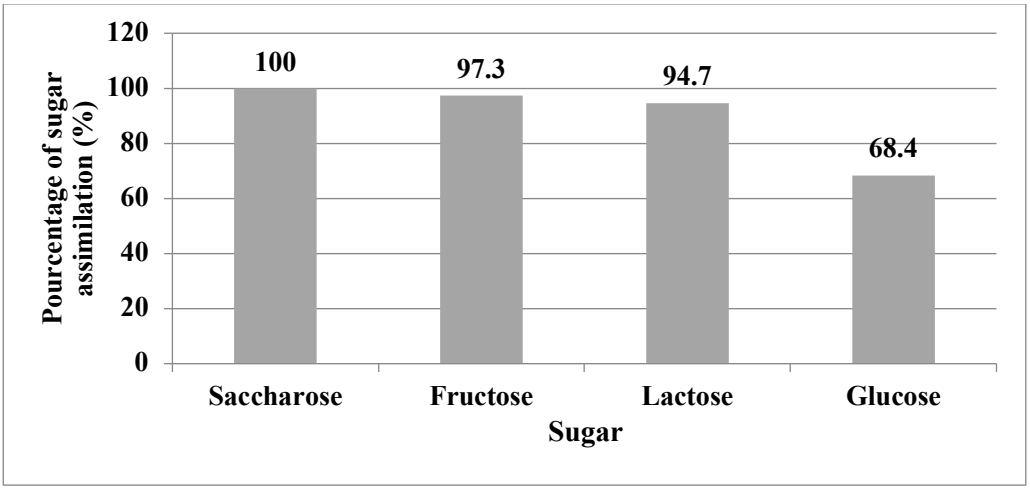

Figure 5. Histogram representing the percentage (%) of sugar uptake by isolated microorganisms.

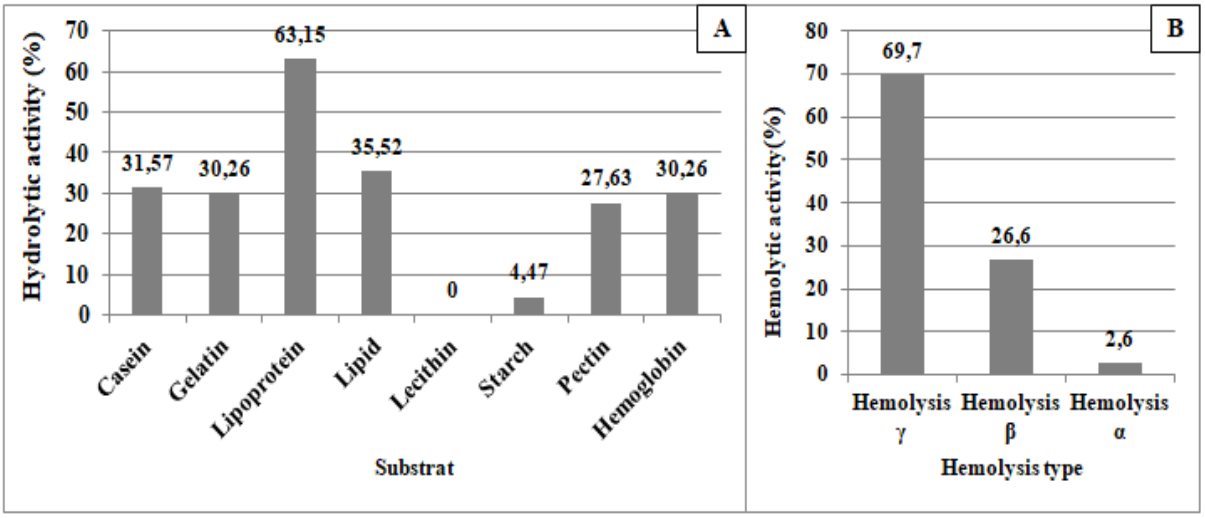

Figure 6. Histogram of hydrolytic activity of all strains involved in soft rot (A- hydrolytic activity by substrate type, B- hemolysis type by percentage of hemolytic activity).

3.2. Identification of isolated microorganisms

Based on the results presented in Tables 1 and 2, we observed that the isolated species belong mainly to the following six (06) genera in the three phylums:

- Yeasts: *Candida* and *Trichosporon*;
- Fungi: *Cladosporium* and *Botrytis*;
- Bacteria: *Staphylococcu*s and *Neisseria*.

Indeed, most of isolated and identified microorganisms belong to the fungi kingdom. Yeasts represent the dominant species in the division of Ascomycota, *with* the species *Candida kefyr* and *Trichosporon sp.* Consistent with our findings, Desbordes (2003) found that

the genus *Trichosporon* is among pectinolytic yeasts that infect mostly vegetables. On the other hand, Rawat (2015) also found that *Candida* and its related genera cause human infections, but there is no literature that classifies *Candida kefyr* as a plant pathogen. This led us to assume that this species is a contaminant, because it known to be a human pathogen (Sanchis et *al.,* 2016). These yeasts have been more frequently isolated from tomato, tangerine and apple. Second, the most abundant Fungi are represented by the species *Cladosporium sp* isolated from mandarins and apples, followed by *Botrytis sp,* isolated mainly from onions. Finally, we note the two genera *Fusarium* and *Penicillium*, contrary to the studies of Abdullah *et al.* (2016) and Selmaoui *et al.* (2017).

This study also revealed the presence of bacteria associated with soft rot disease, the most dominant of which is the genus *Neisseria*. Suprinyan *et al.* (2012) have also isolated this genus from rotten fruit waste destined for biogas production. In addition, the species *Staphylococcus xylosus* isolated from strawberries and tomatoes is the second most common bacterial species; compared to Park *et al.* (2019).

However, we also found species such as *Photobacterium damselae* and *Vibrio vulnificus,* generally pathogens of raw seafood (Rivas *et al.,* 2013; Copin *et al.,* 2015). Their presence in our rotten fruits and vegetables is explained by contact with other seafood infected with these pathogens, or contamination by washing water. It is remembered that these identifications are relatively brief and certainly require a molecular study that may reveal other more likely species.

Indeed, yeast and Fungi have been found to infect fruit much more because they are rich in nutrients and water (Moss, 2008). In addition, the variation of the pH spectrum in fruits and vegetables, presents a competitive factor to promote the growth and infection of the latter by yeasts and Fungi, but not by bacteria (Warnasuriya et *al.,* 1985). Because low pH values (2.2 to 5) prevent or delay the growth of bacteria, yeast and mould are able to grow in these pH ranges (Desbordes, 2003).

Furthermore, some species infect only one type of fruit or vegetable, such as *Candida parapsilosis* that has been isolated from the eggplant, and *Staphylococcus xylosus* isolated primarily from strawberries. In addition, other species are found in several fruits and vegetables, as is the case with the two (02) yeasts: *Candida kefyr* and *Trichoporon sp*.

3.3. Pathogeicity test

After 24 hours of incubation of the pectinolytic strains sown on the three (03) samples (zucchini, tangerine, tomato), we observed that the most samples showed symptoms of soft rot (Table 3). Moreover, pectinolytic strains PS2 (*Trichosporon sp*), PS9 (*Staphylococcus xylosus*), PS13 (*Trichosporon sp*) and PS19 (*Geotrichum capitatum*) caused harsh soft rot.

3.4. Antimicrobial activity of common liquid disinfectants

In the last part of this work, we agreed to test some tools for controlling the spread of these pectinolytic plant pathogens, using *in-vitro* test by three antimicrobial agents. The antimicrobial test is based on the spread of traditionally antimicrobial agents used for cleaning and disinfecting vegetables and fruits in households and food industries (bleach, white vinegar, sodium bicarbonate).

Based on the results obtained (Table 4), we noted that, depending on the diameter of the inhibition zone, the most effective disinfectant is vinegar, then bicarbonate, which gave a very significant antimicrobial activity, except for the bacterium PS10: *Staphylococcus xylosus*. The analysis of variance (Annex Table 1, 2 and 3)

indicated a non-significant difference for disinfectants activities (P>0.05).

However, for diluted bleach, almost no inhibition zones were found, even at double and triple concentrations, meaning that pectinolytic strains are resistant to this disinfectant. Consistent with our findings, Fong *et al.* (2011) found that acetic acid and low pH of vinegar inhibits the growth of many pathogens, while sodium bicarbonate inhibits fungi growth with a very limited spectrum, probably because its alkalinity is not enough to eliminate all germs present on fruits and vegetables.

However, bleach activity was almost negligible, contrary to FDA expected results, even after doubling and tripling the concentration. This makes us think of two probabilities: the first is that pectinolytic microorganisms have developed resistance to chlorine compounds, due to the massive use of pesticides in the culture phase (Gava et *al.,* 2018). The second probability is that the concentration used is too low to have a total inhibition of pectinolytic microorganisms (WHO, 2007).

4. Conclusions

In the light of the results obtained, it appears that of the 76 samples of rotten fruits and vegetables marketed in the town of Tamanghasset: 49 yeasts, 14 Fungi and 13 bacteria have been isolated and characterized. The most abundant species were *Candida kefyr* (tomato, mandarin); *Cladosporium sp* (mandarin, apple); *Botytis sp* (onion); *Staphylococcus xylosus* (strawberry) and *Neisseria cinerae* (mandarin).

The results of the phenotypic tests carried out on the microorganisms in question have shown that soft rot is mainly of fungal origin, but this does not exclude the presence of bacteria. Isolated pathogens have a very diverse enzymatic equipment, whose key enzyme in phytopathology is pectinase, which has a role in the pathogenicity of our strains. Furthermore, the production of other enzymes that are not necessarily involved in phytopathology, such as: hemolysin and lipoproteinase, by most of isolates identified, are an attractive results that can confirm the opportunism of these non-pectinolytic pathogens and their involvement in this disease. Therefore, it is mandatory to go towards preventive actions, by the use of common disinfectants, of which the most profitable in our study is white vinegar.

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Annex : Analysis of variance

Table A1. Bicarbonate's activity

Source	DDL	Sum of squares	Average of squares	F	Pr > F
Model	20	447.524	22.376	6.293	**0,0001**
Error	42	149.333	3.556		
Total corrected	62	596.857			

Table A2. Bleach's activity

Source	DDL	Sum of squares	Average of squares	F	Pr > F
Model	20	444.317	56.683	27.260	**0,0001**
Error	42	84	2		
Total corrected	62	528.317			

Table A3. White vinager's activity

Source	DDL	Sum of squares	Average of squares	F	Pr > F
Model	20	1133.651	27.260	27.260	**< 0,001**
Error	42	87.333			
Total corrected	62	1220.984			

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EFFECT OF STORAGE CONDITIONS ON PHYSICAL PROPERTIES CHANGING OF COMPRESSED KIWIFRUIT

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ABSTRACT

In this research, the effect of edible coatings such as grape juice, date juice and white berry juice, moisture in two levels of 90 and 95%, light bulb in two modes of light and without light and storage period of 5, 10 and 15 days on the characteristics the weight, volume and density of the kiwi fruit were investigated and all experiments were performed with three repetitions. For statistical analysis of the obtained data, SAS software was used by factorial experiments and in a completely randomized design. According to the results, the effects of storage period and edible coatings factors on all independent factors had a significant effect. For all dependent factors, the best values were observed in grape syrup coating, which had the least changes in weight, volume and density. The greatest changes in weight, volume and density have also been observed in white berry cover. Also, the light and moisture factors had a positive effect on changing volume and density in kiwi fruit. The highest rate of change was 9.38% for weight loss, 4.68% for volume reduction and finally, the highest percentage for density reduction was 7.5%. In general, among the coatings used, the use of grape juice as a coating has caused the least amount of changes in weight, volume and density, and the light bulb factor has had a positive effect on volume and moisture on density.

1. Introduction

Kiwifruit belongs to the Actinidiaceae family, Actinidia genus and it is one of the garden products (Mohammadian & Eshaghi Teymoori, 1999) .In today's world, is used other uses from kiwifruit that other uses are like canned kiwifruit, pulp a kiwifruit, kiwifruit frozen, drinks kiwifruit, materials extracted from kiwifruit (such as protease, mucilage, kiwifruit aroma) and dried kiwifruit slice (Romero-rodr, 2014). Kiwifruit is a rich source of vitamin C and black kernels in kiwifruit has vitamin D, also kiwifruit has other vitamins like A, B1,B2, B3 and other vitamins in B group and it has folic acid, polyphenols, iron and copper and phosphorus (Gall et al. 1994; Jolie et al. 2010).The phenolic compounds by antioxidant properties prevent from low density lipoprotein oxidation that this happen, prevent from free radical in fruit (Azadbakht & Vahedi Torshizi, 2020; Galvis Sánchez et al. 2003; Lu et al. 2010). On the other hand, agriculture products are under effect of various processes and factors from harvest time until consumption time. This process can be simple process like cleaning, separating, washing, moving and weighing or complementary and transformational processes that have effect on product properties (Torshizi and Azadbakht 2020; Azadbakht et al., 2019). Therefore physical, mechanical, chemical and biological properties cognition and how to maintain or change them for the intended

purposes of the process can be useful for saving quantitative and qualitative properties product. Every year, many agricultural and horticultural products waste in various stages, especially post-harvest stage, so that, the amount of these wastes in third world countries are more than industrial countries due to inattention to the principles of storage of agricultural products under development and evolution of scientific methods of storage and damages caused by pests. Mechanical damages to crops between harvest and consumption are the main quality loss factors and marketability (Azadbakht et al., 2020; Azadbakht, Vahedi, et al., 2019). If the damage to agricultural products on farms and gardens is increased, it will cause a large amount of waste in agricultural products, so that according to the World Food Organization, the amount of this wastage is in Latin American countries to 33% and 40% in Africa. Mechanical damage to agricultural products between harvest and consumption are the main factor in reducing the quality and marketability. In general, the damage reduces the quality of the product and increases its waste due to corruption (Yurtlu and Erdoğan 2005; Wei 1998). Also, the appearance of fruits affects their value in the market. Therefore, it is important to control the fruits well after harvest to prevent any physical damage, which will maintain the proper appearance of the fruit and increase exports (Ganiron, 2014). Different researchers have reported on the effects of coating on different characteristics of fruits, Dalir et al (2018) did an experimental on effect coating by gum seeds contain cinnamon extract in persimmon storage that the results showed using of this coating was saved qualitative properties in storage period and sensorial properties of persimmon fruit decreased lower than without coating mode. Hasani et al (2012) investigated effect of edible coating on basic oil containing cheese protein and rice seed oil for saving kiwifruit physical and chemical properties that stated using of edible coating had better weight loss and hardness than control sample (Hassani et al., 2012). Pradhan et al (2009) had researched effect moisture content on physical properties in jatropha fruit and observed that using of different moisture content changed density and some physical properties like length, width and thickness in jatropha fruit (Pradhan et al., 2009). Azadbakht et al (2019) studied on effect of loading and storage period in pear fruit on some physical properties have reported that storage period had a direct relationship by weight loss percentage and volume loss percentage and increasing storage period increased weight and volume loss (Azadbakht, Vahedi, et al., 2019). Aguiló-Aguayo et al (2013) investigated effect of light and storage period on amount of physical and mechanical properties in tomato, the physical result showed that use of fluence light caused decreasing firmness and weight percentage at storage period and was a meaningful different between sample control and the sample by light mode (Aguiló-Aguayo et al., 2013).

The aim for this research is the investigation physical properties in kiwifruit at different situation; because it is a damage-sensitive fruit and the physical properties change under storage period and these specifications are an important factor in the marketability and maintenance of this product. Also using of coating for storage period increase the durability of this product and according to the high importance of fruit appearance quality, in this investigation, effective use of a coating, light mode, and storage period is investigated on maintaining the quality of weight, volume and density of kiwifruit, because weight changes are caused by the removal of moisture in the fruit, this can reduce the marketability of the fruit.

2. Materials and methods

2.1. Sample preparation

The medium-sized kiwifruit (Hayward cultivar) with length, width, and thickness about 61.5, 49 and 43.5 mm respectively, were prepared from a garden in Golestan province, Iran. They were immediately brought to the laboratory of the Biosystems Mechanical Engineering Department at Gorgan University of Agricultural Sciences and Natural Resources. 108 kiwifruits with uniform appearance and size were selected in the commercial maturity stage and with completely healthy skin. They were

then subjected to quasi-static loading. The purpose of using quasi-static pressure was to investigate the effect of the desired factors in the damaged fruits on the desired parameters. Obviously, damaged fruits are most prone to physical tissue damage. Then, the fruits were completely immersed in pasteurized solutions of grape juice, dates and berries (at a concentration of 10% by weight for 1 min) and then dried in air.

The samples were placed in containers and transferred to the refrigerator with the ability to change light conditions and ambient humidity (Figure 1.A). Some of the samples were placed inside an oven dryer to measure humidity and measured according to standards (Azadbakht & Vahedi Torshizi, 2020). The amount of moisture measured for kiwifruit was 82.1±0.5%. The samples were then prepared to measure their chemical properties.

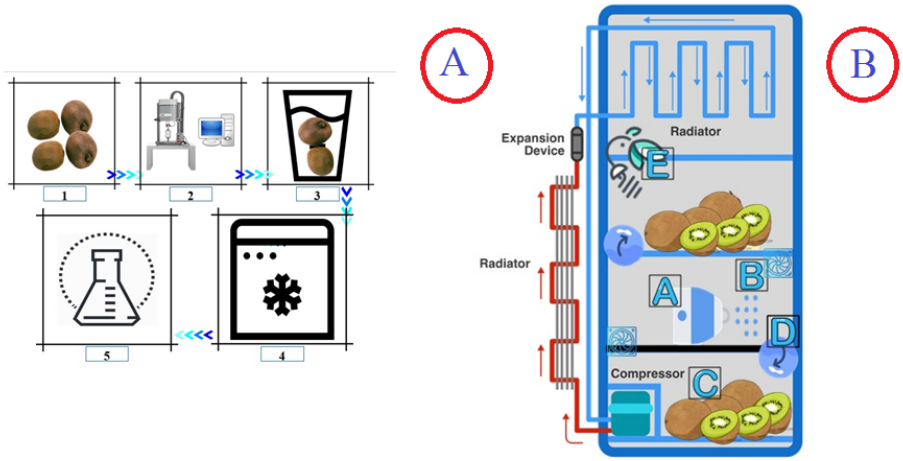

Figure 1. Overview of the test steps
Part A:
1) preparation of samples; 2) application of quasi-static load; 3) immersion of samples in solutions; 4) storage of samples in the refrigerator; 5) measurement of chemical properties of samples.
Part B:
Schematic of the refrigerator and related facilities
A) Ultrasonic humidifier module and water tank; B) Air conditioning techniques; C) Location of screens; D) Air direction; E) Low consumption refrigerator

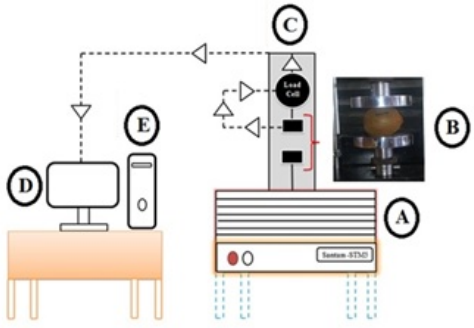

Figure 2. Static quasi-load diagram of kiwifruit
(A) The force-deformation device (Indestrone). (B) Jaw's thin edges. (C) Load Cell. (D) Computer. (E) Information save.

2.2. Quasi-static loading

To apply the quasi-static load on the wide edge, a force-deformation device with the brand name Santam-STM5 with a load of 500 Newtons was used. Two circular plates were used to test the pressure (Figure 2). This test was performed at a speed of 5mm/s with a power of 30N in three repetitions. To perform this experiment, the kiwifruit was placed horizontally between two plates and subjected to quasi-static pressure (Azadbakht, Vahedi Torshizi, et al., 2019).

2.3. Sample storage conditions

To store kiwifruit samples, a refrigerator equipped with a humidifier system with the ability to adjust the overall humidity of the refrigerator's internal environment was used (Figure 1.B). The desired moisture was prepared by an ultrasonic humidifier module 400mL.h (with 24v DC voltage, 19W power, and 450mA current) and was uniformly integrated into the refrigerator by two channels and two fans with dimensions of 10×10 cm. The moisture emission module depends on different levels of the independent moisture factor and has a humidity control sensor to start working by reducing the amount of moisture. The refrigerator used had internal dimensions of 60×80×60 cm and had two separate and completely equal parts. Each part was completely insulated in terms of light, and in one part of it, a low-energy lamp was placed. The energy-saving lamp was powered by city electricity and had a power of 30W. The brightness of the lamp was 1800 lumens and affected the specimens throughout the storage period. The distance between the light source and the samples was 30 cm. Samples were stored for 5, 10, and 15 days.

2.4. Investigate the physical properties

In this research have investigated weight, volume and density properties for kiwifruit at first, after coating all samples weight measured by a scales accuracy 0.01 and for measuring samples volume used fluid displacement method and it was done before coating, at finally the density of all samples was calculated by dividing the mass by the volume. Due to the reduction in experiment error, all samples were selected in the same size and then all samples were placed at packs and kept for 5, 10 and 15 day at storage. Then, during the 5-day storage period, the samples were taken out of the storage and the weight, volume and density were measured again. This was also done for the 10 and 15 day storage periods. Finally, the percentage of change in weight, volume and density of the samples was calculated compared to the first day of storage and the percentage of changes in weight, volume and initial density was obtained.

2.5. Statistical Analysis

First, the fruits were subjected to quasi-static loads and three different coatings were applied to them, including solutions containing grape juice, dates, and berries. The kiwifruit specimens were then stored. Warehouse conditions included 90% and 95% humidity, ambient light conditions were completely dark and light with the use of energy-saving lamps and storage periods of 5, 10, and 15 days. After the storage period, the qualitative properties of the kiwifruit samples were measured, weight loss, volume loss and density loss percentage. All experiments were performed in three replications and the results were analyzed using factorial experiments and in a completely randomized design using SAS statistical software.

3. Results and discussions

The ANOVA results for weight loss percentage, volume loss percentage and density loss percentage have been showed in table 1. According to this table, the interaction coating and storage period factors signified at the statistical level 1% and the light independent factor didn’t has a meaningful effect for weight loss percentage, volume loss percentage and density loss percentage in kiwifruit after storage. Also for the moisture content amount was obtained a statistical level 1% just for density loss percentage in kiwifruit and interaction moisture×light hadn’t meaningful effect for any dependent factors and interaction moisture×coating signified for density loss percentage at the statistical level 1% and the interaction light×coating only signified for

volume loss percentage at the statistical level 1%. At finally effect light×storage period was meaningful for volume loss percentage and for coating×storage time was obtained significant effect for every dependent factors.

Table 1. Variance analysis of weight loss percentage, volume reduction percentage and density reduction percentage.

Independent factors	WLP		VLP		DLP	
	MS	F value	MS	F value	MS	F value
Moisture content	0.328	1.18ns	0.067	0.82 ns	62.77	160.29**
Light	0.004	0.02ns	0.070	0.86 ns	0.003	0.01 ns
Edible coating	45.45	163.65**	16.46	199.47**	23.61	60.30**
Storage period	229.64	826.75**	33.39	404.61**	157.18	403.88**
Moisture content× Light	0.0006	0ns	0	0 ns	0.023	0.06 ns
Moisture content × Edible coating	0.053	0.19 ns	0	0 ns	4.36	11.14**
Moisture content× Storage period	0.015	0.06 ns	0	0 ns	7.33	18.73
Light× Edible coating	0.020	0.07 ns	1.12	13.57**	0.155	0.40 ns
Light× Storage period	0.004	0.01 ns	0.4	4.964*	0.128	0.33 ns
Edible coating× Storage period	8.56	30.83**	5.96	72.27**	4.711	12.03**

WLP= Weight loss percentage, VLP= volume loss percentage, DLP= Density loss percentage, MS=Mean square. ** Significant at the statistical level of 1%, * Significant at the statistical level of 5%, ns no significant

3.1. Weight loss percentage

Considering Table 1, it was concluded that the independent factors of vegetation cover and storage period had a significant effect on the dependent variable of weight loss percentage at the level of 1%. Also, due to the large amount of average squares (MS) obtained, the independent storage period factor has a greater impact than other independent factors on the amount of weight loss percentage.

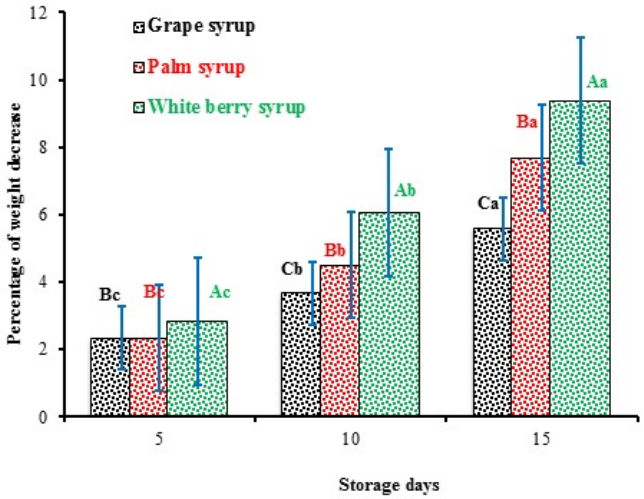

Figure 3. Interaction coating and storage time on amount weight loss percentage.
Similar large letters indicate no meaning in a fixed storage period, similar small letters indicate no meaning in a fixed edible coating.

Figure 3 shows the results of the interaction of storage time and cover the various covenants on the percentage of weight loss. According to the figure, it can be said that with the increase of storage days, the percentage of weight loss has increased. The reason for this is the removal of moisture during storage for kiwifruit. Also, the use of coverage has had a significant effect on reducing the percentage of weight loss. According to the figure, except for 5-day storage, which did not show a significant difference between grapes and dates, there was a significant difference between all coatings in the two storage periods of 15 and 10 days. From between using of three coating in this investigation, the grape coating had the lowest weight loss and this happen showed that this coating had positive effect on weight loss.

This result was similar result by García an et al (1995) on apple fruit that stated the turgor pressure is a reason for the changing weight loss in storage; also cell's wall is a semipermeable membrane for water and fruit ingredients and based on Osmotic property, water can move through cell's wall into cell, while fruit ingredients don't move and will stay in cell's wall (Singh et al., 2014). Furthermore, water infiltration inside cell causes swelling and turgor pressure on the inside of the cell wall and this pressure creates a stress between cells in inside cell wall that if this stress to the texture and cell wall be more stress than cell wall tension, the cell wall will change and the cell wall failure will begin and by changing in cell wall, the weight fruit will change and decrease (Alvarez et al., 2000). Any changes in the turgor pressure, subsequently changes the mechanical properties of the cell wall and consequently all the tissue. In other words, over time and during product storage, by decreasing the turgor pressure at the cellular level, the stresses due to mechanical blows in the product tissue are reduced and therefore the product's vulnerability is reduced (Garíca et al., 1995). The another factor for weight loss is decomposition of starch or cell wall polymer compounds that it is a factor in removing moisture of fruit and as a result, it reduces fruit weight that Tucker (1993) expressed this reason (Tucker, 1993). The highest weight loss percentage was obtained at the 15-day storage period in white berry coating with amount 9.388% and the lowest amount weight loss percentage was in the 5-day storage period in grape coating with amount 2.335%.

3.2. Volume loss percentage

Considering Table 1 and comparing the mean squares (MS) of the two factors of coverage and storage, it was concluded that the storage time factor had a greater effect than the coverage factor on the percentage of volume reduction.

The result for interaction storage period and edible coating have showed in figure 4..A According this figure an incremental trend for the percentage reduction in the volume of kiwifruit samples during storage has been. There were a significant difference between all storage periods for all coatings. So according the figure, the edible coating has created a significant difference for volume loss percentage at a fix storage period, In all of storage period were a significant result between all edible coating and grape syrup had the best and lowest changing volume for kiwifruit, however can explain that the most of kiwifruit texture has a water texture and during the storage, this water will be remove due to environmental factors and it has direct effect on weight and volume loss for fruits (Strik & Cahn, 1998).

On the other hand, creating pressure and loading increase the rate of destruction of the internal structure and will increase the loss of interstitial water in the product that this happen reduces the volume of product (Harker & Hallett, 1994). The highest amount for volume loss was 4.468% at storage period by 15 days and white berry coating, also the lowest amount had obtained at grape syrup coating and 5 days by 0.82% changing for volume loss. This result was similar by Azadbakht et al on investigation pear volume (Azadbakht, Vahedi, et al., 2019).

The interaction light and edible coating on volume loss have been observed in figure 4.B and based on this figure, the light in turn on mode had decreased volume loss, however the turn on mode for light didn't have a significant different. Also when the light was turn off,

between all of coating has obtained a significant different and for turn on mode wasn't significant different between grape and palm syrup, but these had significant different by white berry coating. The maximum and minimum amount for volume loss were 2.652 and 1.059 % that showed in turn off mode by white berry coating and turn on mode by grape syrup coating for kiwifruit. In figure 4.C have showed interaction light and storage period for volume loss percentage that the storage period dependent showed by increasing storage period the amount volume loss had a significant different and according this figure, using light mode dependent didn't has any significant different through 5 and10 day of storage period, but in 15 day of storage, the light mode had a significant different for turn on and turn off mode. The highest volume loss amount was 2.967% at 15 day of storage period by turn off mode and the lowest amount observed at 5 day of storage period and turn on mode by 0.951%.

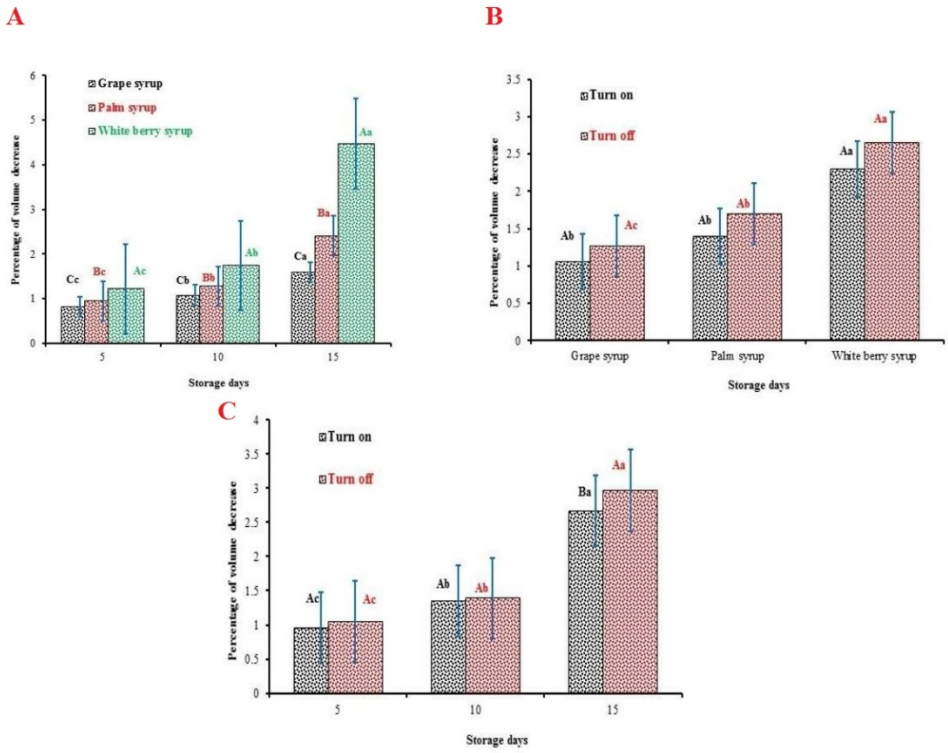

Figure 4. Interaction different conditions on amount volume loss percentage.
A) Interaction coating and storage time on amount density loss percentage; B) Interaction coating and light on amount density loss percentage; C) Interaction light and storage time on amount density loss percentage. Similar large letters indicate no meaning in a fixed storage period, similar small letters indicate no meaning in a fixed moisture content.

3.3. Density loss percentage

According to Table 1, it was concluded that independent factors of fruit moisture content, vegetation cover and storage period had a significant effect on the dependent variable percentage of density reduction at the level of 1%. Also, due to the large amount of mean squares (MS) obtained, the independent storage period factor has a greater effect than the other independent factors on the amount of density reduction percentage.

The density loss percentage amount for interaction storage period and edible coating is showed in figure 5.A and according this figure can explain that increasing storage period increased the density loss percentage in kiwifruit

and was a significant different between all storage period's days. Also in fifth day of storage day wasn't a significant different between all coatings and in tenth day of storage period has showed a significant different between coatings and for fifteenth day, between palm and white berry syrup coating didn't obtained a significant mode. In figure 5.B has been showed interaction storage period and moisture content on density loss percentage. This figure showed that the increasing storage period increased the density loss percentage and between all of storage period's day were a meaningful different and also for each storage period's day were a meaningful different between moisture level, Also the moisture level had a positive effect on density changing in kiwifruit and prevented from more decreasing density. The interaction effect for storage period and moisture content has been showed at figure 5.C that it showed, using edible coating had a meaningful effect on density loss and using grape syrup coating caused that the lowest density loss obtained for kiwifruit and also in moisture content in 95% didn't obtain any meaningful different for palm and white berry coating but the grape syrup had a meaningful effect between both of moisture content.

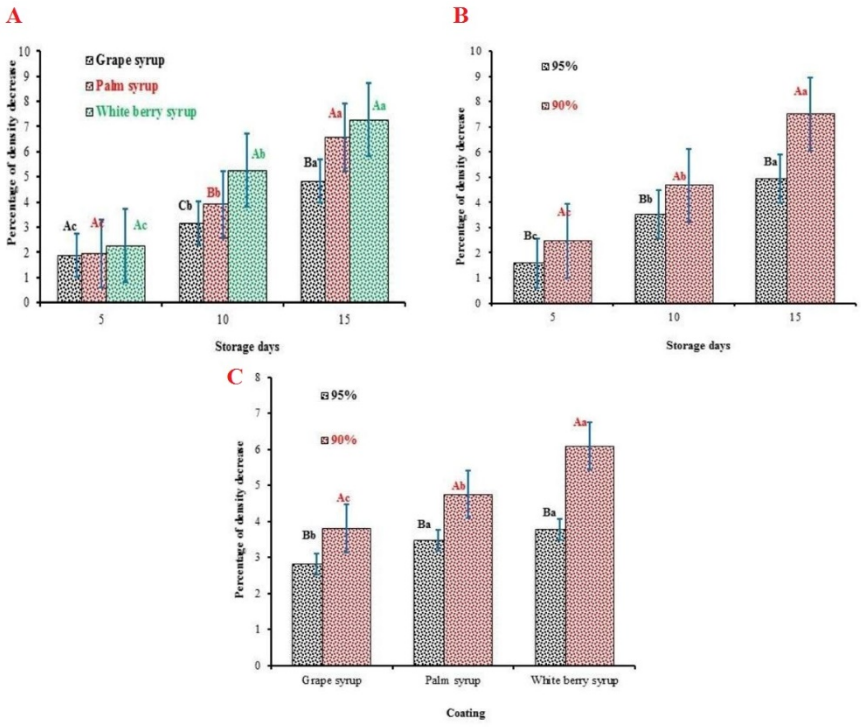

Figure 5. Interaction different conditions on amount density loss percentage.
A) Interaction coating and storage time on amount density loss percentage; B) Interaction moisture and storage time on amount density loss percentage; C) Interaction coating and moisture on amount density loss percentage.
Similar large letters indicate no meaning in a fixed storage period, similar small letters indicate no meaning in a fixed moisture content.

These had a meaningful different by grape syrup that it showed grape syrup had positive effect on decreasing density loss and this coating delayed the kiwifruit ripening and this mode saved the water in fruit and decreased changing texture, so the changing density was lower than other coating (Crisosto et al., 2001). Bakhshi et

at (2011) did an investigation on apple and had a similar result by this research and stated that the pectin destruction between cells cause that the density amount reduce, after the pectin destruction, it distribute around the cell and small walls connect together and this mode create a big space between cells that this happen decrease density amount (Bakhshi Khaniki et al., 2012). The highest and lowest amount density loss were 7.273 and 1.88% by white berry and grape coating respectively.

The reason for this can be stated as follows that the products begin to lose moisture for balancing moisture content with the environment, which it reduces the weight of the samples and according that, the moisture content has a straight relation with density fruit, so by decreasing weight of fruit, the density had decreased that this happen have increased density loss percentage in kiwifruit. And this happen showed that using of injecting moisture at storage environment had a positive and meaningful effect for preventing from density loss in kiwifruit. The maximum and minimum density loss percentage amounts were 7.5% and 1.589% in storage period 15 and 5 respectively.

In comparing every three syrups were a meaningful different between moisture 90 and 95 % and the moisture had a positive effect and increasing moisture has been decreased the density loss. Pradhan et al. (2009) also stated that the use of moisture had an effect on jatropha fruit density (Pradhan et al., 2009).

4. Conclusions

The use of moisture and turn on mode light has not been able to have a significant effect on the rate of weight loss of samples and the interaction of these two factors with each other as well as cover factors and storage time has not been significant for the percentage of weight loss of samples. But the factor of moisture and turn on mode light has had a significant effect on the percentage of decrease in volume and density of kiwifruit. From between using of factors, storage period was the most important than other dependent factors and after storage period, the edible coating was the most effective for preventing kiwifruit changes in this research. Also, the use of edible grape juice cover compared to the other two coatings has been able to have the greatest effect on the process of reducing the physical changes of kiwifruit, and after that, date juice has had a greater effect. Also, the use of moisture had a positive effect on preventing the decrease in the density of the samples and 95% of the moisture in this study was useful for kiwifruit. To reduce the sample volume, light in this study was able to make a significant difference compared to when this light is not used.

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INFLUENCE OF RIPENING CONDITIONS ON SURVIVAL OF *BRUCELLA MELITENSIS* IN TRADITIONAL LIGHVAN CHEESE (EWE MILK CHEESE)

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ABSTRACT

In developing countries, brucellosis is a reported and in most of cases, consumption of raw milk and traditional cheeses contaminated with *Brucella* spp., especially *B. melitensis*, is the main cause of disease. The aim of this study was to evaluate the effects of ripening conditions (ripening temperatures: 4, 9 and 14°C and salt concentrations: 8, 12 and 15%) on survival of *B. melitensis* in traditional Lighvan cheese (a typical Iranian brine-ripened cheese) manufactured with raw ewe's milk during 150 days of ripening. Results showed that the viable counts of *B. melitensis* changed significantly (p<0.01) as a function of storage temperature. *B. melitensis* survived significantly better at 4°C and 9°C than 14°C (p<0.01). All of salt concentrations (8, 12 or 15% NaCl) significantly (p <0.001) affected the inactivation of pathogen. *B. melitensis* had been completely eliminated at the end of ripening period (150 days). Our findings indicated that the using of hurdle technology (the two limiting factor, namely temperature and salt concentration), is a powerful tool to eliminate *B. melitensis* in Lighvan cheese after at least 5 months of ripening.

1. Introduction

Lighvan cheese is the most popular and commonly consumed Iranian traditional cheese with a long history of manufacturing. It is traditionally manufactured in the Lighvan region located in the province of East Azarbaijan, northern-west of Iran. It is a white brined, semi-hard cheese with sour taste, pleasant and very specific flavor and crumbly texture. The cheese is made from raw ewe's milk or mixtures of ewe and goat milk (70/30). Based on traditional technique, the milk is coagulated by rennet (from abomasum of lambs or kids) and is offered for consumption after undergoing 3-12 months of ripening in brine. During ripening process, some changes in the cheese including physic-chemical changes as well as the production of antagonistic compounds by indigenous microbial flora, organic acid and other compounds, could decrease the growth of pathogens (Hanifian & Khani, 2012).

There are many studies involving traditional cheeses manufactured with raw milk

in different countries that illustrate the presence and/or survival of important pathogenic bacteria such as *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and *Escherichia* O157:H7 (Pinto *et al.,* 2009, Jakobsen *et al.,* 2011). The similar pattern of pathogenic bacteria is relevant in Iran, but in our country, raw milk and milk products especially cheese made from unpasteurized milk of sheep and goats is widely recognised as an important source of *Brucella* spp. contamination and a vehicle of brucellosis (Akbarmehr, 2011).

Based on the reports of the Iranian Ministry of Health and Medical Education, brucellosis is a common disease in most regions of Iran (Zamani *et al.,* 2011). Generally, the disease is serious food-borne infection and undulant fever, night sweats, strange odor and severe headache are common symptoms that can observe in patients. Raw milk and cheese manufactured from raw milk of ewe and goats that may harbor *Brucella* spp. (Seleem *et al.,* 2010; Solera, 2010).

Using hurdle technology (temperature, salt concentration and time of ripening) may serve as potential effective methods to eliminate and or reduction of pathogens in raw milk cheeses (Al-Holy *et al.,* 2012). The survival of important food-borne pathogens such as *Yersinia entrocolitica* (Hanifian & Khani, 2012), *Listeria monocytogenes* and *Salmonella typhimurium* (unpublished observations) in Lighvan traditional cheese under various conditions of ripening has been investigated by several authors, but there is little information about the survival of *B. melitensis*. Therefore, in this context, the aim of present work was to evaluate the influence of ripening temperatures (4, 9 or 14°C) and ripening salting (8, 12 and 15%) on survival of *B. melitensis* in traditional Lighvan cheese manufactured with raw milk during 150 days of ripening.

2. Materials and methods

2.1. Materials

Rennet casein was obtained from Meito Sangyo Co., Ltd. (Tokyo, Japan). Brain Heart Infusion (BHI) broth and Peptone Water were used from Merck Company (Darmstadt, Germany). *Brucella* Selective Supplement SR0083, Blood agar, dextrose solution and inactivated horse serum were used to prepare a *Brucella* selective medium. All these media were purchased from Oxoid Co., Ltd. (Hampshire, England).

2.2. Strain and culture preparation

Native *B. melitensis* biovar 1 isolated previously from raw milk was obtained from the Razi Institute for Serums and Vaccines (Tehran, Iran). The strain was maintained in BHI broth containing 25% v/v glycerol at -80°C. For activating, two consecutive subcultures were incubated in BHI Broth at 37°C for 24 h. This work was done in 3 replicates. The overnight culture (from the second subculture) was diluted to achieve an initial inoculation level of approximately 10^5 colony forming units per millilitre (CFU/mL) of milk.

2.3. Cheese manufacturing

Lighvan cheese (8.5 kg) was made with raw milk obtained from Lighvan village, as shown Fig. 1. For this, the cheese milk was examined for the absence of *B. melitensis* contamination prior to the cheese preparation. A *B. melitensis* biovar 1 culture was added to Fresh (pH ~6.6) raw whole ewe's milk after the milking stage and before starting the process of cheese manufacturing concentration of 10^5 colony forming units per millilitre (CFU/mL).

2.4. Ripening conditions

Ripening of the cheese was carried out over a period of 150 days during which samples were taken every 10 days. Two factor experimental design was applied to study the effect of ripening conditions (three temperatures: 4, 9 or 14°C), (three concentrations of NaCl: 8, 12 or 15%) on the survival of *B. melitensis* of the cheese. The experiment was done twice.

2.5. Microbiological analysis

25 grams of each cheese was sampled with 225 mL of 0.1% peptone water in a stomacher bag. The sample was then homogenized in stomacher and diluted it ten-fold serial dilution for plate count enumeration. The plates were incubated at 37 °C for 6 days. After which colonies having the 2-7 mm diameter, are spheroid in shape, moist, slightly opalescent and translucent were counted.

2.6. Statistical analysis

The effects of ripening temperature, salting and time on the survival of *B. melitensis* were evaluated using SPSS (version 16.0) and by Analysis of variance (ANOVA) test. P-value less than 0.05 were considered statistically significant.

3.Results and discussions

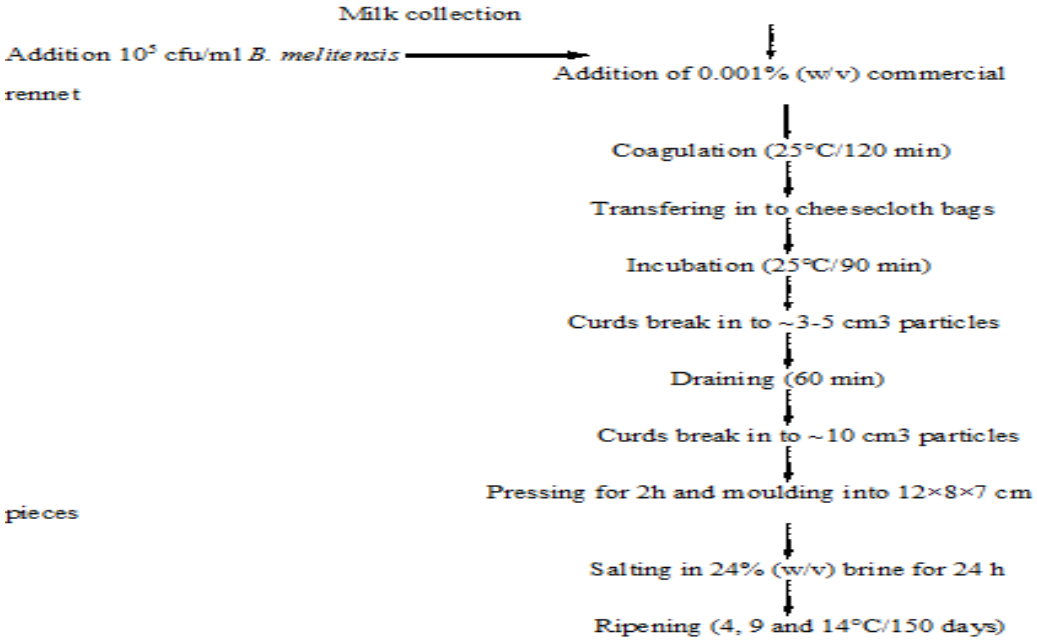

Figure 1. Schematic flowchart of Lighvan cheese production.

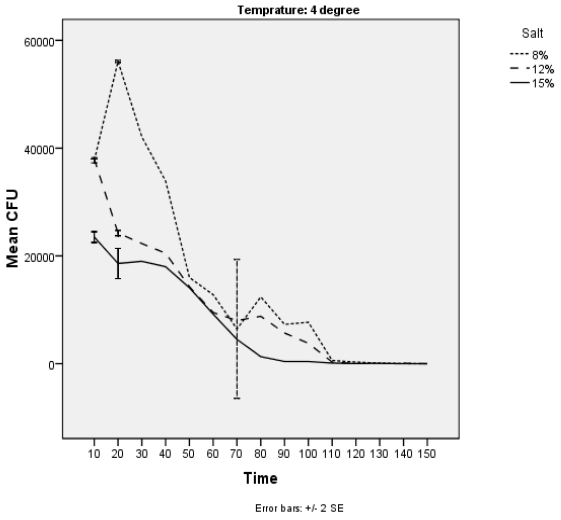

Figure 2. Survival of *B. melitensis* in traditional Lighvan cheese at 4°C during ripening.

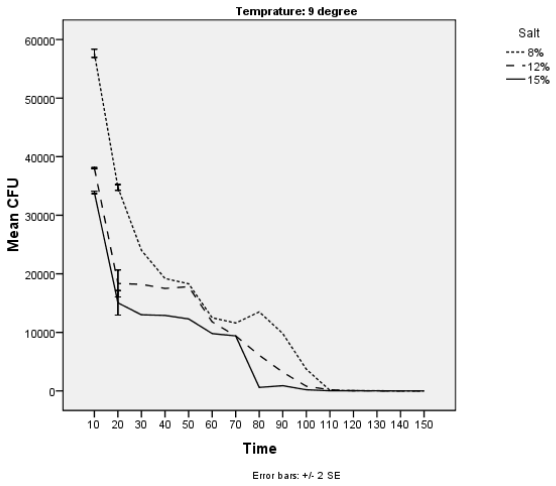

Figure 3. Survival of *B. melitensis* in traditional Lighvan cheese at 9°C during ripening.

Figures 2, 3 and 4 indicated the survival of *B. melitensis* during 150 days of ripening period. *B. melitensis* was not isolated from any of milk samples. In detail, after inoculation, *B. melitensis* populations were reduced most rapidly during the first week of storage (between 34311 (±2624) and 25488 (±2965) log unit) at three temperatures; after that they continually decreased, being below the detection limit (1log CFU/g) at the end of ripening. Temperature had significant effect on *B. melitensis* counts during ripening (Fig 2, 3 and 4). Analysis of variance indicated that numbers of surviving *B. melitensis* differed significantly (p<0.01) with storage temperatures; it was demonstrated that 14°C was more effective than 9°C and 4°C (P<0.01). The mean of the count of *B. melitensis* in traditional Lighvan cheese at 14°C, 9°C and 4°C was 13782, 12794 and 7375 CFU/g, respectively. According to results of Kruskal-Wallis, higher temperature enhanced the sensitivity of *B. melitensis*, suggested that temperature is a crucial factor in decrease of the bacterial populations (P=0.079). Although *B. melitensis* were inhibited at the end of period (150 days) at the three temperatures, they survived in traditional Lighvan cheese for 130 days (14°C) and 140 days (9°C and 4°C). Inhibitory effects of NaCl concentration on the growth of *B. melitensis* are shown in Figures 5, 6 and 7. The results obtained for NaCl concentrations demonstrated that all concentrations (8, 12 and 15% NaCl) significantly (p<0.001) affected the inactivation of pathogen (Fig 5, 6 and 7). Our results indicated that combined effect and interaction of temperature/NaCl significantly (p<0.001) affected the inactivation of pathogen. The results showed that time of ripening significantly influenced (p<0.001) the survival of *B. melitensis* in Lighvan cheese.

In Iran, the incidence of *B. melitensis* in different types of dairy products particularly raw milk and milk products, especially cheese made from raw milk of sheep and goats, has been reported by several of researchers (Akbarmehr, 2011). To our knowledge, evaluation of potential survival of *B. melitensis* during production and ripening of traditional raw ewe's milk cheeses as well as the influence of ripening conditions on the fate of this bacterium in cheese such as Lighvan cheese has never been studied.

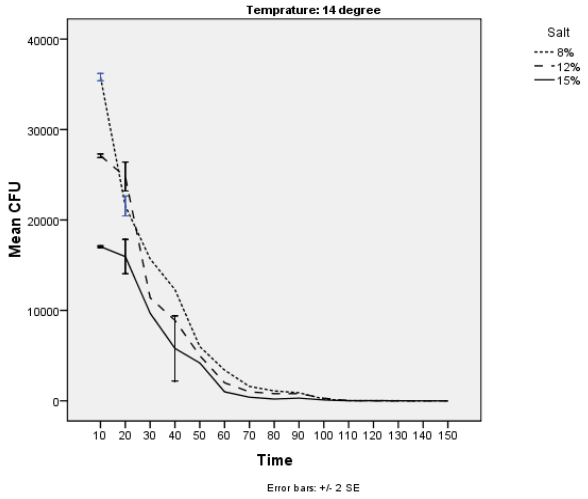

Figure 4. Survival of *B. melitensis* in traditional Lighvan cheese at 14°C during ripening.

According to results of this work, following ripening process, a significant reduction of *B. melitensis* count was observed. Several factors may contribute to reduction of this pathogen during ripening, such as presence of indigenous lactic acid bacteria including *Lactococcus lactis* and *Streptococcus thermophilus* (Hanifian & Khani, 2012, Masoud *et al.,* 2012, Navidghasemizad *et al.,* 2009, Ong *et al.,* 2009). The progressive production of some compounds such as bacteriocin, hydrogen peroxide and volatile compounds by lactic acid bacteria during ripening is well

documented. A number of studies have shown the inhibitory effects of these compounds against food-borne pathogens (Tiganitas *et al.,* 2009, Tamagnini *et al.,* 2008). On the other hand, The pH from 6.6 to 7.4 is the best range for growth and survival of *Brucella* spp., therefore, acidic property (pH) would be a key factor in decrease of survival and growth of *Brucella* spp. in dairy products such as cheese (Ozturkoglu *et al.,* 2005, Zúñiga *et al.,* 2005, Delbes *et al.,* 2006; Falenski *et al.,* 2011). A recent study by Aminifar *et al.* (2010) showed that the pH reduced from 60 to 90 days of Lighvan cheese storage on average from 5.69 to 4.65. Hanifian & Khani (2012), revealed that pH reduction at the end of ripening process could be due to the natural lactic acid bacteria such as mesophilic lactobacilli, thermophilic lactobacilli and lactococci. The quantity levels of these bacteria were in their maximum levels at the end of ripening period. Therefore, acidic property was associated with a significant decrease of the bacterial count during ripening period of Lighvan cheese.

This study demonstrated that *B. melitensis* could survive 130 days (at 4 and 9°C) and 140 days (at 14°C) in traditional Lighvan cheese and this survival are regarded as a serious risk for consumer health. Based on our results, it seems that one of the main methods for complete elimination of *B. melitensis* is brining of Lighvan cheese for a long ripening period, at least 5 months.

With regards to the effect of temperature, it was found that 14°C is more effective than 9°C and 4°C (p<0.01). This result is in agreement with those achieved by Ingham *et al.* (2000), Tamagnini *et al.* (2005) and Callon *et al* (2011). Lower growth of most pathogens such as *B. melitensis* at low temperatures is due to alteration of fatty acid components in cell membrane of bacteria that interfere with membrane fluidity and lead to their death (Al-Holy *et al.,* 2012).

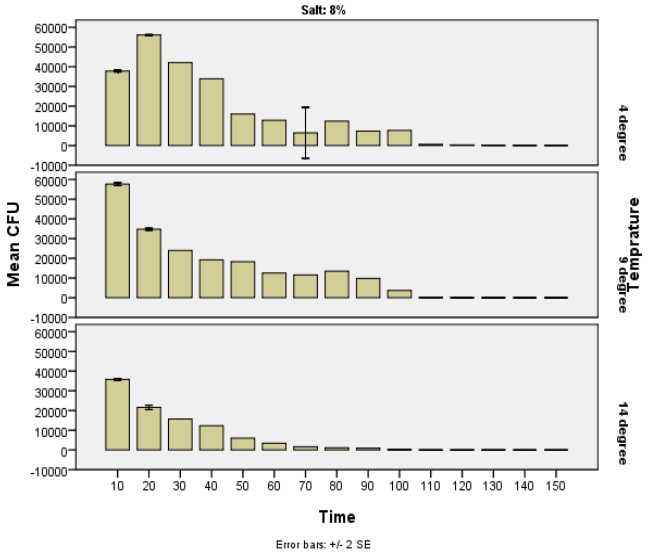

Figure 5. Effect of salt treatment (8%) on the survival of *B. melitensis* in traditional Lighvan cheese at 4, 9 and 14°C during ripening.

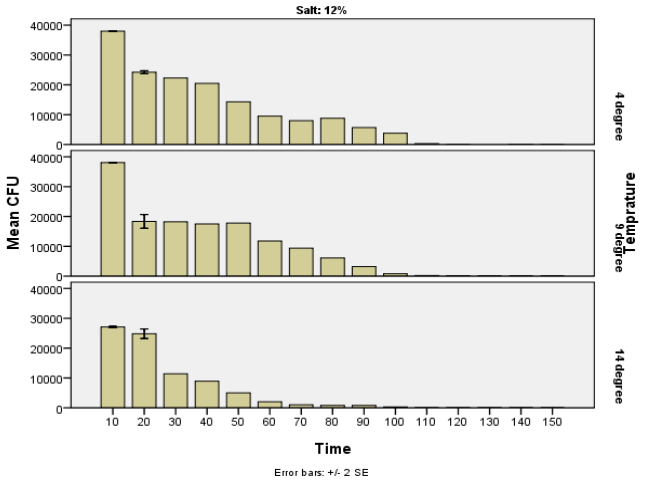

Figure 6. Effect of salt treatment (12%) on the survival of *B. melitensis* in traditional Lighvan cheese at 4, 9 and 14°C during ripening.

In addition to the temperature, the concentration of NaCl could affect the survival of pathogens during ripening period. Therefore, in the present study, effects of ripening salting (8, 12 or 15%) on

survival of B. melitensis also were evaluated. The results showed that the number of B. melitensis reduced drastically by all of concentrations (8, 12 and 15% NaCl) (p<0.001). In addition, Aminifar *et al.* (2010) showed that the salt concentration was increased (approximately 3%) during ripening of Lighvan cheese.

The observed trends for inactivation of pathogens with increasing of osmotic stress posed by NaCl in the present study are in agreement with those achieved by Ozturkoglu *et al.* (2006) on *Listeria innocua* during manufacture and storage of Turkish White Cheese in which the authors indicated the number of *listeria* cells drastically reduced following salt treatment.

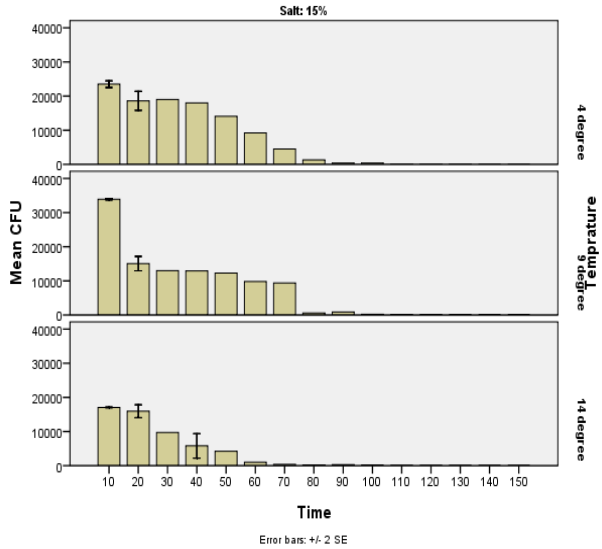

Figure 7. Effect of salt treatment (15%) on the survival of *B. melitensis* in traditional Lighvan cheese at 4, 9 and 14°C during ripening.

4. Conclusions

It can be concluded that the number of *B. melitensis* cells declined drastically during ripening days and eliminated at the end of ripening, but survival of *B. melitensis* during ripening under adverse conditions such as high salt concentrations and high temperatures along with high contamination of raw milk and consequently traditional Lighvan cheese with mentioned bacteria could be a risk for consumer health. Therefore, additional studies are needed to develop rapid methods for detection of *B. melitensis* in such products. In addition, molecular studies are required to fully understand the survival of *B. melitensis* in order to complete elimination of this pathogen. Further investigations are also required to assess the effects of ripening temperatures and ripening salting on sensorial properties of Lighvan cheese.

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DETERMINATION OF NUTRITIONAL AND MINERAL COMPOSITION OF WASTED PEELS FROM GARLIC, ONION AND POTATO

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ABSTRACT

In this research, the results of determining the nutritional and mineral composition of garlic, onion and potato wasted peels were presented. It was found that garlic wasted peels were characterized by the highest content of total dietary fibre (62.10%), total sugars (6.51%), dry matter (80.8%), total ash (7.37%), B (18.0 mg/kg), Al (826 mg/kg), S (1635 mg/kg), K (9081 mg/kg), Ca (20610 mg/kg), Cr (18.40 mg/kg), Mn (35.4 mg/kg), Fe (682 mg/kg), Zn (12.9 mg/kg), Se (0.058 mg/kg) and Mo (1.480 mg/kg). Onion wasted peels were the richest in free fat (0.31%), reducing sugars (3.10%), Na (1021 mg/kg), Mg (1285 mg/kg), P (881 mg/kg) and Cu (4.58 mg/kg). Potato wasted peels contained the highest amount of crude protein (2.67%), digestible carbohydrates (9.5%) and water content (84.3%). The waste materials that were investigated could be used as a source to obtain valuable components presented in large quantities in them.

1.Introduction

Fruit and vegetable peel wastes are commonly generated from both households and food processing industry (Pathak et al., 2016). Unavoidable food supply chain wastes represent a perspective waste as a resource due to its high volume, chemical richness and heterogeneity (Matharu et al., 2016). According to Bhatnagar et al. (2015), agricultural waste materials are economic and environmental friendly due to their chemical composition and their availability in abundance. Waste peels from fruits and vegetables are of great importance as a renewable resource and an agro-industrial waste. Fruits and vegetables wastes and by-products, including peels, which are generated in high amounts during industrial processing, represent a serious environmental problem and need to be managed and utilized. Efforts have been made to improve methods and ways of reusing fruits and vegetables wastes. Agro industrial waste often is utilized as feed or fertilizer. Valorizing the biocomponents in by-products from fruit and vegetable industries is perspective and important way of reusing these waste materials (Bhatnagar et al., 2015).

Hameed and Ahmad (2009) investigated the potential of garlic peel, agricultural waste, for the removal of methylene blue from aqueous solution (Hameed and Ahmad, 2009). Prasad Reddy and Rhim (2014) extracted cellulose micro-fibres and cellulose nanocrystals from garlic skins – underutilized agricultural by-products (Prasad Reddy and Rhim, 2014). Chhouk et al. (2017) used carbon dioxide expanded ethanol method to extract phenolic compounds from garlic husk, which was regarded as agricultural waste (Chhouk et al., 2017). Huang G.-g. et al. (2019) used garlic peel as the precursor for carbons via hydrothermal carbonization and KOH

activation for CO_2 adsorption (Huang G.-g. et al., 2019).

Ng et al. (1999) studied the effects of extrusion-cooking on the physico-chemical parameters and microstructure of cell walls of onion waste (Ng et al., 1999). Coventry et al. (2002) showed the potential of composted onion waste for decreasing the inoculum density of soil infested with *Sclerotium cepivorum* on a small-scale basis (Coventry et al., 2002). Roldán et al. (2008) studied bioactive, antioxidant and antibrowning properties of onion by-products stabilized by different treatments (Roldán et al., 2008). In the study by Kiassos et al. (2009), response surface methodology was implemented in order to optimize recovery of polyphenols from onion solid wastes (Kiassos et al., 2009). In their work, Moussouni et al. (2010) presented the use of a crude peroxidase homogenate from onion solid waste as a biocatalyst for the synthesis of the natural aurone, aureusidin (Moussouni et al., 2010). According to Albishi et al. (2013), onion skin may serve as a promising source of natural antioxidants for the development of food systems or value-added products (Albishi et al., 2013). Angeleska et al. (2013) studied the biocatalytic activity of a crude peroxidase extract from onion solid waste on the reaction between catechol and heterocyclic 1,3-dicarbonyl compounds (Angeleska et al., 2013). Choi et al. (2015) studied the potential of enzymatic hydrolysis for glucose production and quercetin extraction from onion skin waste (Choi et al., 2015). In their study, Katsampa et al. (2015) used onion solid wastes as a source to produce antioxidant polyphenol- and pigment-enriched extracts by ultrasound-assisted solid-liquid extraction (Katsampa et al., 2015). Benítez et al. (2017) obtained fibre concentrates from onion by-products, studied their effects on glucose adsorption, as well as their composition, physicochemical properties, alpha-amylase activity and starch digestibility (Benítez et al., 2017). Nile S.H. et al. (2017) reported a significant amount of quercetin and quercetin glycosides in onion solid waste with antioxidant and enzyme inhibitory activities against urease and xanthine oxidase (Nile S.H. et al., 2017). Kim H.M. et al. (2017) investigated the production of valuable rare sugars and bioethanol from onion juice residue for efficient use the waste material (Kim H.M. et al., 2017). According to Maiti et al. (2017), naturally abundant bio-waste onion skins could be a promising bio-piezoelectric material for harvesting green energy from mechanical and biomechanical activities (Maiti et al., 2017). The results of work by Nile A. et al. (2018) indicated that onion solid waste and the extracted flavonols had potential beneficial effects (Nile A. et al., 2018). Munir et al. (2018) used subcritical water extraction to recover bioactive phenolic compounds from waste onion skin (Munir et al., 2018). Baldassarre et al. (2018) valorized onion skins (an under-utilized agricultural by-product) towards enzymatic pectic oligosaccharides production with high hydrolysis performance (Baldassarre et al., 2018). Campone et al. (2018) developed a green analytical procedure for the analysis of phenolic compounds in outer dry layers of brown skin onion (Campone et al., 2018). Kim S.-W. et al. (2019) developed an emerging hurdle technology for maximizing extraction efficiency of quercetin from onion waste (Kim S.-W. et al., 2019). According to Mondal et al. (2019), onion peel dust can be effectively utilized as an adsorbent for the removal of nitrate from aqueous solutions (Mondal et al., 2019). Alonso-Lemus et al. (2019) prepared non-noble metal electrocatalysts from onion skin wastes (Alonso-Lemus et al., 2019).

Madhumithah et al. (2011) studied the utilization of vegetable wastes, including potato wastes, for the production of protease by *Aspergillus niger* (Madhumithah et al., 2011). In the study by Hossain M.B. et al. (2014), steroidal alkaloids were extracted from potato peel waste by ultrasound assisted extraction and solid liquid extraction (Hossain M.B. et al., 2014). Hossain M.B. et al. (2015) optimized solvent concentration and temperature for recovery of steroidal alkaloids from potato

peels using pressurized liquid extraction assisted by response surface methodology (Hossain M.B. et al., 2015). Lappalainen et al. (2015) modified the starch-containing biomaterial potato peel waste by alkaline hydrolysis and cationization like native starch (Lappalainen et al., 2015). Liang et al. (2015) produced lactic acid by anaerobic fermentation of potato peel waste in a sequencing batch reactor inoculated with undefined mixed culture from a municipal wastewater treatment plant (Liang et al., 2015). Liang and McDonald (2015) studied the feasibility of biogas production from potato peel waste and its lactic acid fermentation residue in a batch anaerobic digestion process under mesophilic condition (Liang and McDonald, 2015). In the work by Jacob and Banerjee (2016), an aquatic weed *Pistia stratiotes* has been established as an efficient co-substrate for industrial potato waste (Jacob and Banerjee, 2016). Chintagunta et al. (2016) produced ethanol in short incubation time by employing co-culture of *Aspergillus niger* and *Saccharomyces cerevisiae* to potato waste through simultaneous saccharification and fermentation, and investigated the possibilities of utilizing the residue after ethanol fermentation for further enrichment as biomanure (Chintagunta et al., 2016). In their work, Du and Li (2016) demonstrated the capability of microbial fuel cell in simultaneous potato waste reduction and electricity generation as well as the significant effects of mixed treatment with cooked potato (Du and Li, 2016). In another their work, Du and Li (2017) showed the effect of mixed feeding of anaerobically cultured waste activated sludge on the performance of microbial fuel cell for direct treatment of solid potato waste (Du and Li, 2017). Kumar et al. (2016) developed a microwave irradiation based process using a solid acid catalyst for the production of glucose from potato peel waste (Kumar et al., 2016). According to Gupta et al. (2016), potato plant wastes can be an adsorbent for the removal of cationic dyes (methylene blue and malachite green) from aqueous solution (Gupta et al., 2016).

Abdelraof et al. (2019) investigated the bacterial cellulose production by using potato peel waste hydrolysate by green environmental friendly method (Abdelraof et al., 2019). According to Ben Atitallah et al. (2019), potato peels waste is an attractive by-product to produce bioethanol using a newly isolated yeast strain *Wickerhamomyces anomalus* (Ben Atitallah et al., 2019). Chohan et al. (2020) optimized bioethanol production from potato peel waste using simultaneous saccharification and fermentation process (Chohan et al., 2020). In the work by Ghinea et al. (2019), the physico-chemical characteristics of fruit and vegetable waste, including potatoes, were determined in order to develop a model for food waste composting (Ghinea et al. 2019). Li et al. (2019) evaluated the possibility and controlling strategy of acidogenic fermentation of wasted potato at different pH values (Li et al., 2019). Yang J.-S. et al. (2019) used ultrasound-microwave assisted extraction combined with HCl for extraction of pectin from potato pulp by response surface methodology (Yang J.-S. et al., 2019). Zhang Y. et al. (2019a) studied the feasibility of potato processing waste as a co-substrate for co-digestion with microalgae *Chlorella vulgaris* in batch biochemical methane potential tests (Zhang Y. et al., 2019a). Zhang Y. et al. (2019b) investigated the feasibility of using potato processing waste as a feedstock for co-digestion with a marine microalgae (*Tisochrysis lutea*) during semi-continuous anaerobic study (Zhang Y. et al., 2019b).

In the work by Díaz et al. (2017), vegetable supermarket wastes, including potatoes, have been treated by acid, thermal and enzymatic hydrolisis procedures with the purpose to maximise the concentration of fermentable sugars in the final broth (Díaz et al., 2017). Mu et al. (2017) used anaerobic granular sludge as an inoculum for co-digestion of potato waste and cabbage waste in batch and semi-continuous mesophilic modes for enhanced methane production (Mu et al., 2017). Rodríguez Amado et al. (2014) optimized the extraction of antioxidants from potato peel

waste by three variables (processing-time, temperature and ethanol concentration) (Rodríguez Amado et al., 2014). In their work, Vázquez and Guerra Rodríguez (2011) elucidated using computer simulation if the biotechnological production of transglutaminase by *Streptomyces mobaraensis* from hydrolysates of potato wastes is feasible (Vázquez and Guerra Rodríguez, 2011). Zhang Z. et al. (2015) prepared activated carbon (a low cost and highly efficient adsorbent) from waste potato residue (Zhang Z. et al., 2015). Wei et al. (2015) prepared green biocomposites from polyhydroxybutyrate and potato peel waste fermentation residue fibres (low-value waste byproduct from a fermentation process of potato peel waste) and studied the potential applications of the potato peel waste fermentation residue fibres in agriculture and horticulture as biocomposites (Wei et al., 2015). Wijngaard et al. (2012) optimized the solid-liquid extraction and pressurized liquid extraction of antioxidant from industrial potato peel waste by response surface methodology (Wijngaard et al., 2012). In the review by Wu (2016), several advanced potato peel processing technologies were introduced, their advantages and disadvantages were analyzed from the standpoint of popularization and economy (Wu, 2016). Riciputi et al. (2018) determined the phenolic contents in industrial potato by-products obtained after potato processing by using response surface methodology (Riciputi et al., 2018). Xie et al. (2018) investigated the structural characteristics, physicochemical properties and morphology of pectin from potato peel waste treated with high hydrostatic pressure and high pressure homogenization (Xie et al., 2018). Yang X. et al. (2018) prepared a novel biochar with a microscale spherical shape from the zero-cost feedstock, potato peel waste, via a two-step thermal process (Yang X. et al., 2018).

The main objective of the present work is determination of nutritional and mineral composition of wasted peels from garlic, onion and potato. This would be useful in further investigations to look for opportunities to utilize these waste materials.

2. Materials and methods

2.1. Materials

In this research, wasted peels from garlic, onion and potato were used as tested material. The samples were collected from the local market and were examined in the SGS Bulgaria Ltd, Laboratory Varna. Garlic, onion and potato wasted peels were analyzed for the following parameters: free fat, crude protein, total dietary fibre, digestible carbohydrates, total sugars, reducing sugars, water content, dry matter, total ash, mineral composition.

2.2. Methods

2.2.1. Determination of free fat

The method for determining free fat is based on the extraction of fat from the product with an organic solvent (petroleum ether) in a Soxle apparatus, evaporation of the solvent and weight determination of the mass of the extracted fat.

2.2.2. Determination of crude protein

The test is carried out by the Kjeldhal method – mineralization of the sample with concentrated sulfuric acid at a temperature of 420ºC (the organic-bound nitrogen is mineralized to ammonium ion), alkalization and distillation of the liberated ammonia in boric acid solution, titrimetric determination of distilled ammonia with hydrochloric acid, calculating the protein content of ammonia quantity at a factor of N=6.25.

2.2.3. Determination of total dietary fibre

The sample is gelatinized with α-amylase, the proteins are digested with protease and the starch is digested with amyloglucosidase. Dietary fibre is precipitated with ethanol. They are separated by vacuum filtration and degreased. The sample is dried and the fibres are determined by weight. The result is adjusted for protein and ash.

2.2.4. Determination of carbohydrates (digestible)

Determination of digestible carbohydrates is performed by calculation method. Analytical

data are required for all other nutritional components of the product concerned – water content, protein, fat content, total ash, total dietary fibre. Carbohydrate content is calculated as a percentage of the total mass.

2.2.5. Determination of reducing sugars

The principle of the method for determining of reducing sugars consists in the quantitative reduction of the Cu^{2+} contained in the Luff solution, from the reducing sugars in the product to the Cu^{1+} and the oxidation of the latter by iodine solution and residual titration of iodine with sodium thiosulphate solution.

2.2.6. Determination of total sugars

Total sugars are determined by the algorithm of reducing sugars after conversion of sucrose to reducing sugars with hydrochloric acid.

2.2.7. Determination of water content

The water content is determined by azeotropic distillation – the sample was boiled in toluene, the separated water vapor condensed in a refrigerator and captured in a graduated receiver. The process continues until the water is completely separated. The water content is calculated from the reported amount of water taken away from the mass of the sample.

2.2.8. Determination of dry matter

The amount of dry matter is calculated after azeotropically determining of the water content.

2.2.9. Determination of total ash

Determination of total ash involves complete ash of the sample at 500-550ºC and weight determination of the ash content.

2.2.10. Determination of mineral composition

The sample is mineralized with a mixture of nitric acid and hydrogen peroxide (or hydrochloric acid), under pressure or by incineration in a muffle furnace at 450ºC. After decomposition, the sample solution is adjusted to the correct volume and the content of metals and non-metals is determined directly by ICP-OES/ICP-MS.

Methodologies well-described in details could be also found in the work by Baloch et al. (2015).

3.Results and discussions

3.1. Nutritional composition of garlic, onion and potato wasted peels

In Table 1, the results for the nutritional composition of garlic, onion and potato wasted peels were presented. The free fat content was highest in onion wasted peels (0.31%), followed by garlic wasted peels (0.22%) and potato wasted peels (0.15%). The crude protein content was similar in garlic wasted peels (2.61%) and potato wasted peels (2.67%). The crude protein content was lower in onion wasted peels (1.92%) than in wasted peels from garlic and potato. The amount of total dietary fiber was significantly higher in garlic wasted peels (62.10%) than in onion wasted peels (16.02%) and potato wasted peels (2.53%). Digestible carbohydrates predominated in potato wasted peels (9.5%), followed by garlic wasted peels (8.5%) and onion wasted peels (4.7%).

Table 1. Nutritional composition of wasted peels from garlic, onion and potato

Parameter, %	Garlic wasted peels	Onion wasted peels	Potato wasted peels
Free fat	0.22 ± 0.01	0.31 ± 0.02	0.15 ± 0.01
Crude protein	2.61 ± 0.15	1.92 ± 0.15	2.67 ± 0.15
Total dietary fibre	62.10 ± 3.11	16.02 ± 0.80	2.53 ± 0.25
Carbohydrates (digestible)	8.5 ± 1.7	4.7 ± 0.9	9.5 ± 1.9
Sugars (total)	6.51 ± 0.25	4.29 ± 0.25	1.68 ± 0.25
Sugars (reducing)	1.36 ± 0.25	3.10 ± 0.25	0.53 ± 0.06
Water content	19.2 ± 0.3	75.3 ± 0.3	84.3 ± 0.3
Dry matter	80.8 ± 0.3	24.8 ± 0.3	15.8 ± 0.3
Total ash	7.37 ± 0.22	1.71 ± 0.05	0.88 ± 0.03

The richest in total sugars were garlic wasted peels (6.51%), followed by onion wasted peels (4.29%) and potato wasted peels (1.68%). The amount of reducing sugars was highest in onion wasted peels (3.10%), followed by garlic wasted peels (1.36%) and potato wasted peels (0.53%).

The richest in water content were potato wasted peels (84.3%), followed by onion wasted peels (75.3%) and garlic wasted peels (19.2%). The highest dry matter content was found in garlic wasted peels (80.8%), followed by onion wasted peels (24.8%) and potato wasted peels (15.8%). Garlic wasted peels were the richest in total ash (7.37%), followed by onion wasted peels (1.71%) and potato wasted peels (0.88%).

Compared to our results for garlic wasted peels, Pathak et al. (2016) obtained 5.84% moisture and 8.47% ash content in garlic peels (Pathak et al., 2016).

According to Kallel et al. (2014), garlic husks can be used as an easily accessible source of natural bioactive compounds. Kallel et al. (2014) obtained 3.52 g/100g moisture, 16.65 g/100g ash, 8.43 g/100g protein, 0.86 g/100g lipid, 62.23 g/100g total dietary fibre content in garlic husk (Kallel et al., 2014).

Onion wastes include onion skins, outer fleshy scales and roots generated during industrial peeling, as well as undersized, malformed, diseased or damaged bulbs (Benítez et al., 2011). According to Salak et al. (2013), subcritical water treatment can be used to potentially solubilize and hydrolyze onion waste (a low cost feedstock) into valuable compounds. The conversion and solubility of carbohydrates were increased by this method (Salak et al., 2013).

Benítez et al. (2011) obtained the following values for the chemical composition of industrial onion wastes from two cultivars: in outer scales (6.3% and 7.5% dry matter; 9.3% and 8.3% crude protein; 5.6% and 4.6% total ash), in top-bottom (13.2% and 18.0% dry matter; 15.6% and 15.0% crude protein; 8.6% and 8.2% total ash), and in brown skin (51.9% and 50.8% dry matter; 2.3% and 2.4% crude protein; 10.6% and 9.3% total ash) (Benítez et al., 2011).

According to Pereira et al. (2017), dried onion waste had 9.41% moisture content, 1.48% ash, 36.56% reducing sugars (Pereira et al., 2017).

Unlike our results (Table 1), Mukherjee et al. (2008) obtained the following values for the proximate composition of potato peel in terms of crude protein (16.72%), crude lipid (0.6%), crude fibre (7.8%), ash (7.0%) (Mukherjee et al., 2008).

Sharoba et al. (2013) obtained lower moisture content (3.58%), higher total ash content (6.92%), higher fat content (2.25%), higher protein content (12.16%) and higher total dietary fibre content (73.25%) in potato peels (Sharoba et al., 2013) compared to our results (Table 1).

Our results for potato wasted peels (Table 1) were lower than results obtained by Liang et al. (2015) for chemical composition of potato peel waste from two different potato processing plants: carbohydrate content (39.9% and 51.3%), protein (22.4% and 22.8%), lipids (2.6% and 2.1%), ash (8.5% and 11.0%) (Liang et al., 2015).

Liang and McDonald (2015) obtained 39.3% carbohydrates and 2.0% lipids content in potato peel waste (Liang and McDonald, 2015). These values were higher than results obtained by us (Table 1).

Ravi et al. (2018) obtained the following nutritional values of potato waste: water (6.00%), crude ash (6.30%), crude protein (10.45%), crude fat (0.62%), crude fibre (2.50%) (Ravi et al., 2018).

The value of dry matter of potato wasted peel (15.8%) (Table 1) was very similar to the value of dry matter content in potato peel (16.3%) obtained by Hossain M.E. et al. (2015). According to Hossain M.E. et al. (2015), potato peel was characterized with 13.0% crude protein, 12.5% crude fibre, 9.0% ash (Hossain M.E. et al., 2015).

According to Jacob et al. (2016), potato wastes characterized with 84% moisture, 10%

ash, 43% carbohydrate, 8.75% protein content (Jacob et al., 2016).

3.2. Mineral composition of garlic, onion and potato wasted peels

The results for the mineral composition of garlic, onion and potato wasted peels were presented in Table 2.

In terms of mineral composition, the highest calcium content was found in garlic wasted peels (20610 mg/kg). The second content element in garlic wasted peels was potassium (9081 mg/kg). In onion wasted peels, the highest was the calcium content (5134 mg/kg), followed by potassium (2918 mg/kg).

Potassium content predominated in potato wasted peels (4959 mg/kg). The calcium content of potato wasted peels (130 mg/kg) was significantly lower than that of wasted peels from garlic and onion. The richest in sulfur were garlic wasted peels (1635 mg/kg), followed by onion wasted peels (1172 mg/kg) and potato wasted peels (390 mg/kg). The richest in magnesium were onion wasted peels (1285 mg/kg), followed by garlic wasted peels (950 mg/kg) and potato wasted peels (325 mg/kg).

Table 2. Mineral composition of wasted peels from garlic, onion and potato

Parameter, mg/kg	Garlic wasted peels	Onion wasted peels	Potato wasted peels
B	18.0 ± 10 rel.%	7.75 ± 10 rel.%	1.21 ± 10 rel.%
Na	123 ± 5 rel.%	1021 ± 5 rel.%	17.6 ± 10 rel.%
Mg	950 ± 5 rel.%	1285 ± 5 rel.%	325 ± 5 rel.%
Al	826 ± 5 rel.%	23.8 ± 10 rel.%	62.7 ± 10 rel.%
P	721 ± 5 rel.%	881 ± 5 rel.%	378 ± 5 rel.%
S	1635 ± 5 rel.%	1172 ± 5 rel.%	390 ± 5 rel.%
K	9081 ± 5 rel.%	2918 ± 5 rel.%	4959 ± 5 rel.%
Ca	20610 ± 5 rel.%	5134 ± 5 rel.%	130 ± 5 rel.%
Cr	18.40 ± 10 rel.%	0.990 ± 15 rel.%	<0.050
Mn	35.4 ± 10 rel.%	7.23 ± 10 rel.%	2.03 ± 10 rel.%
Fe	682 ± 5 rel.%	36.7 ± 10 rel.%	41.6 ± 10 rel.%
Cu	2.09 ± 10 rel.%	4.58 ± 10 rel.%	1.67 ± 10 rel.%
Zn	12.9 ± 10 rel.%	11.7 ± 10 rel.%	4.32 ± 10 rel.%
Se	0.058 ± 20 rel.%	<0.050	<0.050
Mo	1.480 ± 10 rel.%	0.470 ± 15 rel.%	<0.050

Sodium content was significantly higher in onion wasted peels (1021 mg/kg) than in wasted peels from garlic (123 mg/kg) and potato (17.6 mg/kg). Aluminum content was much more prevalent in garlic wasted peels (826 mg/kg) compared to potato wasted peels (62.7 mg/kg) and onion wasted peels (23.8 mg/kg).

Wasted peels from onion were the most abundant in phosphorus (881 mg/kg), followed by garlic wasted peels (721 mg/kg) and potato wasted peels (378 mg/kg). The richest in boron were garlic wasted peels (18.0 mg/kg), followed by onion wasted peels (7.75 mg/kg) and potato wasted peels (1.21 mg/kg).

A significant difference was found in the chromium content. While garlic wasted peels contained 18.40 mg/kg chromium, onion wasted peels contained only 0.990 mg/kg chromium, and in potato wasted peels, chromium content was below the detectable minimum (<0.050 mg/kg).

Garlic wasted peels were the richest in manganese (35.4 mg/kg) compared to wasted peels from onion (7.23 mg/kg) and potato (2.03 mg/kg). Iron content was the highest in garlic wasted peels (682 mg/kg), followed by potato wasted peels (41.6 mg/kg) and onion wasted peels (36.7 mg/kg).

The copper content predominated in onion wasted peels (4.58 mg/kg), followed by garlic

wasted peels (2.09 mg/kg) and potato wasted peels (1.67 mg/kg). The zinc content of wasted peels from garlic and onion was 12.9 mg/kg and 11.7 mg/kg, respectively, and 4.32 mg/kg in potato wasted peels.

Very low selenium content was found in garlic wasted peels (0.058 mg/kg), while in wasted peels from onion and potato, selenium content was below the detectable minimum (<0.050 mg/kg). In wasted peels from garlic and onion, molybdenum content was found to be 1.480 mg/kg and 0.470 mg/kg, respectively, while in potato wasted peels molybdenum was below the detectable minimum (<0.050 mg/kg).

The results we found showed that the content of the first five elements in wasted peels from onion and potato decreased in the following order: Ca>K>Mg>S>Na and K>S>P>Mg>Ca, respectively (Table 2). The results we obtained differed from those obtained by Asquer et al. (2013), Khattak and Rahman (2017) and Kuppusamy et al. (2017).

According to Asquer et al. (2013), the first five minerals in onion wastes, sorted by decreasing amount, were as follows: K>Ca>Mg>Na>Zn, and in potato wastes: K>Ca>Al>Mg>Zn (Asquer et al., 2013). According to Khattak and Rahman (2017), the content of the first five minerals in potato peels decreased as follows: K>P>Na>Mg>Ca (Khattak and Rahman, 2017).

According to Kuppusamy et al. (2017), the elements P, K, Ca, Mg, S, Na decreased in the following order in onion peels and in potato peels, respectively: Ca>Na>Mg>K>S>P and K>Na>P>S>Ca=Mg (Kuppusamy et al., 2017).

According to Benítez et al. (2011), the amounts of minerals they studied in industrial onion wastes from two cultivars decreased as follows: in outer scales (Zn>Fe>K>Mn>Ca>Mg>Se and Fe>Zn>K>Mn>Ca>Mg>Se), in top-bottom (Fe>Zn>Mn>Ca>K>Mg>Se and Fe>Zn>Mn>K>Ca>Mg>Se), and in brown skin (Fe>Ca>Zn>Mn>K>Mg>Se and Fe>Mn>Ca>Zn>K>Mg>Se) (Benítez et al., 2011).

According to Kallel et al. (2014), the mineral content in garlic husk decreased in the following order: Ca>K>Na>Mg>Fe>Mn>Zn>Cu (Kallel et al., 2014).

4. Conclusions

The results obtained in this study showed that in terms of nutritional composition garlic wasted peels were characterized by the highest content of total dietary fibre (62.10%), total sugars (6.51%), dry matter (80.8%), total ash (7.37%). Onion wasted peels were the richest in free fat (0.31%) and reducing sugars (3.10%). Potato wasted peels contained the highest amount of crude protein (2.67%), digestible carbohydrates (9.5%) and water content (84.3%). In garlic wasted peels, the content of the elements decreased in the following order: Ca (20610 mg/kg), K (9081 mg/kg), S (1635 mg/kg), Mg (950 mg/kg), Al (826 mg/kg), P (721 mg/kg), Fe (682 mg/kg), Na (123 mg/kg), Mn (35.4 mg/kg), Cr (18.40 mg/kg), B (18.0 mg/kg), Zn (12.9 mg/kg), Cu (2.09 mg/kg), Mo (1.480 mg/kg) and Se (0.058 mg/kg). In onion wasted peels, the content of the elements decreased as follows: Ca (5134 mg/kg), K (2918 mg/kg), Mg (1285 mg/kg), S (1172 mg/kg), Na (1021 mg/kg), P (881 mg/kg), Fe (36.7 mg/kg), Al (23.8 mg/kg), Zn (11.7 mg/kg), B (7.75 mg/kg), Mn (7.23 mg/kg), Cu (4.58 mg/kg), Mo (0.470 mg/kg). The content of Se in onion wasted peels was below the detectable minimum (<0.050 mg/kg). In potato wasted peels, the content of the elements decreased in the following order: K (4959 mg/kg), S (390 mg/kg), P (378 mg/kg), Mg (325 mg/kg), Ca (130 mg/kg), Al (62.7 mg/kg), Fe (41.6 mg/kg), Na (17.6 mg/kg), Zn (4.32 mg/kg), Mn (2.03 mg/kg), Cu (1.67 mg/kg), B (1.21 mg/kg). The contents of Cr, Se and Mo in potato wasted peels were below the detectable minimum (<0.050 mg/kg). The waste materials that were researched in this work could be used as a source to obtain valuable components presented in large quantities in them.

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OPTIMAL CONCENTRATION OF PREBIOTIC RAFFINOSE TO INCREASE VIABILITY OF *Lactobacillus acidophilus, Lactobacillus bulgaricus, Streptococcus thermophilus*

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ABSTRACT

Raffinose is an oligosaccharide consisting of three monosaccharide units, namely galactose, glucose, and fructose which are linked by α- (1-6) glycosidic bonds. Raffinose can be obtained mainly from soybeans, green beans, cabbage, broccoli, beets, asparagus, and wheat. Oligosaccharides such as raffinose can be a source of prebiotics because they are not enzymatically hydrolyzed in the stomach and small intestine so they can reach the large intestine. The use of raffinose in Indonesia is still limited due to its relatively high price. This is inversely proportional to Indonesia's abundance of natural sources of raffinose. The purpose of this study was to analyze the optimal concentration of raffinose to increase the viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*. The design used was a completely randomized design (CRD) with fixed variables being the concentration of raffinose and the independent variables were the viability of lactic acid bacteria, the pH value, and the total titrated lactic acid levels. *L. acidophilus* was the most sensitive and fast-growing probiotic with high viability in modified MRSB media with the addition of raffinose, followed by *S. thermophilus* and *L. bulgaricus*. The optimal viability of *L. acidophilus* occurred at the addition of 0.3% raffinose with an incubation time of 24 hours. Meanwhile, for *L. bulgaricus* and *S. thermophilus* the optimal viability occurred at the addition of 0.5% raffinose with an incubation time of 24 hours.

1.Introduction

Probiotics are functional food ingredients in the form of live microbes that benefit the health of the host (humans) by increasing the balance of microflora in the gut (Schrezenmeir and de Vrese, 2001, Mohammadi et al., 2013; Saad et al., 2013; Pham and Mohajeri, 2018; Gibson et al., 2017). The microorganisms best known as probiotics are lactic acid bacteria such as Lactobacillus sp., Streptococcus sp., and Bifidobacteria, widely used in yogurt and other dairy products (Schrezenmeir and de Vrese, 2001; Mohammadi et al., 2013; Gibson et al. ., 2017). Probiotics are not permanently in the host's body, so they must be consumed regularly (Saad et al., 2013; Pham and Mohajeri, 2018; Gibson et al., 2017). *L. acidophilus* and *L. bulgaricus* are lactic acid bacteria in the form of rods, gram-positive, non-spore-forming, 0.6-0.9 µm wide and 1.5-6.0 µm long (Gomes and Malcata, 1999; Adamberg et al., 2014). The growth of *L. acidophilus* and *L. bulgaricus* can occur in the temperature range 35°C-45°C, the optimal pH for growth is in the range 5.5-6.0. *L.*

acidophilus and *L. bulgaricus* are homofermentative, producing 0.3-2.0% of a metabolic product in the form of DL-lactic acid in milk (Schrezenmeir and de Vrese, 2001; Mohammadi et al., 2013; Gibson et al., 2017).

The distribution of *L. acidophilus* and *L. bulgaricus* is influenced by several environmental factors including pH, oxygen availability, substrate specifications, and interactions between bacteria (Schrezenmeir and de Vrese, 2001, Mohammadi et al., 2013; Gibson et al., 2017). *L. acidophilus* and *L. bulgaricus* are non-pathogenic and even act as health promoters in the gastrointestinal tract and genital (Gomes and Malcata, 1999; Adamberg et al., 2014). *S. thermophilus* is a lactic acid bacterium in the form of cocci with a diameter of 0.7-0.9 μm that forms chains, including the Gram-positive, non-spore, thermoduric group (Yerlikaya and Ozer, 2014). *S. thermophilus* produces an L (+) lactic acid configuration and does not ferment maltose. *S. thermophilus* and *L. bulgaricus* are lactic acid bacteria used in the fermentation of milk into yogurt (Das et al., 2019; Yerlikaya and Ozer, 2014).

Prebiotics are food ingredients that cannot be digested and can have a positive effect by increasing the growth and activity of a number of probiotic bacterial species in the large intestine, so as to maintain the health of the human digestive tract (Schrezenmeir and de Vrese, 2001; Roberfroid et al., 2010, Pham and Mohajeri, 2018). Food that is not digested by the upper digestive tract is able to reach the colon in an intact state so that it can be used as the main substrate for the growth of probiotic bacteria (Cummings et al., 2001; De Sousa et al., 2011. Ose et al., 2018).

Oligosaccharides such as raffinose can be a prebiotic source because they are not enzymatically hydrolyzed in the stomach and small intestine so they can reach the colon (Cummings et al., 2001; Roberfroid et al., 2010, Palacio et al., 2014, Carlson et al. , 2017). The use of raffinose in Indonesia is still limited because Indonesia still imports raffinose so the price is relatively high. This is inversely proportional to Indonesia's abundance of natural sources of raffinose, so it is necessary to study the effect of the prebiotic raffinose on probiotic viability.

Raffinose ($C_{18}H_{32}0_{16}$) is an oligosaccharide consisting of three monosaccharide units, namely galactose, glucose, and fructose which is linked by α (1-6) glycoside bonds (Amorim et al., 2020b, Teixeira et al., 2012, Martínez-Villaluenga et al., 2005). The chemical structure of raffinose is α-D-galactopyranosil- (1,6) -α-D-glucopyranosil- (1,2) -β-D-fructofuranoside (Adamberg et al., 2018). Raffinose is a non-reducing trisaccharide, dissolves in pyridine, but is difficult to dissolve in alcohol (Teixeira et al., 2012, Martínez-Villaluenga et al., 2005). Raffinose can be obtained mainly from the Leguminaceae (soybeans, green beans) and several other plants such as cabbage, broccoli, beets, asparagus, wheat (Amorim et al., 2020a, Martínez-Villaluenga et al., 2005).

Raffinose can be used as a prebiotic source for digestive tract health (Martínez-Villaluenga et al., 2005). Therefore, it is necessary to research to determine the optimal concentration of raffinose in increasing the viability of *L. acidophilus, L. bulgaricus, S. thermophilus*. In addition, research on the effect of variations in raffinose concentrations on the viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* has never been done before. The purpose of this study was to analyze and determine the optimal concentration of raffinose as a carbon source and prebiotic to increase the viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*.

2. Materials and methods

2.1. Materials

The research materials were *Lactobacillus acidophilus, Lactobacillus bulgaricus, Streptococcus thermophilus* obtained from IPB Culture Collection (IPBCC), MRSA media (deMan Rogosa and Sharpe Agar, Oxoid), MRSB media (deMan Rogosa and Sharpe Broth, Oxoid), and modified MRSB media which formulated from glucose (Merck), yeast extract (BactoTM), beef extract (Himedia), bacto peptone (Merck), sodium acetate (Sigma), KH_2PO_4 (Merck), $MgSO_4.7H_2O$ (Merck),

$MnSO_4.H_2O$ (Merck), ammonium citrate tribasic (Sigma), Tween® 80 (Merck), agar powder (Merck), raffinose (Sigma).

2.2. Preparation of standard raffinose stock solutions

Standard raffinose stock solution was prepared at a concentration of 5% (w/v). Raffinose is weighed as much as 10 g and dissolved in 200 mL of distilled water, then shaken until homogeneous. Further dilution was carried out to obtain raffinose concentrations of 0.1%, 0.3% and 0.5% (w/v).

2.3. Preparation of *Lactobacillus acidophilus, Lactobacillus bulgaricus, and Streptococcus thermophilus* cultures

Isolates of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* were cultured as much as 2% on MRSB medium (deMan Rogosa and Sharpe Broth, Oxoid) and incubated at 37°C for 24 hours. It was taken as much as 2% to be suspended on modified MRSB media.

2.4. Effect of raffinose concentrations on the viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*

The isolates of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* were each inoculated as much as 2% on MRSB media with the addition of raffinose concentrations of 0%, 0.1%, 0.3%, and 0.5%. Each treatment was inoculated with 3 replications and incubated at 37°C for 0, 6, 12, 18, and 24 hours. The viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* was calculated for the length of incubation time (0, 6, 12, 18, and 24 hours) at 37°C. Furthermore, dilution is made with sterile distilled water according to the incubation time. Incubation for 0 hours was carried out by diluting up to 10^4. Incubation for 6 hours was carried out by diluting up to 10^5. Incubation for 12 hours was carried out by diluting up to 10^6. Incubation for 18 hours was carried out by diluting up to 10^7. Incubation for 24 hours was carried out by diluting up to 10^8. Next, taking 0.1 mL of the sample in two the last dilution was to be inoculated on MRS agar plate with the spread plate method, and then incubated at 37 ° C for 48 hours. The number of colonies of *Lactobacillus acidophilus, Lactobacillus bulgaricus, Streptococcus thermophilus* growing was counted and expressed in Colony Forming Units/ mL.

2.5. Measurement of pH

The value of the degree of acidity (pH) was measured for each treatment with the concentration of raffinose 0%, 0.1%, 0.3% and 0.5% after the incubation period of 0, 6,12, 18, and 24 hours using a pH meter (TOA). The pH meter was calibrated with a phosphate buffer (pH 6.86) and an acetate buffer (pH 4.00).

2.6. Analysis of the total titrated lactic acid levels

Amount of 25 mL of the sample were taken and put into an Erlenmeyer flask containing 100 mL of distilled water. This mixture is added with the PP (phenolphthalein) indicator to test the total lactic acid by 2 to 3 drops. The sample is titrated with 0.1 N NaOH solutions until the color changes to pink, which indicates the end point of the titration has been reached.

2.7. Analysis statistic

The research data were processed statistically using analysis of variance (ANOVA) followed by Duncan's test at the 5% significance level if the results obtained were significantly different between samples using SPSS 17.0 software. The design used was a completely randomized design (CRD) with fixed variables being the concentration of raffinose and the independent variables were the viability of lactic acid bacteria, the pH value and the total titrated lactic acid levels.

3.Results and discussions

3.1. Effect of raffinose concentration on the viability of *Lactobacillus acidophilus*

Table 1 show that *Lactobacillus acidophilus* without the addition of raffinose (control) began to experience a significant increase in viability at 6 and 12 hours incubation. Meanwhile, at the

incubation time of 18 and 24 hours the viability of *Lactobacillus acidophilus* tended to remain. In the 6 and 12 hour incubation period *L. acidophilus* undergoes an exponential phase, where there is significant growth due to rapid cell division, while in the 18 to 24 hour incubation period, the growth has entered a stationary phase, where growth begins to slow down and eventually experiences a number. the same number of bacteria that grow with the number of bacteria that die. This situation is due to the reduced supply of nutrients, the accumulation of toxic metabolites such as bacteriocins and organic acids, and changes in pH that become acidic (Zainuddin et al., 2008). It is undeniable that if the supply of nutrients is reduced, the metabolic activity will also decrease, so that the number of cells will decrease, the accumulation of toxic compounds produced by bacteria which cause death for bacterial cells (Yusriyah & Agustini, 2014).

The addition of 0.1% raffinose showed the same results as the control, except that the growth was more significant, because the number of cells produced was higher than the control. The addition of raffinose by 0.3% and 0.5% had a significant effect on increasing the viability of *L. acidophilus* cells compared to controls and addition of 0.1% raffinose. The growth of L. acidophilus with the addition of rafinosa 0.3% and 0.5% continued to increase even until the incubation time of 24 hours. This happens because raffinose is a prebiotic compound that can be used specifically by *L. acidophilus* as a source of carbon and a source of nutrients in its viability. *L. acidophilus* produces the enzyme glycopyranosidase which is able to hydrolyze the α- (1,6) glycosidic bonds that connect galactose compounds with glucose, and also produces fructofuranosidase enzymes which can hydrolyze β - (1,2) - glycosidic bonds between glucose and fructose (Gänzle & Follador, 2012). This causes *L. acidophilus* to be able to use galactose, glucose and those produced from the hydrolysis of raffinose fructose more effectively as a carbon source for its viability.

Table 1. The results of the viability analysis of *Lactobacillus acidophilus* (CFU/mL) at several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%)

Incubation time (hours)	Viability of *L. acidophilus* (log CFU/mL)			
	Raffinose 0%	Raffinose 0.1%	Raffinose 0.3%	Raffinose 0.5%
0	7.59±0,32^{a}	7.38±0,24^{a}	7.32±0,33^{a}	7.90±0,45^{b}
6	8.28±0,25^{b}	8.01±0,43^{b}	8.32±0,19^{b}	8.27±0,16^{b}
12	8.85±0,18^{c}	9.08±0,15^{c}	8.88±0,12^{c}	8.88±0,23^{c}
18	8.92±0,27^{c}	9.45±0,22^{d}	9.55±0,37^{d}	9.05±0,12^{c}
24	8.90±0,11^{c}	8.77±0,27^{c}	10.10±0,29^{e}	9.61±0,36^{d}

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

The addition of 0.3% raffinose was the most optimal treatment in stimulating the growth of *L. acidophilus* compared to other treatments because it was able to produce the highest increase in viability. Meanwhile, the addition of 0.1% raffinose tended to have a relatively insignificant effect on the increase in the viability of *L. acidophilus* and was comparable to the control treatment. This is due to the addition of raffinose which is still too little in number

Table 2. Total levels of lactic acid titrated *Lactobacillus acidophilus* with several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%) for 24 hours

Incubation time (hours)	Total levels of lactic acid titrated *L. acidophilus* (%)			
	Raffinose 0%	Raffinose 0,10%	Raffinose 0,30%	Raffinose 0,50%
0 jam	0,33±0,03^{a}	0,45±0,01^{c}	0,49±0,03^{c}	0,36±0,02^{b}
6 jam	0,38±0,02^{b}	0,49±0,02^{c}	0,54±0,02^{d}	0,38±0,02^{b}
12 jam	0,45±0,01^{c}	0,52±0,02^{d}	0,68±0,04^{f}	0,46±0,02^{c}
18 jam	0,47±0,03^{c}	0,54±0,01^{d}	0,72±0,02^{f}	0,54±0,01^{d}
24 jam	0,49±0,01^{c}	0,63±0,02^{e}	0,80±0,01^{g}	0,60±0,03^{e}

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

During the 24-hour incubation period for *L. acidophilus* bacteria, there was a significant increase in the total levels of lactic acid ($p < 0.05$), especially in the 0.3% raffinose addition treatment when compared to other treatments (Table 2). The increase in the total levels of lactic acid caused a decrease in the pH value from the range of 7.0 to 4.8 (Figure 1).

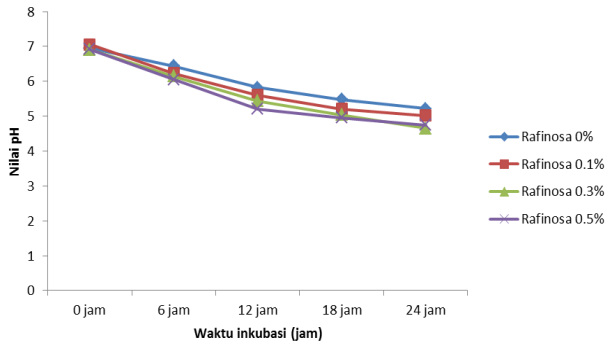

Figure 1. The pH value of *Lactobacillus acidophilus* with several variations in the concentration of raffinose (0%; 0.1%; 0.3%; 0.5%) for 24 hours

Lactic acid compounds as short-chain fatty acids can be used as a carbon source by *Lactobacillus acidophilus* for growth through the β-oxidation metabolic pathway. Apart from lactic acid, several other short-chain fatty acid compounds are also produced including acetic acid, propionate acid, and butyric acid as a result of the metabolism of raffinose by *Lactobacillus acidophilus*. The effects of short-chain fatty acid production and increased viability of probiotics are beneficial, among others, increasing intestinal function, calcium absorption, lipid metabolism, and reducing the risk of colon cancer (Macfarlane & Cummings, 1999).

After entering the stationary phase and death, most of the lactic acid raffinose fermentation will undergo alcohol fermentation by the enzyme lactate dehydrogenase. The higher the total colony count (CFU/ mL) indicates a significant increase in the viability of *Lactobacillus acidophilus*. The increase in lactic acid production during fermentation has a positive correlation with the increase in the viability of *L. acidophilus*. The ability of a carbohydrate source to be fermented is related to the enzymatic hydrolysis system by bacteria (Moreno et al., 2017). Fructofuranosidase is an enzyme that hydrolyzes the fructose group from raffinose at the β- (1,2) end position so that it contributes to fructan metabolism. Some of the factors that affect raffinose fermentability include the structure of the saccharide (level of molecular branching and glycosidic bonds formed), degree of polymerization (length of carbon chains) (Gänzle & Follador, 2012).

Raffinose has a simpler saccharide structure and a low degree of polymerization with a short carbon chain length, making it easier for *L. acidophilus* to ferment it when compared to other prebiotic sources such as inulin, FOS (fructo oligosaccharide), GOS (galacto oligosaccharide), and resistant starch (Gänzle & Follador, 2012). One of the benefits of raffinose as a prebiotic is that it can increase the growth of probiotic bacteria by lowering the intestinal pH to an optimal level, which is influenced by the physicochemical properties of bile acids (Gänzle & Follador, 2012). High concentrations

of raffinose prebiotics can decrease the solubility of bile acids which can reduce their toxicity. The character of raffinose which cannot be digested directly in the human gastrointestinal tract causes raffinose to act as a prebiotic (Date et al., 2014).

3.2. Effect of raffinose concentration on the viability of *Lactobacillus bulgaricus*

Table 3 shows that the higher the concentration of raffinose added, the viability of *Lactobacillus bulgaricus* also increased significantly. The highest increase in the viability of *L. bulgaricus* based on the results of the study was the addition of 0.5% raffinose concentration with an incubation time of 24 hours. Raffinose consists of several types of sugar, namely galactose, glucose, and fructose which can be metabolized by bacteria into lactic acid during the fermentation process. The more raffinose available, the more substrate *L. bulgaricus* can hydrolyze into pyruvic acid which can then be converted into other organic acids such as lactic, propionate, butyric, and acetic acid. The longer the incubation time, the higher the viability of *L. bulgaricus*. The longer incubation time will give *L. bulgaricus* time to ferment raffinose into simple sugars such as glucose, galactose, and fructose to then undergo the process of glycolysis to pyruvic asthma and with the help of the enzyme lactic dehydrogenase, it is converted to lactic acid and growth energy (Yusriyah & Agustini, 2014).

Lactobacillus bulgaricus with control treatment experienced a significant increase in viability at 6 and 12 hours incubation time, but at 18 and 24 hours, incubation time the viability tended to enter the stationary phase. This also happened to the addition of 0.1% raffinose. This shows that *L. bulgaricus* at the incubation period of 6 hours and 12 hours experienced a log phase, namely the bacterial cell phase growing exponentially. During the log phase each cell divides to form two cells, each of which will divide again and so on as long as there are sufficient nutrients in the medium and the environment supports bacterial growth. Whereas at the 18 hours and 24 hour incubation time, the growth has entered a stationary phase towards death, wherein this phase there is no bacterial growth, but most of the bacteria die because the nutrients are depleted. This is due to the addition of the prebiotic raffinose which is too little.

The addition of raffinose by 0.3% and 0.5% had a very significant effect on the viability of *Lactobacillus bulgaricus* cells compared to other treatments. The viability of *L. bulgaricus* on the addition of raffinose 0.3%, and 0.5% continued to increase even at the incubation time of 24 hours. This occurs because the prebiotic raffinose as a carbon source is used optimally by *L. bulgaricus*, so that at the 18 hours and 24 hour incubation time, the viability of *L. bulgaricus* continues to increase and is still in an exponential phase.

During the 24-hour incubation period in the fermentation of raffinose by *L. bulgaricus*, the total lactic acid levels increased significantly ($p < 0.05$) (Table 4). The increase in the total levels of lactic acid caused a significant decrease in the pH value from the range of 7.0 to 5.0 (Figure 2). This significant increase shows that *L. bulgaricus* bacteria are able to produce fructofuranosidase enzymes with high activity so that they can hydrolyze raffinose into glucose, galactose, and fructose monosaccharides which will then be converted into lactic acid products by the enzyme lactic dehydrogenase. The higher the amount of raffinose concentration added to the modified MRS media had an impact on the increase in the total accumulated levels of lactic acid so that a significant decrease in the pH value also occurred with the length of the incubation time.

Table 3. The results of the viability analysis of *Lactobacillus bulgaricus* (CFU/mL) at several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%)

Incubation time (hours)	Viability of *L. bulgaricus* (log CFU/mL)			
	Raffinose 0%	Raffinose 0.1%	Raffinose 0.3%	Raffinose 0.5%
0	6.93±0,14[a]	6.84±0,15[a]	7.38±0,41[b]	7.41±0,25[b]
6	7.49±0,22[b]	7.34±0,22[b]	7.83±0,17[c]	7.76±0,14[c]
12	7.68±0,18[c]	7.75±0,16[c]	8.21±0,26[d]	8.27±0,18[d]
18	7.72±0,10[c]	8.01±0,28[d]	8.69±0,23[e]	8.62±0,28[e]
24	7.89±0,15[c]	8.33±0,13[d]	9.31±0,14[f]	9.61±0,24[f]

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

Table 4. Total levels of lactic acid titrated *Lactobacillus bulgaricus* with several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%) for 24 hours

Incubation time (hours)	Total levels of lactic acid titrated *Lactobacillus bulgaricus* (%)			
	Raffinose 0%	Raffinose 0,10%	Raffinose 0,30%	Raffinose 0,50%
0	0,39±0,02[a]	0,45±0,01[b]	0,54±0,01[c]	0,56±0,02[d]
6	0,43±0,02[b]	0,48±0,01[c]	0,63±0,03[d]	0,63±0,02[d]
12	0,48±0,03[c]	0,50±0,02[c]	0,75±0,02[e]	0,72±0,03[e]
18	0,50±0,01[c]	0,57±0,02[d]	0,82±0,01[f]	0,88±0,03[g]
24	0,54±0,02[c]	0,63±0,01[d]	0,90±0,03[g]	0,99±0,01[h]

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

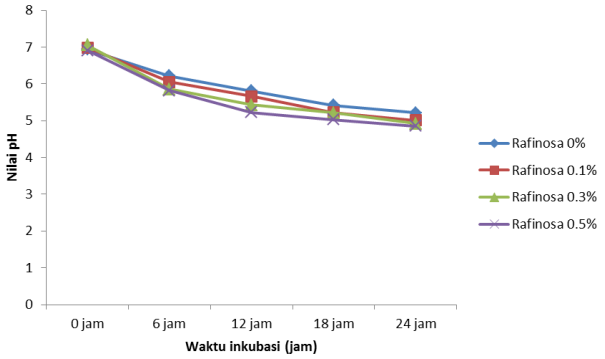

Figure 2. The pH value of *Lactobacillus bulgaricus* with several variations in the concentration of raffinose (0%; 0.1%; 0.3%; 0.5%) for 24 hours

The change in pH value is caused by the formation of organic acids with the main product being lactic acid. The fermentation of carbohydrates by lactic acid bacteria produces organic acids such as lactic and acetate which make the surrounding pH acid so that pathogenic organisms are unable to live (Mohammadi et al., 2013). Thus the change in pH to acid will cause an antimicrobial effect for pathogenic microbes, on the other hand, lactic acid bacteria can still live in an acidic environment with an optimum pH of 3–5. Another mechanism of this antimicrobial property is that lactic acid bacteria also produce antimicrobial peptides such as bacteriocins. Bacteriocins have inhibitory properties because the polypeptides contained can combine with pathogenic bacterial cell membrane proteins so that the cell membrane cannot function properly in terms of selecting molecules in and out of cells (De Sousa et al., 2011).

Lactobacillus acidophilus, Lactobacillus bulgaricus, and *Streptococcus thermophilus* are lactic acid bacteria that are homofermentative, so they can produce lactic acid as the majority product of carbohydrate fermentation and a small portion of acetate via the hexose diphosphate (HDP) pathway or also known as Embden-Meyerhoff, Pathway 2012; Cummings,

et al., 2001). Lactic acid bacteria which are heterofermentative produce lactic acid from carbohydrate fermentation through the hexose monophosphate (HMP) pathway and the pentose phosphate pathway (Gänzle & Follador, 2012). Most of the lactic acid formed during the fermentation process is converted into acetic acid, propionate, and butyric acid through the acetyl-CoA pathway (Gänzle & Follador, 2012; Cummings, et al., 2001).

3.3. Effect of raffinose concentration on the viability of *Streptococcus thermophilus*

Similar to *L. acidophilus* and *L. bulgaricus*, the growth of *Streptococcus thermophilus* was strongly influenced by the concentration of raffinose added to the modified MRSB medium ($p < 0.05$). This is indicated by the increasing growth of the total number of *Streptococcus thermophilus* colonies during the 24-hour incubation period of 1-2 log (CFU / mL) of *Streptococcus thermophilus* colonies in the 0.1%, 0.3%, and 0.5% raffinose treatment (Table 5). Table 5 shows that the higher the raffinose concentration is given, the impact on the increased viability of *Streptococcus thermophilus*. In addition, along with the increasing incubation time, the viability of S. thermophilus also increased for each treatment tested. The highest increase in viability of *S. thermophilus* occurred in the addition of 0.5% raffinose treatment. The increase in total *S. thermophilus* started at the incubation time of 6 hours and then continued to increase even until the incubation time of 24 hours. This shows that at that time it was an exponential phase where the growth of *S. thermophilus* took place optimally due to the availability of sufficient nutrients and environmental conditions that supported its growth.

The decrease in pH during raffinose fermentation by *Streptococcus thermophilus* was influenced by the activity of these bacteria in hydrolyzing raffinose to lactic acid (Figure 3 and Table 6). The production of lactic acid as a result of rhinoceros metabolism caused a significant decrease in the pH value from the range of 7.0 to 4.5. This is related to the increasing number of lactic acid bacteria populations that use raffinose as a carbon source for their growth. The more carbon sources that can be metabolized, the more lactic acid is produced so that the pH will automatically be lower. Streptococcus sp. is responsible for decreasing the initial pH of fermented milk to around 5.0. Then the type of Lactobacillus sp. is responsible for further decreases until the pH reaches 4.5. S. thermophilus also produces the enzyme fructofuranosidase which hydrolyzes raffinose into fructose, glucose, and galactose which are then converted into lactic acid products by the enzyme lactic dehydrogenase (Gänzle & Follador, 2012; Cummings, et al., 2001).

Table 5. The results of the viability analysis of *Streptococcus thermophilus* (CFU/mL) at several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%)

Incubation time (hours)	Viability of *S. thermophilus* (log CFU/mL)			
	Raffinose 0%	Raffinose 0.1%	Raffinose 0.3%	Raffinose 0.5%
0	7.15±0,30^{a}	7.20±0,11^{a}	7.57±0,12^{a}	7.51±0,11^{a}
6	7.64±0,21^{b}	7.70±0,26^{b}	7.80±0,24^{b}	7.66±0,14^{b}
12	7.74±0,18^{b}	7.86±0,14^{b}	8.20±0,18^{c}	8.19±0,20^{c}
18	7.94±0,24^{c}	8.24±0,20^{c}	8.41±0,15^{c}	8.77±0,16^{d}
24	8.37±0,16^{c}	8.32±0,15^{c}	8.84±0,21^{d}	9.39±0,18^{e}

Note: Different letters indicate significantly different values with a real level of 95%, ($\alpha = 5\%$), after a statistical test was carried out with the Duncan test on SPSS 17.0

Table 6. Total levels of lactic acid titrated *Streptococcus thermophilus* with several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%) for 24 hours

Incubation time (hours)	Total levels of lactic acid titrated *Streptococcus thermophilus* (%)			
	Raffinose 0%	Raffinose 0,10%	Raffinose 0,30%	Raffinose 0,50%
0	0,38±0,02^{a}	0,40±0,02^{a}	0,49±0,02^{d}	0,49±0,01^{d}
6	0,42±0,02^{a}	0,44±0,02^{a}	0,68±0,03^{e}	0,68±0,01^{e}
12	0,56±0,01^{c}	0,48±0,01^{b}	0,72±0,01^{e}	0,87±0,02^{g}
18	0,59±0,01^{c}	0,54±0,01^{b}	0,85±0,02^{f}	0,98±0,02^{h}
24	0,64±0,02^{c}	0,63±0,02^{c}	0,99±0,01^{e}	1,17±0,02^{g}

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

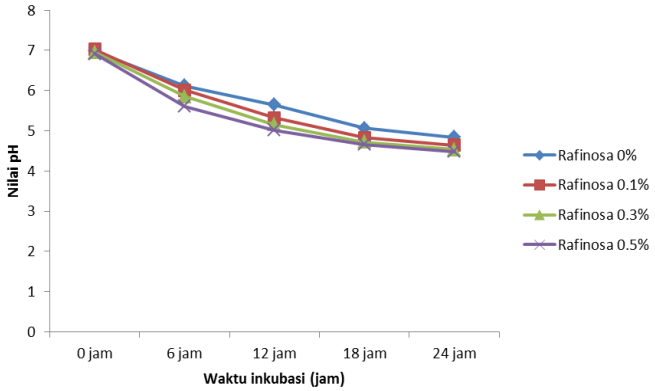

Figure 3. The pH value of *Streptococcus thermophilus* with several variations in the concentration of raffinose (0%; 0.1%; 0.3%; 0.5%) for 24 hours

The benefits of short-chain fatty acids from raffinose metabolism on health, among others, are that these compounds can be absorbed by the intestinal mucosa and play a role in meeting energy needs (Mueller et al., 2016). Lactic acid will make the intestinal conditions acidic so that pathogenic bacteria that cannot stand the acid will die. Acetic acid will be metabolized in muscle, kidney, heart, and brain cells (Rastall, 2013). Propionic acid is a gluconeogenic precursor that suppresses cholesterol synthesis in the liver. *Lactobacillus plantarum* can reduce blood pressure, fibrinogen, and LDL cholesterol and raise HDL cholesterol (De Sousa et al., 2011). Meanwhile, butyric acid is the main energy source for colonocytes, where butyrate is metabolized by the colonic epithelium and functions as a regulator of cell growth and differentiation (Mueller et al., 2016). In addition, butyric acid plays an important role in preventing cancer (Rastall, 2013).

Raffinose fermentation is a source of energy for probiotic bacteria such as Lactobacillus sp. and Bifidobacterium. Along with the energy requirements for growth, the availability of carbohydrates will decrease, so that protein and amino acids will become the dominant metabolic energy sources for probiotic bacteria in the colon (Cummings et al., 2001). This will cause an increase in pathogenic bacteria in the intestine because protein and amino acids are the main nutrient sources for pathogenic bacteria (Adamberg et al., 2014). Therefore, the consumption of raffinose as a carbohydrate compound that is difficult to digest is needed to maintain the balance of the microflora in the intestine. This is what ultimately led to the concept of prebiotics. In the production of short-chain fatty acids, raffinose compounds will be hydrolyzed by the enzyme fructofuranosidase produced by *L. acidophilus, L. plantarum*, and *S. thermophilus* into fructose, glucose, and galactose (Adamberg et al., 2018). Furthermore, fructose, glucose, and galactose undergo a glycolysis process to become pyruvic acid (Teixeira et al., 2012). In the subsequent metabolic process, pyruvic acid will be converted into lactic acid, acetic acid, propionic acid, butyric acid, and CO_2 (Amorim et al., 2020b). *Lactobacillus acidophilus* is the most sensitive lactic acid bacteria and is able to grow quickly and has high viability because it can grow optimally on MRSB media with the addition of 0.3% raffinose. Then followed by *S. thermophilus* and *L. bulgaricus* which were able

to grow optimally and had high viability in the addition of 0.5% raffinose.

4. Conclusions

Raffinose has potential as a prebiotic that provides a carbon source for growth and increases the viability of the probiotics *Lactobacillus acidophilus, Lactobacillus bulgaricus,* and *Streptococcus thermophilus*. *L. acidophilus* was the most sensitive probiotic and was able to grow rapidly and significantly in modified MRSB media with the addition of raffinose, followed by *S. thermophilus* and *L. bulgaricus*. The optimal viability of *L. acidophilus* occurred at the addition of 0.3% raffinose with an incubation time of 24 hours. Meanwhile, for *L. bulgaricus* and *S. thermophilus*, the optimal viability occurred at the addition of 0.5% raffinose with an incubation time of 24 hours. Further research is needed to analyze the content and types of short-chain fatty acids (acetic, propionic, and butyrate) produced during raffinose fermentation by the probiotics *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*.

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COMPARISON OF DIFFERENT EXTRACTION METHODS FOR THE DETERMINATION OF *PITURANTHOS SCOPARIUS* ESSENTIAL OILS: CHEMICAL COMPOSITION, ANTIMICROBIAL AND ANTI-INFLAMMATORY ACTIVITIES

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ABSTRACT

The essential oils of *Pituranthos Scoparius* obtained by hydrodistillation (HD), microwave assisted extraction (MAE) and steam distillation (SD) were investigated for their chemical components, oil yield diversity and microbial activity. As a result of this investigation, the anti-inflammatory activity of the essential oils is reported here for the first time. The essential oils extracted from *P. Scoparius* were analyzed by GC/MS. Sixty-three compounds were identified, representing (87.69 %) in HD, (82.45 %) in MAE, and (88.94 %) in SD of the essential oils' total compositions. The predominant compounds identified were Dillapiol (16.38-37.21%), α-Pinene (0.48-10.84%) and Myristicin (4.21-9.37%). The antimicrobial activity of the essential oils was Identified using the disk diffusion method against seven bacteria strains and two yeasts. An appreciable antimicrobial activity was observed against *sarcina lutea*, and weak activity was observed against *staphylococcus epidermidis*, *saccharomyces cerevisiae* and *candida albicans*. The anti-inflammatory activity of the essential oils was Identified using the carrageenan induced edema method. The essential oils extracted by HD, MEA and SD showed an anti-inflammatory activity comparable to Diclofenac. The results reveal that the method of extraction of *P. scoparius* influences the chemical composition and anti-inflammatory activity of the essential oils.

1. Introduction

The genus *Pituranthos* is a member of the Apiaceaes family and consists of more than 20 species (Ozenda, 2004). *P. Scoparius* (Coss & Dur) Benth & Hook is a plant endemic to the Saharan region of North Africa (Quezel and Santa, 1962) known as "Tattayet" in Tamahaq (Tuareg language) and "Guezzah" in Arabic.

P. Scoparius is a perennial aphyllous plant. The stems are 40-80 cm tall. The flowers, often with peduncle, white petals and marrow veins, are bunched in lateral umbels that are fairly spread out. However, due to the high temperature of the Central Sahara from which the plants used in this experiment were obtained, the species used did not contain leaves, flowers, or seeds (Quezel and Santa, 1962). *P. Scoparius* is used in traditional Algerian medicine for many purposes. In infusion, the aerial part is used as a remedy for digestive difficulties, diabetes, hepatitis, asthma and rheumatic diseases (Ould el hadj *et al.*, 2003; (Hammiche

and Maiza, 2006) and also as an additive flavoring (Boukef, 1986; Benchelah et al, 2000). The powdered stems are used against reptile bites (Benchelah et al., 2000; Abdellah and Sahki, 2004).

Previous research has shown that *P. Scoparius* is an important source of essential oils, with a considerable variation in major compounds depending on the part used, the plant maturity, the climate conditions and the extraction methods employed (Vernin *et al.*, 1999; Vérité and Nacer, 2003; Smaili *et al.*, 2011; Gourine *et al.,* 2011; Lograda *et al.*, 2013; Hammoudi *et al.*, 2015; Belkacemi *et al.*, 2015; Chikhoune *et al.*, 2016; Ksouri *et al.*, 2017; Malti *et al.*, 2018, Attia *et al.*, 2011). In addition, Sabinene and α-pinene were identified as major compounds of most of *P. Scoparius* essential oils (Smaili *et al*, 2011; Lograda *et al*, 2013; Chikhoune *et al.*, 2016; Malti *et al.*, 2018; Attia *et al*, 2011). However, α-pinene was not identified in samples from some Algerian Saharan regions (Ghardaia, Bechar and Tamenrasset) (Belkacemi *et al.*, 2015; Ksouri *et al.*, 2017; Malti *et al.*, 2018) and Sabinene had not been reported in other studies on the same species (Vernin *et al.*, 1999; Vérité and Nacer, 2003; Gourine *et al.,* 2011; Hammoudi *et al.*, 2015; Belkacemi *et al.*, 2015; Ksouri *et al.*, 2017). Alternatively, samples from Ghardaïa, Laghouat, Bechar, Tamenrasset and Batna were characterized by very high contents of limonene (Gourine *et al.,* 2011; Ksouri *et al.*, 2017; Malti *et al.*, 2018). Bornyl Acetate was the dominant component of *P. Scoparius* essential oils from the Oum El Bouaghi and Tamenrasset regions (Vernin *et al.*, 1999; Hammoudi *et al.*, 2015). Other compounds were reported only in some samples and with appreciable amounts, such as: 7-methoxy-3-methyl-1-H-isichromen-1-one and methyl propene in P. *Scoparius* from Ghardaia (Belkacemi *et al.*, 2015), 6- methoxyelemicine in samples from Ghardaïa, Bechar and Batna (Malti *et al.*, 2018), Apiol in samples from Oum El Bouaghi (Vernin *et al.*, 1999) and Δ-3-Carene only in the Tunisian essential oil (Attia *et al*, 2011). Other essential oils were also characterized by a high presence of Myristicin (Vérité and Nacer, 2003; Smaili *et al.*, 2011; Gourine *et al.,* 2011; Malti *et al.*, 2018). These reported results confirmed the existence of different chemotypes in *P. Scoparius* essential oils.

Several studies reported the significant effect of the extraction methods on the yields and the chemical compositions of essential oils (Lucchesi *et al.*, 2004: Elyemni *et al.*, 2019; Lo Presti *et al.*, 2005). Our work was based on the chemical variability of *P. Scoparius* essential oil as well as the antimicrobial and the anti-inflammatory activities depending on the extraction methods. To the best of our knowledge, this is the first study Dedicated to extractions using the microwave assisted hydrodistillation is reported here for the first time. In addition, no pharmacological studies on the anti-inflammatory activity of essential oils were previously conducted. The aim of our work is to check the variation of the chemical composition, antimicrobial and anti-inflammatory activities of the essential oils using three extraction methods (hydrodistillation, micro-wave and steam distillation).

2. Materials and methods

2.1. Samples

The aerial parts of *P. Scoparius* were collected from the Algerian Sahara (Taessa, Tamanrasset) in July of 2012. The harvested plants were washed and then allowed to dry in the shade in a dry and ventilated place. Global Positioning System (GPS) technology was used to record the location: latitude (23º05′64″ N), longitude (5º30′79,7″ E) and altitude (1748 m). Identification of plant was performed in the Botany laboratory of the National Forest Research Institute in Tamanrasset (NFRI).

2.2. Essential oils

2.2.1. Hydro-distillation (HD)

(200 g) of plant were placed in a flask. The flask was then filled with (4L) of water. The flask containing the plant and water was brought to a boil for 4hours using Clevenger-type apparatus (Council of Europe, 2010). The

essential oil-laden water vapors then passed through the refrigerant, condensed and fell into a separating funnel. The water and oil separated by density difference. All samples were collected and subsequently stored (sealed brown vial) at 4°C in the dark for further experimentation. The yield, based on the dry weight of the sample, was calculated.

2.2.2. Microwave-assisted extraction (MAE)

Microwave-assisted extraction was used at atmospheric pressure at (850 W, 2450 MHz) power for 50 minutes (Beoletto *et al.*, 2016), using a laboratory microwave for extracting the essential oil (M937, Samsung, United Kingdom). (100 g) of aerial part was placed in a (1L) flask with (100 ml) of water and connected to the Clevenger-type apparatus located outside the microwave. The essential oil was collected in the same way as mentioned in the HD process. The yield, based on dry weight of the sample, was calculated.

2.2.3. Steam distillation (SD)

The steam was passed through a (5L) flask containing (200 g) of plant for 4 h. The steam was then passed through a coiled tube where it was condensed. The distillate was collected. Finally, the essential oil obtained was separated through decantation using a separating funnel (Pushpangadan *et al.*, 2012). The essential oil was collected in the same way as mentioned in the HD process. The yield, based on dry weight of the sample, was calculated.

2.3. Gas chromatography (GC) and Gas chromatography-Mass spectrometry (GC-MS) analysis

Analyses of the essential oils were carried out using a Hewlett-Packard 6800 GC equipped with an HP5-MS capillary column (30m x 0.32mm x 0.25 m) film thickness. The oven temperature was kept at 60°C for 8 min initially, then gradually increased to 250°C at 2°C/ min and kept again at 250°C for 20 min. The injector and detector temperature were 250 °C and 280 °C, respectively. The carrier gas was helium used at flow rate of 1.2 mL/min. 1µl of sample was injected in split mode at a ratio of 1:70, where the detection ionization energy was 70 eV. For GC-MS analysis, the same above-mentioned conditions for GC were applied. The mass scan range was 29-550 Uma. The percentage composition of the oils was expressed as peak area by integration from the total ion current. The percentage of each compound was computed using the normalization method from the GC peak area, calculated as mean value of three injections, without using correction factors. The identification of the compounds was based on the comparison of retention indices with those reported in the literature. Retention indices were calculated using the Van Den Dool and Kratz equation. The series of n-alkanes (C_5-C_{28}) were injected in the same conditions. The EI–mass spectra of essential oils were compared with those of mass spectra library (NIST and Wiley 7N library) and the literature (Adams, 2007).

2.4. Pharmacological activities

2.4.1. Animals

Strains of albino mice of both sexes, weighing (20-25g) were used for pharmacological studies. The animals were obtained from the Department of Pharmacotoxicology, SAIDAL Antibiotical-Medea, Algeria. The animals were housed in cages under standard laboratory conditions (12-hour light/dark cycle at 25°C). The animals were divided into groups of five and fasted for 12 hours before the experiment.

2.4.2. Acute toxicity assay

The acute toxicity test was performed using the Lorke method (Lorke, 1983). Mice were divided into several groups of five. (0.5 mL) of (2000 mg / kg) of essential oils were administered orally. The control group was treated in the same manner without administering essential oil. The mice were observed for 14 days. The mice that died in each group were counted for lethal dose (LD50) determination.

2.4.3. Anti-inflammatory activity

The anti-inflammatory activity was determined based on the carrageenan induced edema test (Levy, 1969). Groups of five mice each were treated orally with (0.5 mL) of

essential oils (50 mg/kg, 100 mg/kg and 150 mg/kg). After 30 minutes, (0.025 mL) of (1%) carrageenan solution (diluted with 0.9% physiological water) was injected into the right hind paw planter surface of the mice. Positive control received (50 mg/kg, 100 mg/kg and 150 mg/kg) of Diclofenac. After 3.30 hours, the animal suffers euthanasia and their right and left paws were removed and weighed with a precision scale. The percentage of edema was calculated according to the following equation:

% Edema = 100 × (Average weight of left paws - average weight of right paws) ÷ Average weight of left paws.

The percentage reduction of edema in the treated mice relative to the control was calculated according to the following equation:

% Reduction of edema= 100 × (% Edema control - % Edema test) ÷ % Edema control.

2.4.4. Antimicrobial activity

2.4.4.1. Microbial strains

Nine stains were used, including five Gram-positive bacteria: *Staphylococcus Aureus* ATCC 6538/P, *Staphylococcus Epidermidis* ATCC 12228, *Enterobacter Faecalis*, *Bacillus Subtilis* ATCC 6633 and *Sarcina Lutea*, two Gram-negative bacteria: *Escherichia Coli* ATCC 11105 and *Pseudomonas Aeruginosa* ATCC 27853 and two yeasts: *Candida Albicans* ATCC 10231 and *Saccharomyces Cerevisiae* ATCC 2601.

The microbial strains belonged to the American Type Culture Collection, except for *Enterobacter Faecalis*, and were supplied from Frantz Fanon Hospital in Blida, Algeria and Pastor Institution in Algiers, Algeria.

2.4.4.2. Agar disk diffusion method

The antimicrobial activity was determined using the disk diffusion method (National Committee for Clinical Laboratory Standards, 1997).The test was performed using the Soja Agar medium for bacterial germs and Sabouraud medium for fungal germs. The microbial suspensions were adjusted to 10^6 CFU/ml (Colony Forming Units) in a sterile saline solution and were streaked over the surface of the plates using a sterile cotton swab in order to get a uniform microbial growth on the test plates. Sterile filter paper disks (6 mm in diameter) were then permeated with (10 uL) of essential oil. Plates were incubated at 37°C for 24h. Penicillin G, Oxalin and Amoxypen (200 mg/mL) were used as standard antibiotics. The antimicrobial activity was evaluated by measuring the diameter of inhibition in mm (diameter of the disc included). Assays were performed in triplicate.

2.5. Statistical analysis

Collected data are expressed as mean ± standard deviation. All tests were performed using IBM SPSS v 23. The comparison between chemical compositions was performed using a multivariate analysis of variance, sustained by a Tukey post hoc test, if significant. The study of biological activity was established by the ANOVA or Kruskal Wallis tests, sustained by Tukey or Mann Whitney U tests, if significant. Differences are considered as significant when ($p<0.05$), highly significant when ($p<0.01$) or strongly significant when ($p<0.001$).

3. Results and discussions

3.1. Yields and chemical composition of essential oils

The extraction of the aerial parts of *P. Scoparius* produced yellow essential oils ranging in yield from 0.20% to 0.53% (w/w) as a function of the extraction method (Table 1). The results showed that the extraction method had a significant influence on essential oil yields of *P. Scoparius* ($p <0.05$). The extraction by HD provided the highest yield (0.53%, 4h) compared to that obtained by SD (0.33 %, 4h), however the lowest yield was obtained using MAE (0.20 %, 0.5h). The results obtained agreed with those reported by several authors (0.25–0.99 %) (Vérité and Nacer, 2003; Lograda *et al.*, 2013; Hammoudi *et al.*, 2015; Ksouri *et al.*, 2017; Malti *et al*, 2018, Attia *et al.*, 2011). However higher oil yields were obtained from some different Algerian Saharan regions in

others studies (1.0-2.8%) (Gourine *et al.,* 2011; Lograda *et al.*, 2013).

Table 1. Variability in the chemical composition of *P. Scoparius* essential oil obtained by different methods

No.	Compounds[a]	RI[b]	RI[c]	HD	MAE	SD
1	*α*-Thujene	914	930	1.94	-	0.49
2	*α*-Pinene	925	939	10.84	0.48	3.28
3	Camphene	937	953	0.16	-	tr
4	Sabinene	967	976	0,88	tr	0.26
5	*β*-Pinene	971	979	2.63	0.27	1.19
6	*β*-Myrcene	989	990	0.38	-	0.14
7	*I*-Phellandrene	1003	1005	0.39	tr	0.18
8	*Δ*-3-Carene	1009	1011	1.08	tr	0.80
9	*α*-Terpinene	1015	1017	0.28	tr	0.25
10	*p*-Cymene	1025	1024	4.89	1.57	4.02
11	Limonene	1028	1029	3.16	0.77	1.73
12	*β*-Ocimene*Z*	1039	1040	2.70	0.32	1.00
13	*β*-Ocimene*E*	1047	1050	0.27	-	tr
14	*γ*- Terpinene	1057	1059	0.56	0.18	0.60
15	*Cis*sabinene hydrate	1064	1068	0.11	tr	tr
16	*α*-Terpinolene	1085	1088	0.24	0.19	0.38
17	*Trans*sabinene hydrate	1095	1097	0.22	-	0.13
18	*β*-Thujone	1113	1114	0.16	-	0.30
19	*α*-Campholenal	1117	1125	-	0.65	-
20	Terpinene-1-ol	1118	-	0.15	-	-
21	*α*-Comphoaldehyde	1123	-	0.37	-	0.53
22	Pinocarveol (trans)	1135	1139	0.69	0.92	0.57
23	*Cis* Verbenol	1138	1140	0.11	0.49	-
24	*Trans* Verbenol	1142	1144	-	-	0.27
25	Penthylbenzene	1154	1156	0.14	-	0.27
26	Pinocarvone	1160	1162	0.55	0.52	0.30
27	4-Terpineol	1176	1177	1.04	1.10	1.36
28	Crypton	1179	-	-	0.22	0.26
29	Cymen-8-ol-para	1183	1183	0.30	0.37	0.44
30	*α*-Terpineol	1189	1189	0.18	-	0.29
31	(-)Myrtenal	1194	1193	0.44	0.37	0.24
32	Myrtenol	1200	1200	0.48	0.36	0.25
33	*α*-Phellandrene epoxide	1204	-	-	0.67	0.70
34	*I*-Verbenone	1206	1205	0.21	0.32	0.34
35	*Trans*Carveol	1218	1216	0.30	0.56	0.41
36	Propanal,2-methyl-3-phenyl	1238	-	-	-	0.41
37	*I*-Carvone	1241	1242	0.13	0.24	0.15

38	Carvotanacetone	1245	1247	tr	0.14	0.12
39	Phellandral	1273	1271	0.10	-	0.20
40	Safranal	1280	-	-	0.68	-
41	*p*-Cymen-7-ol	1287	1290	tr	0.18	0.22
42	Thymol	1289	1290	-	-	0.15
43	Carvacrol	1300	1299	0.30	0.61	0.73
44	*α*-Copaene	1370	1376	0.11	0.18	0.22
45	Methyleugenol	1400	1401	0.25	0.61	0.37
46	*β*-Selinene	1481	1485	0.21	0.11	0.22
47	Myristicin	1523	1520	4.21	9.35	7.15
48	*α*-Calacorene	1533	1545	tr	0.11	0.18
49	*β*-Calacorene	1549	-	0.39	-	-
50	Élémicine	1556	1554	0.14	0.33	0.28
51	1,5-epoxysalvial-4 (14) ene	1560	-	0.62	0.60	0.66
52	Spathulenol	1575	1576	4.27	5.23	4.23
53	Salvia-4 (14)-en-1-one	1586	-	0.44	0.76	0.71
54	Caryophyllene oxide	1600	1581	0.57	-	0.24
55	Dillapiol	1612	1622	16.38	37.21	31.95
56	Isospathulenol	1634	-	0.52	0.18	0.22
57	*β*-eudesmol	1645	1649	9.19	7.10	7.70
58	Butylidenephtalide*Z*	1668	1672	7.15	1.23	6.65
59	Butylidenephtalide*E*	1700	1718	1.86	2.72	2.20
60	3-N-Butyl phtalide	1724	1739	4.46	3.50	2.86
61	Butylidenedihydro-phtalide	1777	-	0.33	-	0.14
62	Hexahydrofarnesyl acetone	1848	-	-	0.32	-
63	Acidepalmitique	1947	-	0.21	0.73	tr
Yield				0.53	0.20	0.33
Monoterpene Hydrocarbons				30.16	3.59	13.94
Oxygenated Monoterpenes				6.22	8.59	9.02
Sesquiterpene Hydrocarbons				5.17	10.36	8.14
Oxygenated Sesquiterpenes				32.13	51.41	45.99
Phtalides				13.8	7.45	11.85
Others				0.21	1.05	tr
Total				87.69	82.45	88.94

[a] Compounds listed according to their elution order on apolar HP5MS ™ capillary,[b]Retention Indices on apolar column (HP5MS), [c] Retention indices of literature on apolar column reported by Adams (2007), tr: trace (<0.1%), −: Not detected, all components were identified by comparison of their retention indices with those of published data (Adams, 2007) and mass spectra with literature data, the MS library (NIST and Wiley 7N library)

GC and GC-MS analyses resulted in the identification of sixty-three compounds representing (82.45-88.94%) of the total composition of the essential oils. As shown in (Table 1), a noteworthy qualitative similarity was observed, but with differences in the abundance of major compounds. The oxygenated sesquiterpenes were found to be the

main chemical group in all samples (32.13-51.41%). The second most frequently identified chemical class in the hydrodistillated sample was monoterpene hydrocarbons (30.16%). This fraction was present in much lower amounts in the samples obtained by SD and MAE (13.94 and 3.59%, respectively). The oxygenated monoterpene compounds were present in lower amounts in all samples (<10%). Among the three extractive processes, an important observation revealed that MAE isolated the highest relative amount of oxygenated sesquiterpenes (51.41%), as compared to SD with (45.99%) and HD with (32.13%). This result, which indicates that oils isolated by Microwave-assisted hydrodistillation are characterized by a higher content of oxygenated compounds, was previously reported on essential oils of three different species (Ocimum *basilicum L.*, *Mentha crispa L.* and *Thymus vulgaris L.*) (Lucchesi *et al.*, 2004). The same finding was obtained on *Rosmarinus officinalis L.* essential oils by (Elyemni *et al.,* 2019; Karakayat *et al.,* 2014; Okoh *et al.,* 2010; Moradi *et al.,* 2018).This may be due to an increase in hydrothermal effects in HD, compared to MAE which uses a lower quantity of water, that is rapidly heated [20, 30].Conversely, Lo Presti, M.et al. reported in their study on the same species (*Rosmarinus officinalis L.*) that the essential oils produced by MAE and HD were characterized by very similar chemical profiles (Lo Presti et al., 2005). Alternatively, a different result was previously published, where the amount of the oxygenated compounds was higher in the hydrodistillated essential oil compared with the oil isolated by microwave distillation from dried *Cuminum cyminum L.*, and *Zanthoxylum bungeanum Maxim* (Wang *et al.,* 2006). This contradiction in results is probably due to the fact that the amount of oxygenated compounds in essential oil is not dependent only on extraction methods, but also on many other factors including: species used, plant maturity, plant part used and harvest site (Mohammedi *et al.*, 2015; Mohammedi *et al.*, 2019).

The major compounds identified in the three essential oils were Dillapiol, β-eudesmol, Myristicin, α-Pinene and Butylidene phtalide Z, but with significant differences in their proportions depending on the extraction method used. Dillapiol, the major compound in all samples, was present at (37.21%) and (31.95%), respectively, for MAE and SD, but only at (16.38%) for HD. In the same way, Myristicin, the second compound in MAE oil (9.35%), amounted to (7.15%) and (4.21%) in the SD and HD samples, respectively. The hydrodistillated oil contained the highest amount of α-Pinene (10.84%), which is present in much lower amount in the other oils obtained by SD and MAE (3.28% and 0.48%, respectively). Both samples extracted by HD and SD contained important amounts of Butylidene phtalide Z (7.15% and 6.65%, respectively) and p-cymene (4.89% and 4.02%, respectively).These compounds account for only (1.23-1.57%) in the oil obtained by MAE. Other major compounds were determined with equivalent amounts in the oils extracted by HD, SD and MAE such as β-eudesmol (9.19%, 7.70% and 7.10%, respectively), Spathulenol (4.27%, 4.23% and 5.23%, respectively) and 3-N-Butyl phtalide (4.46%, 2.86% and 3.50%, respectively). To the best of our knowledge, 3-N-Butyl phtalide has never been identified in *P. Scoparius* essential oils.

The multivariate analysis of variance showed a strong significant effect of methods on major essential oil compounds. ($p<0.001$).Extraction method variation indicates a significant effect on: Dillapiol ($p<0.001$); α-Pinene ($p<0.001$); Butylidene phtalide Z ($p<0.001$); β-Ocimene Z ($p<0.001$); β-Pinene ($p<0.001$); α-Thujene ($p<0.001$); Myristicin ($p<0.001$); Limonene ($p<0.001$); p-Cymene ($p<0.001$); 3-N-Butyl phtalide ($p<0.001$); β-eudesmol ($p<0.001$); Spathulenol ($p<0.001$); Δ-3-carene ($p<0.001$); Butylidene phtalide E ($p<0.001$); 4-terpineol ($p=0.002$). The mean comparison between major compounds was assessed to choose the best extraction method for each Substance. Results show a considerable difference between methods for most

compounds, except for 4-terpineol, for which results indicate no difference in SD vs MAE (p=0.074), as well as Spathulenol, for which results indicate no difference in SD vs HD (p=0.887).

Table 2. Major compounds of *P. Scoparius* essential oils from different origins previously reported.

Country / Region		Used part	Main constituents (%)						Ref
			α-pinene	Sabinene	Limonene	Myristicin	Dillapiol	Other major constituents	
Algeria	Oum El Bouaghi	S	34	-	-	-	-	Apiol (15.0)	Vernin *et al.*, 1999
		d	11	-	-	-	-	Apiol (52.8); Bornyl acetate (21%).	
	Ghardaïa	S	6.8	-	9.8	7.2	-	Germacrene D (12.7); *α*-Phellandrene (7.1); Methyl eugenol (5.9).	Vérité and Nacer, 2003
		d	8.2	-	11.2	11.1	-	Caryophyllene oxide (12.2); *p*-Cymene (7.5); Thymol (5.9).	
	M'sila	F	17.4	7.5	-	24.1	-	*α*-Phellandrene (15.6).	Smaili *et al.*, 2011
	Ghardaïa	P	4.4-11.2	-	32.7-66.5	≤ 31.1	≤ 23.0	*α*-Phellandrene (≤ 6.4); Germacrene D (≤ 6.3).	Gourine *et al.*, 2011
	Djelfa	P	23.7-27.0	-	1.0-7.8	≤ 18.2	≤ 47.3	Bornyl acetate (≤ 9.6); *p*-Cymene (2-6.7); *β*-Pinene (≤ 5.3).	
	Laghouat	P	35.1-35.8	-	7.0-30.0	-	9.9-25.7	Bornyl acetate (3.0-9.5); *β*-Pinene (5.2).	
	M'sila	P	16.4	14.8	-	-	-	Caryophyllene oxide (9.7); *α*-Farnesene (7.7); *α*-Terpinene (5.8).	Lograda *et al.*, 2013
	Batna	P	23.3	18.6	-	-	-	*α*-Terpinene (7.7); *β*-Ocimene-E (7.6).	
	Biskra	P	8.3-13.4	18.9-24.8	-	7.6	6.6-16.8	-	

	Tamenra -set	P	12.1	-	-	-	-	Bornyl acetate (32); Epi-Bicycle sesquiphellandrene (8.4); Eremophilene (8.2); γ-Cadinène (6.3).	Hammoudi *et al.*, 2015
	Ghardaïa	P	-	-	-	12.1	-	7-Methoxy-3-methyl-1-H-isichromen-1-one (10.6); Methyl propene (9.9).	Belkacemi *et al.*, 2015
	Souk Ahras	P	16.3-26.5	23.6-34.4	-	-	-	*p*-Cymene (8.6-10.1); Terpinen-4-ol (4.1 - 9.7); *α*-Thujene (5.3-5.7); *β*-Pinene (≤ 5.5).	Chikhoune *et al.*, 2016
	Tamenra s -set	P	-	-	46.9	-	-	1.8-Cineol (7.6).	Ksouri *et al.*, 2017
	Batna	P	≤ 11.2	≤ 10.5	≤ 22.4	≤ 19.4	≤ 16.8	6-Methoxyelemicine (≤ 58.2).	Malti *et al.*, 2018
	Biskra	P	7.2-11.2	19.9-28.0	-	≤ 20.1	≤ 18	Elemicine (≤ 29.1).	
	Bechar	P	-	32.4	19.1	-	-	6-Methoxyelemicine (27.4).	
	Ghardaïa	P	2.8-17.1	≤ 31.3	9.2-26.7	≤ 13.6	-	6-Methoxyelemicine (≤ 29.4); *α*-Phellandrene (≤ 15.4); *β*-Phellandrene (≤ 7.9); *β*-Pinene (≤ 7.1).	
Tunisia		P	32.0	17.2	-	-	-	Δ-3-Carene (16.9); *α*-Thujene (13.7); Ocimene (9.8).	Attia *et al.*, 2011

P: Aerial parts; S: Stems; F: Flowers; d: seeds; n.r: not reported.

Previous research on the chemical composition of *P. Scoparius* essential oils obtained by HD and SD from different origins is summarized in (Table 2). It is noted that no study on the essential oil of *P. Scoparius* as a function of extraction method has been performed to-date. As mentioned above, a significant qualitative and quantitative

difference in the chemical compositions was observed. As shown in (Table 2), most samples, whether from Tunisia or from different Algerian regions (M'sila, Batna, Beskra and Souk Ahras) were characterized by a high amount α-Pinene (8.3-35.8%) and Sabinene (14.8-34.4 %) (Lograda *et al.*, 2013; Chikhoune *et al.*, 2016; Attia *et al.*, 2011). Otherwise, essential oils obtained from seeds and stems essential oils from Oum El Bouaghi were dominated by apiol (52.8% and 15%, respectively) and α-pinene (11% and 34%, respectively). Bornyl acetate was present with large amount only in the seeds oil (21%) (Vernin *et al.*, 1999). Limonene (32.7-66.5%), Myristicin (up to 31.1%), dillapiol (up to 23%) and α-pinene (4.4-11.2%) were the main compounds of the aerial part essential oil from Ghardaia (Gourine *et al.,* 2011), while, Germacrene D (12.7%) was the major compound of the stems oil and Caryophyllene oxide (12.2%) the major compound of the seeds from same region (Ghardaïa). These two samples were also characterized by similar amounts of limonene, myristicin and α-pinene (6.8-9.8% and 8.2-11.2%, respectively for stems and seeds oils) (Vérité and Nacer, 2003). Samples from Djelfa and Laghouat showed similar chemical profiles, dominated by α-pinene (23.7-35.8%), dillapiol (9.9-47.3%), Limonene (up to 30.0%), bornyl acetate (3.0-9.6%) and β-Pinene (up to 5.3%). However, myristicin was present in high amounts only in the oil obtained from Djelfa (up to 18.2%) (Gourine *et al.,* 2011). Myristicin 24.1 % and α-pinene (14.4 %) were also the primary compounds in the flours oil from M'sila, followed by α-Phellandrene (15.6 %) (Smaili *et al.*, 2011). The essential oils of *P. Scoparius*, isolated from Batna, Bechar and Ghardaïa, contained the same dominant compounds: 6-Methoxyelemicine (0.0–59.6%), sabinene (0.8–55.6%), limonene (0.3–44.0%), myristicin (0.0–32.4%) and α-pinene (0.7 –31.0%). Dillapiol was found as a principal compound only in the oil from Batna (up to 16.8%) (Malti *et al.*, 2018). 6-methoxyelemicine was identified neither in our work, nor in other reports. In the same study, the samples coming from Biskra were dominated by sabinene (19.9-28.0%), elemicine (up to 29.1%) and myristicin (up to 20.1%), while dillapiol and α-pinene were also present with appreciable percentages (1.4-18% and 7.2-11.2%, respectively) (Malti *et al.*, 2018). Limonene (46.9%) was the predominant compound in the sample from Tamenrasset, followed by 1.8-Cineol (7.6) (Ksouri *et al.*, 2017). In contrast, α-pinene (12.1%), Epi-Bicyclosesqui phellandrene (8.4%), Eremophilene (8.2%) and γ-Cadinene (6.3%) were the main components of the hydrodistillated oil from the same region (Tamenrasset) (Hammoudi *et al.*, 2015). Furthermore, 7-Methoxy-3-methyl-1-H-isichromen-1-one and methyl propene were identified, along with myristicin, in appreciable amounts only in the oil obtained by SD from Ghardaïa (10.6%, 9.9% and 12.1%, respectively) (Chikhoune *et al*., 2016).

3.2. Acute Toxicity Test

The oral administration of *P. Scoparius* essential oils in doses of 2000 mg/kg (limited dose) did not cause any mortality in treated mice for 14 days following the oral administration. According to Hodge and Sterner scale (Hodge, 1943), *P. Scoparius* essential oil can be classified as non-toxic.

3.3. Anti-inflammatory activity

In the present investigation, the anti-inflammatory activity of Diclofenac and *P. Scoparius* essential oils extracted by HD, MAE and SD was determined based on the carrageenan induced edema test. The results obtained are expressed in triplicate in (Table 3).

Our results showed that all the studied samples exhibited anti-inflammatory activity in a dose-dependent manner. In fact, the concentration had a significant effect on the extraction methods ($p<0.05$) with the exception of MAE ($p = 0.308$). The groups treated with essential oils at a dose of 50 mg / kg showed the lowest inhibition percentages of carrageenan-induced edema (25.31 ± 0.85-40.64 ± 2.36%) compared to the groups treated with doses of 100 mg / kg (27.75 ± 0.53-44.36 ± 2.30 %).

Table 3. Effects of *P. Scoparius* essential oils and Diclofenac on carrageenan-induced hind paw edema in mice

Doses (mg/kg)	Inhibition (%)			
	HD	**MAE**	**SD**	**Diclofenac**
50	$25.31\pm0.85^{c, C}$	$40.64\pm2.36^{a, A}$	$31.37\pm3.38^{b, B}$	$42.69\pm3.04^{a, C}$
100	$27.75\pm0.53^{b, B}$	$44.36\pm2.30^{a, A}$	$33.76\pm4.33^{b, B}$	$63.32\pm1.83^{a, B}$
150	$55.68\pm1.30^{b, A}$	$48.15\pm2.82^{c, A}$	$51.09\pm3.68^{bc, A}$	$69.01\pm1.00^{a, A}$

Values are averages ± standard deviation of triplicate analysis.
Data in the same column having different capital letters are significantly different ($P < 0.05$) among different concentrations.
Data in the same row having different lower-case letters are significantly different ($P < 0.05$) among different essential oil extraction method.
Results are ranked in ascending order; a>b>c; A>B>C.

The maximum activity was observed at 150 mg / kg for all samples (48.15 ± 2.82-55.68 ± 1.30%). The percentage of inhibition of Diclofenac (50, 100 and 150 mg / kg), taken as a standard drug, also increased from (42.69 ± 3.04%) to (69.01 ± 1.00%), with increasing dose. At a dose of 50 mg / kg, HD was less effective than the sample obtained by SD (25.31 ± 0.85 vs 31.37 ± 3.38%). However, using MAE, the essential oil showed the highest inhibition percentage (40.64 ± 2.36%). The last value was comparable to that obtained with Diclofenac (42.69 ± 3.04%) and had no significant difference with the standard drug ($p = 0.743$). The same order was observed using samples at a dose of 100 mg / kg (27.75 ± 0.53, 33.76 ± 4.33% and 44.36 ± 2.30%, respectively for HD, SD and MAE). The results showed that the anti-inflammatory activity of HD vs SD at the dose of 100 mg / kg has no significant difference ($p = 0.667$). The same is true for MEA and Diclofenac ($p = 0.099$). In contrast, at a dose of 150 mg / kg, the maximum activity was obtained in HD (55.68 ± 1.30%) and presented no significantly difference from SD ($p=0.147$).

Based on these results, it can be asserted that *P. Scoparius* essential oils have anti-inflammatory activity, with significant difference depending on the method of extraction used. This important anti-inflammatory activity of *P. Scoparius* essential oils can be attributed to their content of dillapiol, β-eudesmol, myristicin, Butylidene phtalide Z and α-Pinene. These results validate the use of *P. Scoparius* as an anti-inflammatory in traditional Algerian medicine.

3.4. Antibacterial assays

The antimicrobial activity of essential oils was determined against seven bacteria strains and two yeasts using the disk diffusion method. This activity is estimated based on the diameter of inhibition zones. Results are presented in (Table 4) for different extraction methods.

Our data showed that *P. Scoparius* essential oils generally possessed a weak to moderate activity against yeasts (inhibition zone from 7.1±0.1 to 13.6±0.4 mm) as well as gram-positive bacteria (inhibition zone from 8.2±0.1 to 21.8±0.3 mm).

S. lutea was the most affected by *P. Scoparius* essential oils; the oil extracted by HD showed the largest zone of inhibition (inhibition zone = 21.8±0.3 mm) as compared to those obtained by MAE (inhibition zone = 19.8±0.7 mm) and by SD (inhibition zone = 14.7±0.6 mm). Similarly, the tested oils exhibited weak activity against *S. aureus* (inhibition zone: 8.2±0.1-11.0±0.1 mm) and *B. subtilus* (inhibition zone: 9.1±0.6-11.2±0.2 mm), while *S. epidermidis* was more affected by the oils obtained by HD and SD (inhibition zone ≈ 12.7 mm). Dillapiol had no effect against *S. aureus* (Ferreira *et al.,* 2016). In another study;

Dillapiol had no effect against gram-negative bacteria and weak effect against gram-positive bacteria (Eftekhar *et al.,* 2014). This limited effect of *P. Scoparius* essential oils against *S. aureus* could be due to their high content of dillapiol.

These results were previously reported by Ksouri, A. *et al.* on *B. bastilus* (inhibition zone = 8.3±1.1 mm) while *S. aureus* was more resistant to *P. scoparius* essential oil (inhibition zone = 20.0±3.0 mm) (Ksouri *et al*., 2017). Kiram, A. *et al.* who worked on *P. scoparius* from Biskra (Southeast Algeria) reported stronger oil activity against the same bacterial strain, *S. aureus* [Kiram *et al.,* 2013).

Table 4. Antimicrobial activity of *P. Scoparius* of essential oils used three different methods extractions.

Microorganisms	Inhibition zone (mm)					
	Essential oil			Standard antibiotics		
	HD	MAE	SD	Penicillin G	Oxalin	Amoxypen
Gram- positive bacteria						
S. aureus	$8.2\pm0.1^{b,F}$	$8.2\pm0.1^{b,D}$	$11.1\pm0.1^{a,B}$	40	40.6	38
S. epidermidis	$12.4\pm0.3^{a,C}$	$10.4\pm0.2^{a,C}$	$12.1\pm0.6^{a,AB}$	14.6	21.8	17.6
S. lutea	$21.8\pm0.3^{a,A}$	$19.8\pm0.7^{a,A}$	$14.7\pm0.6^{b,A}$	>45	>45	>45
B. subtilus	$11.2\pm0.2^{a,D}$	$9.1\pm0.6^{b,D}$	$9.5\pm0.1^{b,C}$	35	40	34
Gram-negative bacteria						
E. faecalis	-	-	-	12	25	26
E. coli	-	-	-	31	16.9	40
P. aeruginosa	-	-	-	-	17	19
Yeast						
C. albicans	$11.5\pm0.3^{b,D}$	$11.3\pm0.2^{b,B}$	$13.4\pm0.2^{a,A}$	-	-	-
S. cerevisiae	$13.6\pm0.4^{a,C}$	$7.1\pm0.1^{b,E}$	$12.0\pm0.1^{a,A}$	-	-	-

Values are averages ± standard deviation of triplicate analysis, zone of inhibition in mm ± standard deviation beyond well diameter (6 mm), data in the same column having different capital letters are significantly different ($P < 0.05$) among different microorganisms, data in the same row having different lower case letters are significantly different ($P < 0.05$) among different essential oil extraction method, results are ranked in ascending order; a>b; A>B>C>D>E>F, -: not action

Alternatively, *P. scoparius* essential oils showed no activity (inhibition zone = 0 mm) against gram-negative bacteria *E. faecalis*, *E. coli* and *P. aeruginosa*. Unlike gram-positive bacteria, it is known that gram-negative bacteria have a very high resistance to essential oils due to their wall made up of a second external barrier (Okoh *et al*., 2010; Dhouioui *et al*., 2016). The same results were obtained by other authors who reported that *P. Scoparius* essential oils generally showed limited activity against *P. aeruginosa*, *E coli* and *E. faecalis* (Ksouri *et al.,* 2017; Ferreira *et al.,* 2017).

Our findings displayed a strong significant difference against *S. aureus* and *S. cerevisiae* between MEA and SD ($p<0.001$), and no significant difference between HD and MEA against strains ($p>0.05$), except for *B. subtilus* and *S. cerevisiae*. The comparison of HD vs SD showed that the extraction method influenced the antimicrobial activity except for *S. epidermidis* and *S. cerevisiae* ($p <0.05$).This could be due to the high content of hydrocarbon monoterpene since the antimicrobial activity of the essential oil could be linked to the presence of α-pinene (Bourkhiss *et al.,* 2007; Abi-ayada *et al.,* 2011; Amarti *et al*., 2011)); although minor molecules can also produce antibacterial activity, contributing to the activity of essential oils (Okoh *et al.,* 2010).

4. Conclusions

The effects of extraction methods on *P. Scoparius* essential oil compositions and their anti-inflammatory and antimicrobial activities are investigated here for the first time. The chemical compositions of the essential oils obtained are qualitatively similar, but with significant differences in the abundance of major compounds. *P. Scoparius* essential oils are non-toxic and exhibit important anti-inflammatory activity and weak antibacterial activity. The extraction method significantly affected the anti-inflammatory and antibacterial activities. It is very important to continue this research and find a correlation between the extraction techniques and their effects on the chemical composition and biological activities.

5. References

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EFFECT OF DRYING ON PHYSICAL AND FLOW PROPERTIES OF BANANA POWDER

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ABSTRACT

Due to a lack of knowledge on the physical characteristics and flowability of the powders, it makes it difficult to handle or design the process, equipment, and handling machinery for powders. The research was inducted to investigate the flow characteristics and flowability of the unripe (green) banana powders, which were prepared by using two different drying methods, viz. sun drying, and hot air drying. A comparative analysis of physical characteristics and flow properties by using the Brookfield Powder Flow Tester (PFT) was done between the hot air-dried and sun-dried powder at an equal moisture content of 6.61% (wet basis), and these results showed a trend that sun-dried powder has a better flow characteristic than the hot air-dried unripe banana powder, which was found to more cohesive at equal moisture contents. Hopper design showed that hot air-dried powder of unripe banana requires higher hopper outlet dimension and hopper half angle than sun-dried powder for mass flow through the conical-shaped hopper. The knowledge of the physical and flow properties of unripe banana powder would be useful in designing hoppers for the equipment in food industries.

1. Introduction

The banana (*Musa acuminate*) is a highly nutritious fruit that is produced and consumed all over the world. The ripen banana and unripe mature banana are rich in carbohydrate and fiber content. Besides these, it also contains phenolic acid, resistant starch, minerals, and vitamins. The unripe banana is readily available at a low cost hence can be processed into various products, which can be preserved for an extended period as ripening banana is very perishable. Thus unripe banana acts as a good potential for industrialization as a highly qualified food product and food diet (Yani, Arief, & Mulyanti, 2013). The processing of unripe bananas involves the preparation of banana powder, peel powder, and fried chips (Singh, Kaushik, & Gosewade, 2018).

The unripe banana powder (flour) is prepared using different drying methods such as hot air drying, sun drying, freeze-drying, and vacuum drying. On an industrial scale, sun drying and hot air drying techniques are used on a large scale. The different drying techniques used to form powder show different flow properties. The drying process removes water from food material, which may induce some changes in color, texture, density, porosity, sorption characteristics, and quality of dehydrated products (Mirhosseini & Amid, 2013). The shelf life of green banana powder is longer than the fruit as it has lower water activity. The green banana powder is characterized by a good starch source, dietary fiber, resistant starch, and antioxidant compounds. Therefore, the green banana powder is widely used in the baking and confectionery industries for the preparation of cakes, puris, parathas, toffees, banana snacks, and crackers (Morin & Briens, 2013). The

unripe banana powder is available in the market. It can be used as an alternative for wheat or gluten in various food products such as weaning food, diet supplements, nutritional supplements, etc. It can be utilized in the preparation of vermicelli combination with a wheat powder. Increasing demands for green banana powder leads to a study of flow behavior properties of the powder for industrial purposes such as blending powders for new formulations, transfer of powders from silos, drums, hoppers, etc., feeding to packaging machines, compaction, and fluidization. The flowability, which indicates the ability of the powder to flow, is considered as one of the fundamental properties of the bulk solid. Flowability includes the Hausner ratio, Carr's index, compressibility, internal friction, wall friction, etc., properties altogether (Jenike, 1964; Kamath, Puri, & Manbeck, 1994).

The food manufacturing and processing units face several problems while handling, storing, and packaging the powders. The standard flow function will help us to determine the flowability or flow behavior of powder products that are handled on an immense scale in the manufacturing or processing industries. These flow properties can be correlated to their various physical properties, viz. internal strength, gravity flow rate, and bulk density. The flowability is affected by physical properties which are used for designing equipment for handling (belt conveyor, screw conveyor, pneumatic conveyor, gravity conveyor, etc.), storing (bin, silo, hopper, bunkers, sacks, etc.), and processing (mixing, bagging, drying, milling, etc.) (Berry, Bradley, & McGregor, 2015; Slettengren, Xanthakis, Ahrné, & Windhab, 2016).

The paper covers a complete understanding of the powder flow behavior of green banana powder prepared by using two different drying methods, namely solar drying and hot air drying, with consideration of hopper design.

2. Materials and methods

2.1. Preparation of unripe mature banana powder

The mature green bananas of the Robusta variety were purchased from the local market of Rourkela, India. The bananas were washed by using potable water to clean them from dust and dirt. The bananas were peeled manually and were dipped into distilled water to avoid browning. The bananas were sliced into a thickness of 1 cm manually by using the slicer. The slices of bananas were treated for 10 minutes by using a 0.5 % (W/V) citric acid solution (Singh, Suvartan, & Sukriti, 2017). After treatment, the slices were drained from the solution and were distributed into two equal lots for drying.

Table 1. Nomenclature

Nomenclature	
SEM	Scanning Electron Microscope
d_{50}	Particle size below which 50% of material volume exists – median, μm
CI	Compressibility Index, %
HR	Hausner Ratio
FF	Flow function curve
σ_1	Major Principle Consolidation Stress (MPC), KPa
σ_c	Unconfined Failure Strength (UFS), Kpa
ε	Consolidation stress, Kpa
C	Cohesion, Kpa
δ_c	Critical angle of internal friction, º
δ_e	Effective angle of internal friction, º
ϕ_w	Effective angle of wall friction, º
ρ_{lb}	Loose poured bulk density, Kg/m^3
ρ_p	Particle density, Kg/m^3
ρ_c	Critical bulk density, Kg/m^3
ρ_t	Tapped bulk density, Kg/m^3
D	Hopper outlet dimension, m
θ	Hopper half-angle, º
L*, a*, and b*	Color values
R^2	Coefficient of determination

The first lot was sundried for 12 hours at the atmospheric condition. The relative humidity varied from 31 to 35%, and temperature approximately changed from 32 to 40ºC in May. The second lot was subjected to hot air drying at 50º C for 24 hours in Hot Air Oven Tray Dryer

(Khera Instruments Pvt. Ltd., India). The hot air was circulated at a speed of 0.4 m/sec over unripe banana slices (Sonawane et al., 2021).

After the completion of drying, each lot was brought to room temperature. Each lot was ground for 2 minutes at the speed of 12000 rpm by using the mixer grinder (Philips, India). After grinding, the powder was sieved by using the ISS 20 sieve to get a uniform size fine powder. Both the powders were kept in a desiccator at 27±2ºC for 24 hours to equalize the moisture content of powders by moisture migration. The moisture content of unripe banana powder was measured as per the standard method (AOAC, 2002). Then fine powder was packaged into high-density polyethylene (HDPE) bags, stored at 25±2ºC temperature for further experiments (Bezerra, Rodrigues, Amante, & Silva, 2013). Thus each powder is ready for measuring its flow properties.

2.2. Physical properties of unripe banana powder

Malvern Mastersizer 3000 (Malvern Instruments, United Kingdom) was used for the particle size determination size of powder particles. The particle size analyzer gives the value of d50, which represents the average particle size of the powder. Scanning Electron Microscope (SEM) (Oxford Instruments, United Kingdom) was also used to analyze particles' shape.

The mass of powder divided by the total volume of powder is called the bulk density of the powder (ρ_b). To measure loose poured bulk density (ρ_{lb}), the powder is allowed to settle freely by gravity without any load (Jaggi, Leaper, & Ingham, 2016). Both bulk density and loose poured density were measured manually by simply pouring the known powder mass in a cylinder and then cross-checked by the readings obtained in the standard flow function test. The following formulae were used for the determination of porosity, Hausner ratio, and Carr's index (Ji, Fitzpatrick, Cronin, Fenelon, & Miao, 2017; Li, Roos, & Miao, 2016a, 2016b):

$$\text{Porosity}=1-(\rho_{lb}/\rho_p) \quad (1)$$

$$\text{Compressibility Ratio}=(\rho_t/\rho_{lb}) \quad (2)$$

$$\text{Carr's Index} = 100\,(1-\rho_{lb}/\rho_t) \quad (3)$$

The particle density of powder was measured by powder density meter (Smart Instruments Company Pvt. Ltd., India), which works on the principle of pycnometer by introducing the helium gas in the sample.

The color values of two powder samples were measured directly using the colorimeter (Hunter Lab Colorimeter, USA). The values of L^*, a^*, and b^* were calculated for each sample by changing the position of the sample through an angle over the instrument (Pathak, Pradhan, & Mishra, 2019; Sonawane, Pathak, & Pradhan, 2020a; Sonawane, Pathak, & Pradhan, 2020b).

2.3. Standard flow function and wall friction test

To measure the standard flow function and wall friction test of the banana powder, the Powder Flow Tester (PFT) (Brookfield, United Kingdom) was used with torsional and axial speeds of 1.0 rev h-1 and 1.0 mm s-1, respectively. PFT works on Jenike's methodology, which involves the application of consolidation stress with given axial and torsional speed through the annular lid to the powder present in the shear cell of annular shape to induce shear in the powder at given consolidation stress (Garg, Mallick, Garcia-Trinanes, & Berry, 2018). The banana powder was loaded into the sample holder, and the test was run using the powder flow software. The flow properties were determined by using a standard vane lid. The measured results and graphs were recorded separately for each of the powders, which were dried by using different methods. The standard flow test consists of 35 runs of consolidation stresses for each sample and takes around 35 minutes to complete the test. It gives the values of normal stresses, bulk density of powder corresponding to an angle of internal friction, unconfined failure strength (UFS), major principal consolidation stress (MPC), critical arching values for hopper design, flow function curve (FF), and cohesion (C). It is used for equipment design and quality

control (Fitzpatrick, Barry, Delaney, & Keogh, 2005).

The standard vane lid was replaced by the wall friction lid for standard wall friction test. The test was carried out by using a new sample of powder for a test of wall friction. The measured results and graphs were recorded separately for both the powder samples, which were dried using different methods. This test takes around 15 minutes and gives values for the effective angle of wall friction (ϕ_w), Hausner Ratio (HR) (compressibility ratio), Carr's Index (compressibility index) (CI), and hopper half-angle (θ) (Crowley, Gazi, Kelly, Huppertz, & O'Mahony, 2014).

2.4. Hopper design

There are two gravity powder flow patterns, namely core flow and mass flow, in the processing industry (Figure 1). Rat-holing and arching are the primary obstructions in designing the hopper for core and mass flow, respectively. For poor flowing powders, the conical hopper with mass flow patterns is generally used on a large scale. To achieve easy discharge of powder, the wall of the conical hopper must be adequately smooth and steep (AMETEK Brookfield Engineering, 2019).

2.5. Statistical analysis

All physical properties of hot air and sun-dried unripe banana powder were determined in triplicates and converted to average values with their standard deviations. The analysis of variance (ANOVA) was performed by using SPSS 20.0 software with Duncan test at the significance level of 5% and presented in the tables.

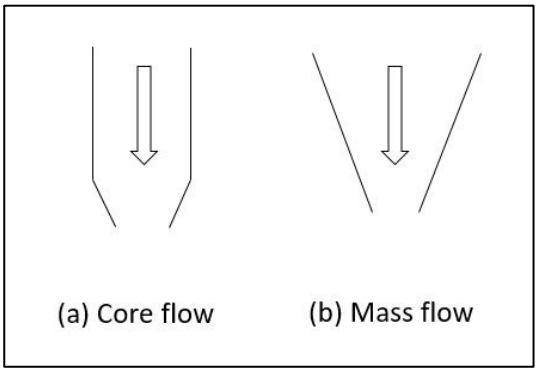

Figure 1. Core and mass flow of powders

3. Results and discussions

3.1. Physical properties of powders

The moisture contents of the hot air and sun-dried unripe banana powder were 6.61% (wet basis). As per the results of the SEM, the geometry of the hot air-dried powder particles was found to be more spheroid and ellipsoid than sun-dried powders (Figure 2). The structural changes in the matrix of slices occurred during its drying processes, and a larger size of particles was formed in sun-dried unripe banana powder during the grinding of slices. The hot, air-dried, unripe banana powder has given a high value of L^* (lightness). While it showed lower values of a^* (red to green) and b^* (yellow to blue) than sun-dried powder. The comparison between color values is shown in Figure 3. The values of d_{50} for hot air and sun-dried powders were 11.5 μm and 17.9 μm, respectively, and it indicates the average particle size of powder (Table 2).

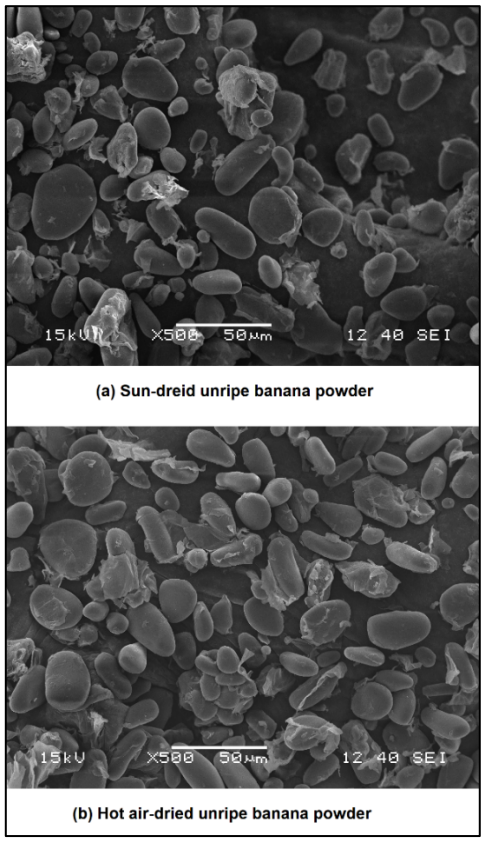

Figure 2. SEM analysis of sun-dried and hot air-dried unripe banana powders

The powder with lower bulk density has higher structural strength and internal friction between particles, which opposes the collapsing of powder when it is poured into a container. On the other hand, the powder with high bulk density has weaker structural strength, allowing collapsing (Abdullah & Geldart, 1999). The particle densities of hot air dried and sun-dried unripe banana powders were 600.2 kg/m3 and 559.5 kg/m3 with a porosity of 0.426 and 0.552, respectively, as shown in Table 2. The bulk density as well as tapped density of hot air-dried powder was higher than sun-dried powder. Thus the hot air-dried unripe banana powder has shown higher structural strength and internal friction.

The average particle size of the hot air-dried powder is lower than the sun-dried powder (Table 2). The cohesiveness of hot air-dried powder has been increased by the van der Waal forces of attraction due to the presence of fine particles in it (Jackson, Sinka, & Cocks, 2007). This causes more resistance to the flow of particles for hot air-dried powder. The Hausner ratio (HR) and compressibility index (CI) of hot air-dried powder were higher as compared to sun-dried powder (Table 2). Table 3 shows the flowability of powders concerning the Hausner ratio and compressibility index values. From Table 3, the hot air and sun-dried powders have shown very poor flowability and poor flowability, respectively.

During the hot air drying process, the glycosidic bonds of starch (polymeric carbohydrates) were broken in large amounts as it is a continuous forced drying technique. In addition to that, there was more retrogradation effect (syneresis) in the starch structure due to the release of water in the hot air drying process. On the other hand, there may be more amount of amylose present in sun-dried unripe banana powder may increase the hardness of starch due to less retrogradation effect and consequently larger particle size of sun-dried unripe banana powder. These reasons have shown less flowability of hot air-dried unripe banana powder than sun-dried unripe banana powder (Mirhosseini & Amid, 2013).

Table 2. Physical properties of the hot air and sun-dried unripe banana powder.

Powder	Hot air dried	Sun-dried
d_{50} (µm)	11.5 ± 1.05	17.9 ± 1.17
ρ_{lb} (kg/m^3)	600.24 ± 15.75	559.59 ± 13.44
ρ_p (kg/m^3)	1248.90 ± 26.17	1024.63 ± 24.31
ρ_t (kg/m^3)	903.27 ± 22.29	776.73 ± 20.44
Porosity	0.53 ± 0.02	0.45 ± 0.02
HR	1.51 ± 0.11	1.36 ± 0.09
CI (%)	0.33 ± 0.01	0.27 ± 0.01
Color values		
L	81.82 ± 2.44	86.40 ± 3.28
a	2.18 ± 0.08	2.16 ± 0.07
b	11.58 ± 0.55	10.74 ± 0.51

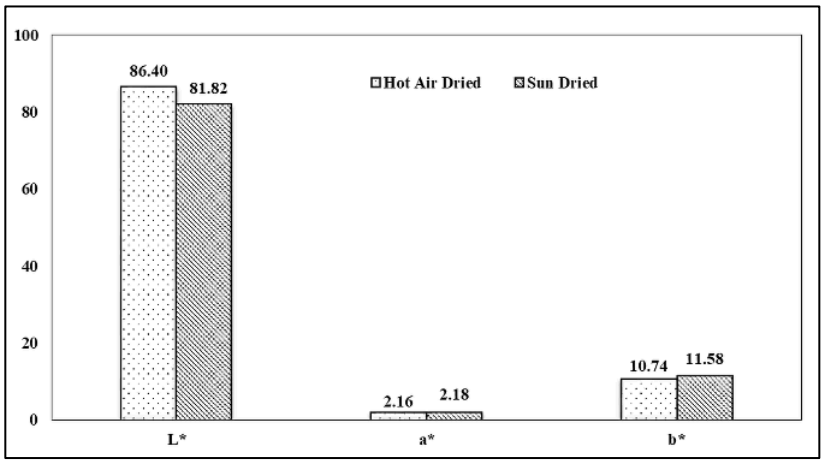

Figure 3. Color values of hot air and sun-dried unripe banana powder.

Table 3. Specification of Carr's Index and Hausner Ratio for flowability.

Flow character	Carr's index (%)	Hausner ratio
Excellent	≤ 10	1.00-1.11
Good	11-15	1.12-1.18
Fair	16-20	1.19-1.25
Possible / Passable	21-25	1.26-1.34
Poor	26-31	1.35-1.45
Very poor	32-37	1.46-1.59
Very, very poor	> 38	> 1.60

Source: Singh & Kumar (2012).

3.2. Yield locus and flow function curve

The five yield loci have given the values of pre-shear stress, unconfined failure strength (UFS), and major principle consolidation stress (MPC). The shear stress value of yield locus was determined by Mohr-Coulomb failure criteria (Jager, Bramante, & Luner, 2015; Crowley et al., 2014). The pre-shear stress values for hot air-dried powder were 0.31, 0.61, 1.21, 2.41, and 4.85 KPa, whereas the pre-shear stresses values for sun-dried powder were 0.31, 0.61, 1.20, 2.41, and 4.85 KPa, as shown in Table 4. The Mohr-Coulomb failure criteria were produced by Brookfield PFT in standard flow test when an instrument was operating in operational stress range.

The plot between major principle consolidation stress (σ_1) and unconfined failure strength (σ_c) is called as flow function curve. It is divided into four regions, namely non-flowing, easy flowing, free-flowing, cohesive, and very cohesive according to Jenike's flowability classification as shown in Figure 2 for the hot air and sun-dried powder (Mistry, 2002). The flowability of powder is given by the flow function curve, and it depends on applied stress (Crowley et al., 2014). The flowability behavior for the hot air and sun-dried powder has been given by the flow function curve from very cohesive to cohesive and cohesive to easy flowing, but sun-dried powder has shown better flowability than hot air-dried unripe banana powder for corresponding major principle consolidation stress (Figure 4). The flow function curve is helpful in hopper design at different fill heights.

3.3. Effective angle of internal friction, angle of wall friction, and bulk density

The variation of bulk density (ρb) for the hot air and sun-dried powder has been shown in Figure 5 concerning major principle consolidation stress. The hot air, as well as sun-dried powder, showed an increase in bulk density as major principle consolidation stress (MPC) increases. The bulk density of the hot air-dried powder was higher than the sun-dried powder for all values of MPC. Thus the hot air-dried powder showed more compactness due to fine particles and more cohesion between particles.

The effective angle of internal friction (δ_e) represents the friction in between the layers of the powder, and it is given by major principle consolidation stress divided by minor principle consolidation stress during steady-state flow. Figure 6 shows the effect of major principle consolidation stress on the effective angle of internal friction. Both cohesive forces, as well as frictional force, contributes to the effective angle of internal friction. For hot air and sun-dried unripe banana powder, the effective internal friction angle was more significant at lower major principle consolidation stress. It decreased with an increase in the consolidation stress.

The hot air-dried powder has given a higher effective angle of internal friction than sun-dried powder. Due to smaller particle size, there would be more particle-particle interaction, and interlocking which leads to more van der Waal effect, which represents cohesive force developed between particles, and this increases the effective angle internal friction for hot air-dried unripe banana powder (Crowley et al., 2014; Mistry, 2002)

Table 4. Flow property test data for the hot air and sun-dried unripe banana powder.

Consolidation endpoint (Yield locus)	Major principal consolidating stress	Unconfined failure strength	Cohesion	Effective angle of internal friction
(KPa)	(KPa)	(KPa)	(KPa)	(°)
Hot air-dried powder				
0.31	0.52 ± 0.02^{a}	0.36 ± 0.01^{a}	0.11 ± 0.01^{a}	49.90 ± 2.57^{a}
0.61	1.01 ± 0.07^{b}	0.54 ± 0.02^{b}	0.16 ± 0.01^{b}	41.10 ± 1.81^{b}
s1.21	1.99 ± 0.12^{c}	0.79 ± 0.04^{c}	0.23 ± 0.01^{c}	37.30 ± 1.37^{c}
2.41	3.94 ± 0.19^{d}	1.11 ± 0.05^{d}	0.34 ± 0.01^{d}	34.50 ± 1.21^{d}
4.85	7.80 ± 0.25^{e}	1.63 ± 0.07^{e}	0.53 ± 0.02^{e}	32.70 ± 1.14^{e}
Sun-dried powder				
0.31	0.55 ± 0.01^{a}	0.37 ± 0.01^{a}	0.11 ± 0.01^{a}	45.40 ± 2.11^{a}
0.61	1.07 ± 0.04^{b}	0.52 ± 0.02^{b}	0.15 ± 0.01^{b}	38.90 ± 2.02^{b}
1.20	2.11 ± 0.09^{c}	0.77 ± 0.02^{c}	0.22 ± 0.01^{c}	$35.00 \pm 2..14^{c}$
2.41	4.15 ± 0.15^{d}	1.08 ± 0.05^{d}	0.33 ± 0.02^{d}	32.04 ± 2.28^{d}
4.85	8.20 ± 0.29^{e}	1.66 ± 0.05^{e}	0.51 ± 0.02^{e}	31.10 ± 1.51^{d}

[a-e] Means within the column with different superscripts are significantly different ($p < 0.05$).

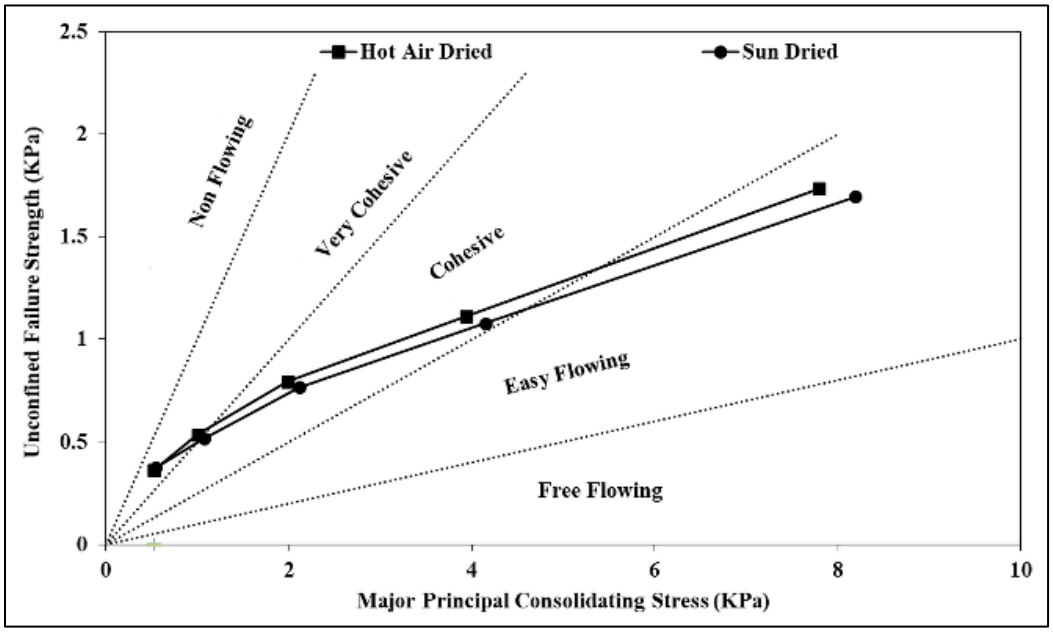

Figure 4. Flow function curve of the hot air and sun-dried unripe banana powder.

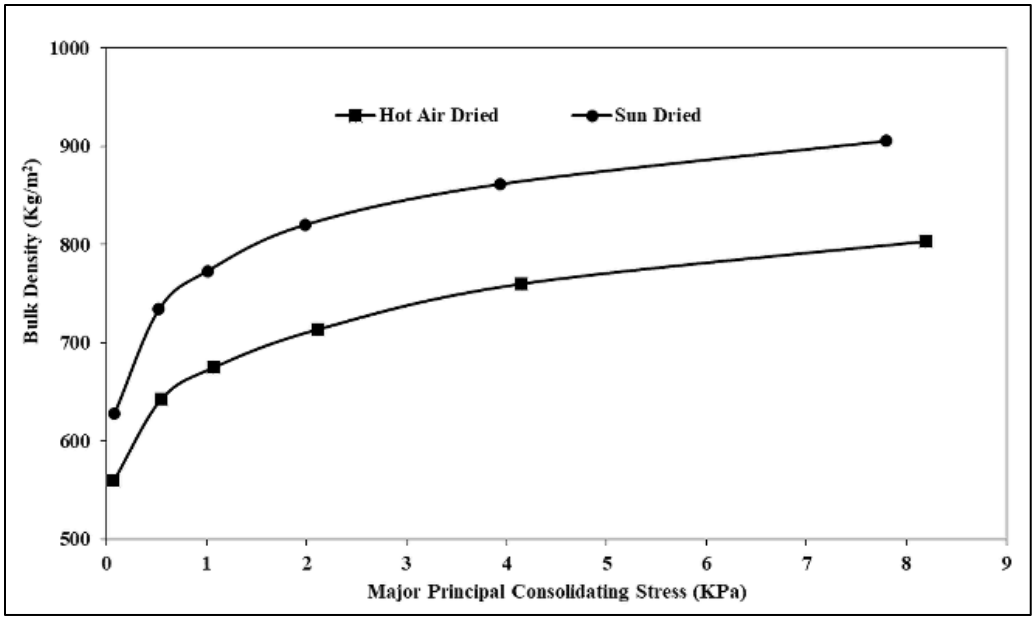

Figure 5. Bulk density as a function of major principle consolidation stress (MPC) for the hot air and sun-dried unripe banana powder.

The wall friction angle (ϕ_w) represents friction between the powder particles and the wall surface at normal stresses, and it is given by the equation (4).

$$\phi_w=\arctan(\mu) \quad (4)$$

Where μ is the coefficient of wall friction, determined by the slope of a straight line at the normal stress passing across the origin to the maximum yield locus, Figure 7 shows that an increase in normal stress also causes an increment in the wall friction angle. Still, the rate of increase in wall friction angle decreases gradually as normal stress increases. The hot air-dried unripe banana powder has shown a higher wall friction angle than sun-dried. The reasons behind the high wall friction angle for hot air-dried powder are smaller particle size and more adhesion between wall and particle of powder.

3.4. Design of hopper for mass flow

The design of a conical shape hopper includes a hopper outlet dimension (D) and hopper half-angle (θ) for mass flow. The Hopper dimension is the outer diameter of the conical hopper through which the flow of solid particles occurs, and the hopper half-angle is an angle made by the hopper wall to the vertical axis. The critical arching represents the design parameters at which powder can't collapse under its self-weight. For the steady flow state of powder, the design parameters of the hopper should be greater than critical arching values at corresponding stresses. Jenike, A.W. (1964) has done foundation work for deriving the equation for conical hopper. The standard flow function gives the data required for hopper design. The equations for hopper dimension (critical outlet diameter) and hopper half angle are given by equations (5) and (6), respectively (Garg et al., 2018).

$$D=(2\times1000\times\sigma_c)/(g\times\rho_c) \quad (5)$$

$$\theta=[90-0.5\arccos((1-\sin\delta_c)/(2\sin\delta_c))]-0.5[\phi_w+\arcsin(\sin\phi_w/\sin\delta_c)] \quad (6)$$

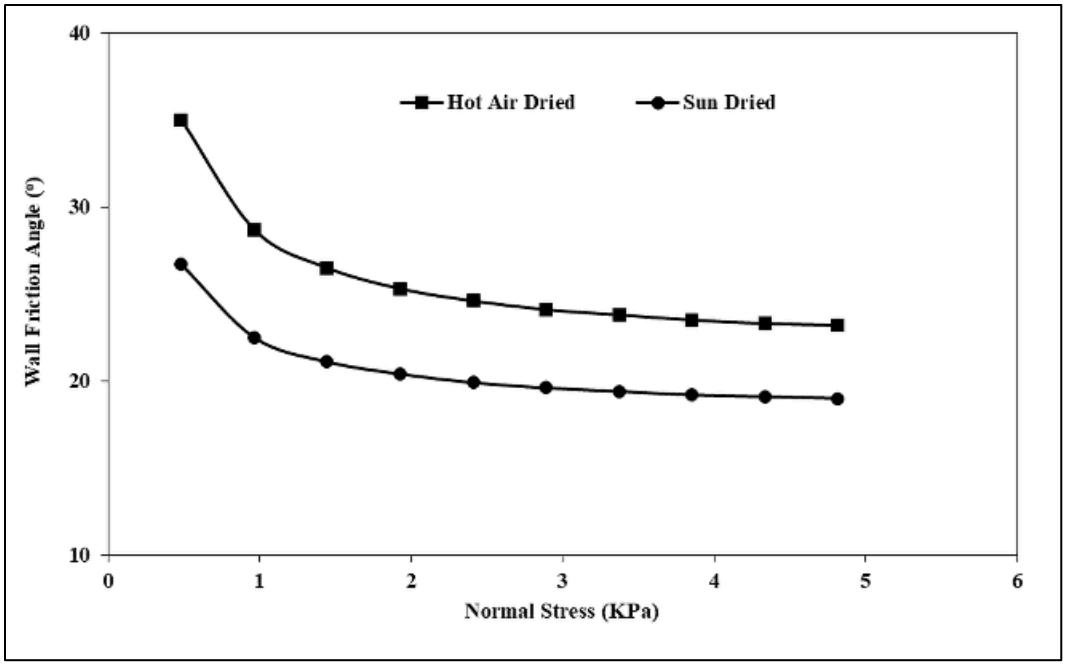

Figure 7. Effective angle of wall friction against normal stress for the hot air and sun-dried unripe banana powder.

Where g is the acceleration due to gravity (m/s2), σ_c is critical consolidation stress, and ρ_c is critical bulk density corresponding to critical consolidation stress. The wall friction angle plays a dominant role in the calculation of the hopper half-angle.

The values of σ_c and ρ_c were taken from the data of bulk density and flow function (FF) graph. The value of ϕ_w was taken from the data wall friction test (Figure 4) at a corresponding value of critical consolidation stress in the standard flow test. The critical arching value of hopper outlet diameter for hot air-dried was 0.116 m at 0.362 KPa; similarly, for sun-dried, it was 0.086 m at 0.300 KPa. Figure 8 shows the graph of hopper outlet versus hopper half-angle. The outlet dimension and hopper half-angle for hot air-dried powder were higher than sun-dried unripe banana powder at corresponding consolidation. This is because of smaller particle size and higher cohesion between particles of hot air-dried unripe banana powder (Table 4).

The linear relation between hopper half-angle and outlet dimension for hot air and sun-dried unripe banana powder is shown in equations (8) and (9).

$$\theta = 36.97D + 15.53 \quad (7)$$

$$\theta = 58.59D + 2.62 \quad (8)$$

Both regression equations have given R^2 values of 0.97, which is acceptable for designing dimensions of the hopper.

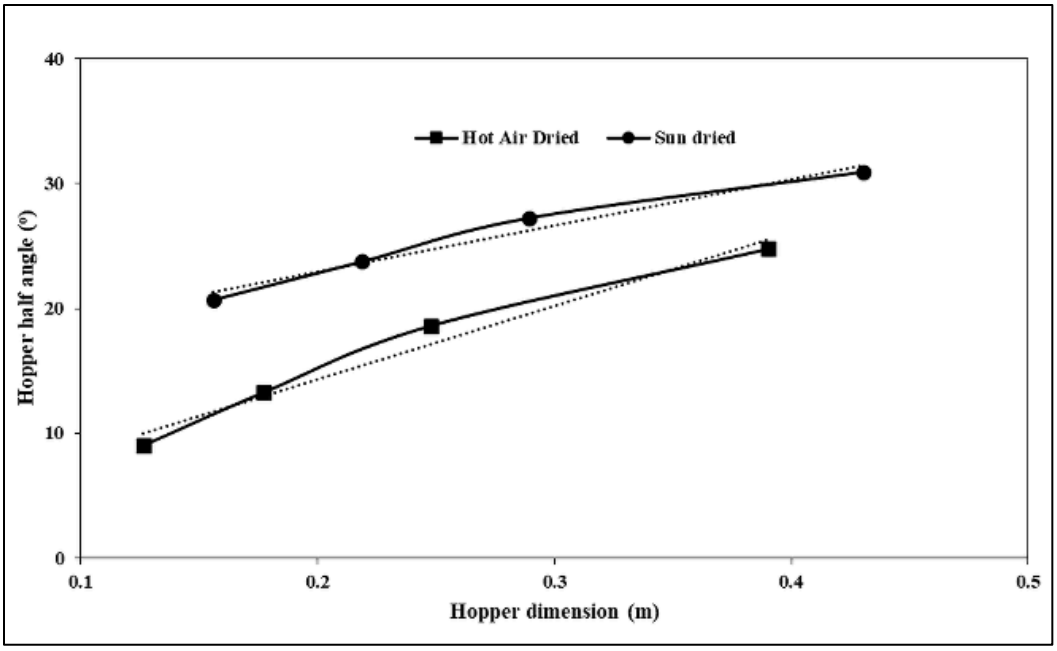

Figure 8. Hopper outlet dimension versus hopper half-angle for the hot air and sun-dried unripe banana powder.

4. Conclusions

The physical characteristics and flowability of unripe banana powders prepared by hot air tray drying and sun drying method were studied at an equal moisture content of 6.61% (wet basis). By comparing the physical characteristics, it was concluded that the hot air-dried powder had a smaller particle size and higher bulk density than sun-dried powder; conversely, the sun-dried powder had a smaller tapped density, porosity, and particle density than hot air-dried powder. From the values of Hausner ratio, compressibility index, and flow function curve, the hot air-dried unripe banana powder was found to be more cohesive and less flowable than sun-dried powder due to structural changes in starch during the drying process. The hot air-dried unripe banana powder required a higher hopper outlet dimension and hopper half angle due to its low flowability than sun-dried powder to achieve desirable mass flow. The drying method for unripe banana powder has shown a more significant effect on the flowability of powders. The results obtained from the inducted research will form an essential database for the manufacturers, processors, processes, and equipment designers for the various unit operations such as handling, storing, and packaging of unripe banana powder.

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INFLUENCE OF PU-ERH TEA EXTRACT ON PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF GERMINATED BROWN RICE

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ABSTRACT

Brown rice is prepared by removing the hull of the rice kernel. Despite the high nutritional value of normal brown rice, it is not widely accepted as it cannot be cooked easily in a conventional rice cooker. However, germinated brown rice (GBR) is easy to cook and the texture is also softer than that of the normal brown rice. The objective of this study was to investigate the physicochemical and antioxidant potential of GBR produced by soaking in different concentrations (0.5–3%, w/v) of Pu-erh tea extracts. The lightness value of the extract-treated GBR was reduced, however, the redness and yellowness values were significantly increased with the concentration of the tea extracts. The GBR samples grown with the extract treatment were enriched with some amino acids such as *γ*-aminobutyric acid although the amount of total free amino acid was reduced in the treated samples. Similarly, the amount of total minerals and DPPH free radical scavenging potential of the extract-treated samples were higher than that of the untreated one. The total polyphenol and/or flavonoid levels of some of the tea-treated GBR samples were also improved. The results indicated that the nutritional and functional properties of brown rice could be enhanced by Pu-erh tea extract treatments.

1. Introduction

Rice supplies daily calories for about half of the world's population. Rice literally means white rice, also known as polished rice. Brown rice is prepared by removing only the outermost layer, the hull, of the rice kernel, and is the least damaging to its nutritional value. However, white rice is produced by further milling and removing the bran and most of the germ layer from brown rice. Brown rice is healthier than polished rice (Dinesh Babu *et al*., 2009). Brown rice has high dietary fiber, rich in vitamin B complex and minerals, and high in fat. Also, it has been reported that brown rice contains high phytic acid, antioxidant, and anti-cancer; it decreases serum cholesterol; and it is considered a low glycemic index food.

Although normal brown rice has high nutritional value, its popularity is low because it cannot be cooked in a conventional rice cooker, however, germinated brown rice (GBR) is easily cooked and the texture is softer than that of brown rice (Komatsuzaki *et al*., 2007; Patil and Khan, 2011). Therefore, GBR could become a popular healthy food. Several studies on GBR indicate that during the process of germination, nutrients in the brown rice change drastically. Not only the contents of existing nutrients are modified but new components are also released due to germination (Spanier *et al*., 2001). GBR

is more beneficial than normal brown rice and white rice, particularly in the prevention of some diet-related diseases, including obesity, type 2 diabetes, and colorectal cancers (Imam *et al*., 2014). An intake of GBR instead of white rice is good for the control of postprandial blood glucose concentration without increasing insulin secretion in subjects with hyperglycemia (Ito *et al*., 2005). Intake of GBR is suggested to protect cell proliferation and apoptosis as well as to prevent heart failure owing to myocardial ischemia (Petchdee *et al*., 2020).

The nutrients like *γ*-aminobutyric acid (GABA), lysine, vitamin E, dietary fiber, niacin, magnesium, vitamin B1, and vitamin B6 are significantly increased in GBR (Spanier *et al*., 2001). They found that regular intake of GBR is beneficial for preventing headaches, relieving constipation, preventing colon cancer, regulating blood sugar level and preventing heart disease. Intake of GABA suppresses blood pressure and improves sleeplessness and autonomic disorder observed during the menopausal or presenile period (Okada *et al*., 2000).

Reports show that different pretreatments and/or cultivation techniques have been employed to enhance the quality of GBR. Apoptotic pathway is found to be activated in the *Lactobacillus acidophilus*-fermented GBR that may prevent preneoplastic lesions of the colon (Li *et al*., 2019). Gamma oryzanols contents are increased in the cold plasma-treated GBR compared to the untreated GBR (Yodpitak *et al*., 2019). Gamma oryzanols is reported to increase the muscle strength (Eslami *et al*., 2014). The GABA content is substantially increased following the cellulase solution treatment to GBR (Zhang *et al*., 2019). Germination of brown rice in red onion solution increases the antioxidant capacity and GABA content as well as makes the rice slightly softer and stickier than that germinated in water (Nakamura *et al*., 2020).

Pu-erh tea is receiving increased attention due to its health benefits for a variety of hypolipidemic, antiobesity, antimutagenic, antioxidative, antitumor, free radical scavenging, and toxicity suppressing activities (Lee and Foo, 2013). Extracts obtained from plant sources have been found to increase the quality of soybean sprouts (Chaikina *et al*., 2009; Kim *et al*., 2017). So far, no reports on the effect of phytochemical-rich Pu-erh tea on GBR have been published. Considering the health benefits of Pu-erh tea and GBR, this study was conducted to investigate the effect of Pu-erh tea on the nutritional and functional values of GBR. This study will provide an insight into the effects of functional food material, such as Pu-erh tea on the quality of GBR.

2.Materials and methods

2.1. Chemicals and materials

Folin-Ciocalteu phenol reagent, quercetin, gallic acid, and 1,1-diphenly-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Sigma-Aldrich Corp, St. Louis, MO, USA) and amino acid standards were obtained from Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). All the chemicals used in this study were of analytical grade.

Brown rice (*Oryza sativa* L.) obtained from a Korean rice cultivar Ilpum Byeo was used in this study. A typical wild Pu-erh tea, produced in the Yunnan province of China, was considered to prepare tea extracts.

2.2. Production of germinated brown rice and sample powder

One kilogram of brown rice was washed with tap water and soaked in tap water alone or three different concentrations (0.5, 1.5, and 3%; w/v) of Pu-erh tea extracts prepared in tap water for 1 h. The sample soaked in tap water alone, 0.5, 1.5, and 3% Pu-erh tea extracts was named GBR-0, GBR-0.5, GBR-1.5, and GBR-3, respectively. After soaking for 1 h, the brown rice samples were put into an incubator (35°C) for 36 h to allow germination. The rice samples were kept in net bags and moistened every 1 h by brief dipping in the respective solutions used for the pre-soaking.

The freshly harvested GBR samples were kept at –70 °C for 24 h before freeze-drying. The freeze-dried samples were ground into powder

using a commercial grinder (HIL-G-501, Hanil Co., Seoul, Korea) and filtered through a 100-mesh sieve.

2.3. Color measurement

The color values of powdered samples were determined following the Hunter's color measurement system as described earlier (Kim *et al*., 2014). The 'L' (lightness), 'a' (redness), and 'b' (yellowness) values were measured using a Chroma Meter (CR-300, Minolta Corp, Tokyo, Japan). A calibration plate (Minolta Corp.; YCIE = 94.5, XCIE = 0.3160, YCIE = 0.330) and a standard plate (Hunter Associates Laboratory Inc., Reston, VA, USA; 'L'= 97.51, 'a'= −0.18, 'b'= 1.67) were considered for standardizing the instrument with D65 illuminant.

2.4. Determination of free amino acid

The free amino acids were analyzed following the method described by Je *et al*. (2005). The powdered sample (1.5 g) was homogenized (12000 rpm, 2 min) with 10 mL of ice-cold 6% (v/v) perchloric acid in an ice bath using an ACE homogenizer (Nissei AM-7, Nihonseikei Kaisha Ltd, Tokyo, Japan) and then kept in ice for 30 min before centrifugation (5000 rpm, 15 min). The supernatant was filtered using a filter paper (Whatman No. 41). The pH of the filtrate was adjusted to 7 using a KOH solution (33%, w/v), and centrifuged (5000 rpm, 10 min). After centrifugation, the precipitate of potassium perchlorate was removed and the pH of the mixture was adjusted to 2.2 using 10 M HCl and then the final volume of the mixture was made 50 mL with distilled water. Two milliliters of the mixture were mixed with 1 mL lithium citrate buffer (pH 2.2) and the free amino acid profile was analyzed using an automatic amino acid analyzer (Biochrom-20, Pharmacia Biotech Co., Uppsala, Sweden).

2.5. Determination of mineral content

The amount of mineral elements was measured using an inductively coupled plasma atomic emission spectrometer (ICP AES: Varian Vista, Victoria, Australia) as described earlier (Skujins, 1998). Five hundred milligrams of powdered sample was digested in a mixture of 65% HNO_3 (15.0 mL) and 35% H_2O_2 (2 mL). The mixture was diluted with an equal volume of distilled water. The amount of different mineral elements was measured after calibrating the ICP AES with known standards.

2.6. Preparation of sample extracts for antioxidant assays

One gram of the powdered sample was extracted in 10 mL of absolute methanol using a shaking incubator (250 rpm, 25°C) for 2 h and the mixture was centrifuged (1660 × *g*, 15 min). The supernatant was filtered through a syringe filter (0.2 µm) and the filtrate extract was used for further analyses.

2.7. Determination of DPPH radical scavenging activity

The DPPH free radical scavenging potential of GBR samples was measured following the methods described earlier (Blois, 1958; Dhungana *et al*., 2015). One hundred microliters of sample extract and freshly prepared 0.05% (w/v) methanolic solution of DPPH were mixed in wells of the 96-well microplate and incubated at room temperature for 30 min under dark condition. After 30 min of incubation, the absorbance values of the reaction mixtures were measured at 517 nm using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific Oy, Vantaa, Finland). The free radical scavenging potential was calculated as follows.

$$\text{Scavenging potential (\%)} = [1 - (S - S_o)/(C - C_o)] \times 100$$

where S, S_o, C, and C_o are the absorbance values of the sample and DPPH, sample and methanol, methanol and DPPH, and methanol, respectively.

2.8. Determination of total polyphenol content

The total polyphenol content (TPC) of GBR samples was determined according to the Folin-Ciocalteau method (Singleton *et al*., 1999) as described by Dhungana *et al*. (2016). Fifty microliters of the sample extracts and 1 mL of 2% (w/v) aqueous Na_2CO_3 were mixed in

microtubes and allowed to react at room temperature for 3 min. After 3 min, fifty microliters of 1 N Folin-Ciocalteau reagent was mixed into the mixture and incubated at room temperature for 30 min under dark condition. The absorbance value of the reaction mixtures was measured at 750 nm using a microplate spectrophotometer (Multiskan GO; Thermo Fisher Scientific). The total polyphenol content of the samples was calculated using the calibration curve drawn using gallic acid (GA) as standard.

2.9. Flavonoid content analysis

The flavonoid content of GBR samples was measured following the procedure described earlier (Zhishen *et al*., 1999; Dhungana *et al*., 2016). One hundred microliters of the sample extracts, 500 µL absolute methanol, 50 µL 10% $AlCl_3$, 50 µL 1 M HCl, and 300 µL distilled water were mixed in microtubes and incubated as in the TPC determination method. After the 30 min incubation, absorbance values of the reaction mixtures were measured at 510 nm using a microplate spectrophotometer (Multiskan GO; Thermo Fischer Scientific). The flavonoid content of the samples was determined using the calibration curve plotted using quercetin as a standard.

2.10. Statistical analysis

Statistical analysis was performed through the analysis of variance using SAS 9.4 (SAS Institute, Cary, NC, USA). The significant differences between samples were determined using the Tukey test ($p<0.05$). The average values of three replicates are reported unless otherwise mentioned.

3. Results and discussions

3.1. Color value of germinated brown rice

The Hunter's color values of GBR were significantly affected by Pu-erh tea extract treatment (Table 1). The lightness value of GBR was the highest in GBR-0 (82.7) and was significantly reduced with the concentration of tea extracts (73.96−78.88). On the contrary, the redness and yellowness values were the highest for GBR-0 (0.82 and 9.14) and significantly increased in the extract-treated samples (1.84−2.77 and 9.47−9.77) with the concentration of the tea extracts, respectively.

Table 1. Hunter's color values of germinated brown rice (GBR) produced after soaking in different concentration of Pu-erh tea extracts

Sample[1)]	Color value[2)]		
	L (lightness)	a (redness)	b (yellowness)
GBR-0	82.7±1.67a[3)]	0.82±0.16d	9.14±0.54d
GBR-0.5	78.88±0.39b	1.84±0.09c	9.470±0.10c
GBR-1.5	75.06±0.43c	2.50±0.03b	9.51±0.07b
GBR-3	73.96±0.28d	2.77±0.03a	9.77±0.07a

[1)] GBR-0: GBR produced after soaking the rice in tap water for 1 h; GBR-0.5: GBR produced after soaking the rice in 0.5% (w/v) Pu-erh tea extract for 1 h; GBR-1.5: GBR produced after soaking the rice in 1.5% (w/v) Pu-erh tea extract for 1 h; and GBR-3: GBR produced after soaking the rice in 3% (w/v) Pu-erh tea extract for 1 h. [2)] L: lightness (100, white; 0, black); a: redness (−, green; +, red); b: yellowness (−, blue; +, yellow). [3)] Values are presented as mean ± standard deviation of three replicates. Values followed by different letters (a, b, c, and d) in the same column indicate significant difference ($p<0.05$, ANOVA, Tukey test).

The effects of Pu-erh tea extracts on the physicochemical characteristics and antioxidant potentials of GBR were investigated in the present study. Visible traits such as the color of a food product are determining factors that affect the willingness of consumers to buy the product (Udomkun *et al*., 2018). The reasons for the variation in the color value of the GBR due to the tea extract treatments were not well understood in the present study.

3.2. Free amino acid content

The effect of tea extract treatments on GBR was less significant across samples when considered the free amino acid content (Table 2). A total of 25 (8 essential, 8 non-essential, and 9 other) amino acids were detected, whereas the amounts of 11 amino acids were non-detectable. The amounts of the essential, non-essential, and total amino acids were higher in the tea extract-untreated GBR than in the treated samples. However, the amount of some amino acids such as GABA was significantly higher in GBR-0.5 (0.59 mg/g) and GBR-1.5 (0.59 mg/g) than in GBR-0 (0.52 mg/g) and GBR-3 (0.51 mg/g).

Calcium plays a role in the activation of diamine oxidase activity that subsequently influences GABA synthesis (Wang *et al*., 2016). High calcium content in Pu-erh tea might have increased the GABA content in two of the tea extract-treated GBR samples. However, the reason for reduced GABA content in GBR-3 could not be justified. Amino acids such as GABA and glycine are associated with learning and memory enhancement; stroke and neurodegenerative disease control; anxiety, sedation, and anticonvulsant relief; and muscle relaxation functions (Mody *et al*., 1994; Oh and Oh, 2004). The GABA-rich foods are beneficial for regulating blood cholesterol and pressure, decreasing insomnia and depression, and relieving pain (Dhakal *et al*., 2012) inhibiting sleeplessness and autonomic disorder observed during the menopausal or presenile period (Okada *et al*., 2000). GABA is also found as advantageous to control diabetes (Reeds, 2000).

Table 2. Free amino acid composition (mg/g of dry weight) of germinated brown rice (GBR) produced after soaking in different concentration of Pu-erh tea extracts

Amino acid	**Sample**[1)]			
	GBR-0	**GBR-0.5**	**GBR-1.5**	**GBR-3**
Essential amino acid				
L-Threonine	0.04±0.01a[2)]	0.04±0.01a	0.04±0.01a	0.03±0.01a
L-Valine	0.11±0.01a	0.09±0.02ab	0.09±0.01ab	0.07±0.01b
L-Methionine	0.03±0.01a	0.01±0.01a	0.02±0.01a	0.02±0.01a
L-Isoleucine	0.06±0.01a	0.05±0.01a	0.05±0.01a	0.04±0.01a
L-Leucine	0.08±0.01a	0.07±0.01ab	0.06±0.02ab	0.05±0.01b
L-Phenylalanine	0.08±0.01a	0.07±0.02ab	0.06±0.01ab	0.05±0.01b
L-Lysine	0.07±0.01a	0.06±0.02a	0.06±0.01a	0.05±0.01a
L-Histidine	0.09±0.02a	0.07±0.01a	0.06±0.01a	0.06±0.01a
Sub-total	0.56	0.46	0.44	0.37
Non-essential amino acid				
L-Asparitic acid	0.04±0.01a	0.03±0.01a	0.04±0.01a	0.04±0.02a
L-Serine	0.09±0.01a	0.08±0.02a	0.10±0.01a	0.09±0.01a
L-Glutamic acid	0.31±0.03ab	0.29±0.02b	0.35±0.01a	0.32±0.02ab
Glycine	0.02±0.01a	0.02±0.01a	0.02±0.01a	0.02±0.01a
L-Alanine	0.15±0.02a	0.14±0.01a	0.15±0.01a	0.14±0.01a
L-Tyrosine	0.10±0.01a	0.07±0.02b	0.06±0.01b	0.05±0.01b
L-Arginine	0.17±0.01a	0.12±0.01b	0.11±0.02bc	0.09±0.01c
Proline	0.05±0.01a	0.05±0.01a	0.04±0.01a	0.04±0.01a
Sub-total	0.93	0.80	0.87	0.79
Other amino acid				
O-Phospho-L-serine	ND[3)]	ND	ND	ND
Taurine	ND	ND	ND	ND
O-Phospho ethanol amine	0.02±0.01a	0.02±0.01a	0.02±0.01a	0.02±0.01a
L-Sarcosine	ND	ND	ND	ND
L-α-Amino asipic acid	0.01±0.01a	0.01±0.01a	0.01±0.01a	0.01±0.01a
L-Citrulline	ND	ND	ND	ND
L-α-Amino-n-butyric acid	0.01±0.01a	0.01±0.01a	0.01±0.01a	0.01±0.01a
L-Cystine	ND	ND	ND	ND

Cystathionine	ND	ND	ND	ND
β-Alanine	0.03±0.01a	0.03±0.01a	0.03±0.01a	0.03±0.01a
D,L-β-Amino isobutyric acid	0.02±0.01a	0.02±0.01a	0.04±0.01a	0.02±0.01a
γ-Amino-n-butyric acid	0.52±0.01b	0.59±0.02a	0.59±0.01a	0.51±0.02b
Ethanolamin	0.01±0.01a	0.02±0.01a	0.03±0.01a	0.01±0.01a
Hydroxylysine	ND	ND	ND	ND
L-Ornithine	0.01±0.01a	0.01±0.01a	0.01±0.01a	0.01±0.01a
1-Methyl-L-histidine	0.03±0.01a	0.02±0.01a	0.02±0.01a	0.02±0.01a
3-Methyl-L-histidine	ND	ND	ND	ND
L-Anserine	ND	ND	ND	ND
L-Carnosine	ND	ND	ND	ND
Hydroxy proline	ND	ND	ND	ND
Sub-total	0.66	0.73	0.76	0.64
Total free amino acid	2.15	1.99	2.07	1.80

[1)] Samples are defined in Table 1. [2)] Values are expressed as mean ± standard deviation of two replicates. Values followed by different letters (a, b, c, and d) in the same row are significantly different ($p<0.05$, ANOVA, Tukey test). [3)] Non-detected.

3.3. Mineral content

Although the amount of total free amino acids was lower in the Pu-erh tea extract-treated GBR samples, the treatment significantly increased many mineral elements along with the total mineral content (Table 3). Out of the eight minerals measured, five were higher in one of the tea extract-treated GBR than in the untreated sample. The amounts of two minerals Ca and Cu were significantly higher in the tea extract-untreated GBR (35.35 and 31.57 mg/kg) than in the extract-treated samples (21.75−26.31 and 12.54−30.39 mg/kg), respectively. K (1417.57−1759.23 mg/kg) was the most abundant mineral in the GBR samples. The amount of total mineral content in the tea extract-treated GBR was substantially higher than that in the untreated sample.

Table 3. Mineral contents (mg/kg of dry weight) of germinated brown rice (GBR) produced after soaking in different concentration of Pu-erh tea extracts

Element	**Sample[1)]**			
	GBR-0	**GBR-0.5**	**GBR-1.5**	**GBR-3**
Ca	35.64±3.87a[2)]	26.31±2.00b	25.69±0.86b	21.75±1.70c
Cu	31.57±0.26a	21.49±0.39c	30.39±0.26b	12.54±0.09d
Fe	35.54±0.16b	34.97±0.52b	43.99±1.48a	22.62±0.16c
K	1417.57±21.13d	1644.99±10.29c	1674.45±4.56b	1759.23±10.21a
Mg	1025.00±11.78a	1043.35±8.95a	967.16±5.01b	984.63±11.76b
Mn	19.45±0.32c	22.05±0.31b	22.16±0.01b	23.89±0.04a
Na	181.23±0.23b	179.91±1.51b	186.86±0.82a	167.19±2.63c
Zn	35.12±0.23b	22.88±0.11c	41.34±0.31a	20.35±0.08d
Total	2781.12	2995.95	2992.04	3012.23

[1)] Samples are defined in Table 1. [2)] Values are expressed as mean ± standard deviation of two replicates. Values followed by different letters (a, b, c, and d) in the same row are significantly different ($p<0.05$, ANOVA, Tukey test).

Like the GABA content, the mineral content of the GBR samples was increased by

soaking brown rice in the mineral-rich Pu-erh tea (McKenzie *et al*., 2010). Similar results of higher zinc content were found in the zinc sulfate-applied soybean sprouts (Xu *et al*., 2012; Zou *et al*., 2014). Minerals, including Fe and Zn, which were increased in some of the Pu-erh tea extract-treated GBR samples, are some of the most commonly lacking elements in human diets (White and Broadley, 2009). Minerals Mg and K are beneficial against hypertension (Houston and Harper, 2008); Fe plays roles in oxygen transport, energy metabolism, mitochondrial respiration, DNA synthesis, and cellular growth and differentiation (Ganz, 2013); Zn contributes to the growth, development, differentiation, DNA synthesis, RNA transcription, and cellular apoptosis (MacDiarmid, 2000).

3.4. DPPH inhibition activities and total polyphenol and flavonoid contents

The antioxidant potentials of the GBR samples were determined through DPPH inhibition activities as well as total polyphenol and flavonoid contents (Table 4). The DPPH free radical scavenging potentials of GBR-1.5 (72.49%) and GBR-3 (76.18%) were significantly higher than that of GBR-0 (62.56%) and GBR-0.5 (62.58%). The total polyphenol content was highest in GBR-0.5 (164.19 μg GAE/g) followed by GBR-0 (152.58 μg GAE/g), GBR-1.5 (143.38 μg GAE/g), and GBR-3 (130.53 μg GAE/g). The flavonoid contents of GBR-1.5 (36.17 μg QE/g) was the significantly lowest among the samples (39.69−41.48 μg QE/g).

Several reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical, and singlet oxygen cause oxidative damage in lipids, proteins, and DNA (Santos *et al*., 2003). Production of excessively high levels of the ROS may harm the cells by lipids peroxidation, proteins oxidation, nucleic acids destruction, enzyme inhibition, programmed cell death activation pathway, and eventually cells death (Mishra *et al*., 2011; Srivastava and Dubey, 2011). The higher levels of biosynthesis of antioxidants in the tea extract-treated samples might be resulted from the high contents of elements like calcium (McKenzie *et al*., 2010) and/or phenolic compounds (Zhang *et al*., 2012; Chen *et al*., 2017) in Pu-erh tea.

Table 4. DPPH inhibition activities and total polyphenol and flavonoid contents of germinated brown rice (GBR) produced after soaking in different concentration of Pu-erh tea extracts

Sample [1)]	DPPH (% inhibition)	Total polyphenol (μg GAE [2)]/g)	Flavonoid (μg QE [3)]/g)
GBR-0	62.56±1.22c[4)]	152.58±3.26b[4)]	39.69±1.88 a
GBR-0.5	62.58±2.05c	164.19±0.72a	41.35±0.68 a
GBR-1.5	72.49±1.64b	143.38±2.68c	36.17±1.59 b
GBR-3	76.18±2.12a	130.53±0.77d	41.48±2.60 a

[1)] Samples are defined in Table 1. [2)] GAE: gallic acid equivalents. [3)] QE: quercetin equivalents. [4)] Values are expressed as mean ± standard deviation of three replicates. Values followed by different letters (a, b, c, and d) in the same column are significantly different ($p<0.05$, ANOVA, Tukey test).

The results of this study are in agreement with that of previous reports of high phenolic contents in the germinated brown rice when the rice was treated with high phenolic-containing onions (Gennaro *et al*., 2002; Griffiths *et al*., 2002; Nakamura *et al*., 2020). Phenolic compounds have antioxidant potentials (Rice-evans *et al*., 1995; Yang *et al*., 2015) that can scavenge harmful free radicals. In addition to phenolic compounds, various enzymatic and non-enzymatic antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase, vitamin C, vitamin E, polyphenols, and carotenoids possess the free radical scavenging potentials. The antioxidant potential of foods is a multifaceted outcome of several factors, such as the partitioning properties of specific

antioxidants, oxidation conditions, and the physical state of the oxidizable substrate (Frankel and Meyer, 2000). Thus, a visible difference in the level of an individual antioxidant such as total polyphenol and/or flavonoid might not always account for higher antioxidant activity, as in the GBR samples with higher DPPH radical scavenging potential but lower total polyphenol and/or flavonoid.

4. Conclusions

The influence of Pu-erh tea extracts on GBR was investigated considering the physicochemical characteristics and antioxidant potentials. The color values of GBR were significantly modified with the tea extract treatments. Although the total free amino acids of the extract-treated GBR samples were lower than the untreated one, the amount of some amino acids like GABA were increased. In addition, the amount of total minerals and antioxidant potentials of the extract-treated GBR samples were higher than that of the untreated one. Overall, the nutritional and functional properties of brown rice could be enhanced by soaking it in Pu-erh tea extracts (0.5–3%, w/v).

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ANTI-CANCER EFFECT OF CURCUMIN ON SURVIVAL AND EXPRESSION OF DNMT1 AND CDH1 GENES IN CELL LINE MIAPACA2

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CDH1.

ABSTRACT

Introduction: Pancreatic cancer is a deadly sinister cancer and the fourth leading reason for death worldwide. DNMT1 is essential for the conservation of the methylation landscape due to its ability to recognize hemimethylated DNA and conserve methylation during somatic cellular division.Ecad play a role in cellular connectivity through extracellular domains, Loss of Ecad Protein, lead to loss of Intercellular Connections, Provides Cell Metastasis. The pharmacological effects of curcumin include inducing apoptosis, anti-cell proliferation, antioxidant and anti-angiogenesis are proved and this compound has the potential to be used in cancer prevention.

Objectives: The current study was performed in order to explore in vitro antitumor activity of curcumin in human pancreatic carcinoma cell line MIAPaCa2.DNMT1 and CDH1 genes expression were examined by quantitative real-time PCR. Finally, the effects of curcumin on viability and DNMT1 gene and CDH1 gene expression status were evaluated.

Method: MiaPaca-2cell line was cultured in monolayers. The cells were treated with curcumin using different concentrations of 2,5, 10, 20, 40, 80 µM for 24, 48 and 72 hours. Viability was checked by MTT assay and DNMT1 and CDH genes expression was evaluated by RT-PCR.

Results: Our results indicate that the level of DNMT1 mRNA expression was decreased after treatment. Expression level of CDH1 mRNA were increased. Data obtained from MTT revealed antiproliferative effects of curcumin for 20,40,80 µM concentrations.

Conclusions: We conclude that cell viability and level of DNMT1 mRNA was decreased after curcumin treatment, and level of CDH1 mRNA was increased. So, These observations suggest that curcumin , a molecule with varied actions, as a supplementary could be developed into an effective chemopreventive and chemotherapeutic agent for pancreatic cancer treatment.

1.Introduction

Pancreatic cancer is a deadly sinister cancer and the fourth leading cause of death worldwide. Despite advances in recent years in the diagnosis and treatment of this cancer, The 5-year survival rate of these patients is below 5% and has a very poor prognosis (Zhang, Liang et al. 2011), (Yin, Wang et al. 2007). By 2030, it is anticipated to be the second murderous cancer in the world (Khan, Zubair et al. 2015). The only way to treat and save these patients is to have surgery and pancreatic resection, but since the disease is diagnosed late, only 10 to 20% of patients are candidates for surgery(Gillen, Schuster et al. 2010). At the time of diagnosis 15% of patients

are in stage 1 or 2, 35% of patients are in advanced stage of the disease and 50% of patients are diagnosed with stage 4 and metastasis(Suker, Beumer et al. 2016). So pancreatic surgery and resection do not help patients much.Their 5-year survival rate is 11% to 25% and only a few months longer will survive (Alexakis, Halloran et al. 2004). Studies on the molecular mechanism of pancreatic cancer display that this disease is related to genetic and epigenetic changes(Hong, Park et al. 2011). Epigenetics is a significant topic introduced as one of the pathways controlling gene expression, determined as the changes in gene expression without any essential changes in sequences(Azad, Kaviani et al. 2013, Rahmani, Azad et al. 2017). Methylation is one of the most substantial epigenetic mechanisms, It involves the addition of a methyl group to the cytosine base after DNA copying(Issa 2007). DNA methylation is carried out by DNA methyltransferase enzymes, It contains three enzymes DNMT1, DNMT3a and DNMT3b. DNMT1 is the significant enzyme for methylation after transcription(Baylin 2005). Epigenetic changes, including DNA methylation, alter gene expression and chromatin structure and changing DNA methylation patterns has a significant role in human cancer, these changes include increased methylation of the CpG islands that can inactivate the tumor suppressor(Kulis and Esteller 2010). Increased dnmt1 gene expression has been observed in most pancreatic cancer patients(Li, Omura et al. 2010).

Ecad glycoprotein encoded by CDH1 gene located on chromosome q22.116 and belong to the cell adhesion molecule (CAM) family. Intracellular and $Ca2^{+}$ dependent protein in epithelial cells and they play a role in cellular connectivity through extracellular domains and communication with next cell cadherins and operate as a tumor inhibitor (Huber, Züllig et al. 2011),(Vesuna, van Diest et al. 2008). Loss of Ecad Protein Function through CDH1 Gene Mutation, Loss of Intercellular Connections, Provides Cell Metastasis(Norton, Ham et al. 2007).

Curcumin is a phenolic compound that is the active compound of the plant Chromoma Longa, known as turmeric. Used as a spice and a compound known in traditional medicine in many countries(Gou, Men et al. 2011). It has anti-bacterial, anti-fungal, anti-yeast, anti-parasitic and anti-cancer effects(Anuchapreeda, Fukumori et al. 2012). The pharmacological effects of curcumin include apoptotic, anti-cell proliferation, antioxidant and anti-angiogenesis and this compound has the potential to be used in cancer prevention(Shehzad, Wahid et al. 2010). In addition to treating cancer as an anti-inflammatory, it is also used to treat Alzheimer's disease and malaria, It targets cell DNA, RNA and cell proteins (enzymes) (Yallapu, Jaggi et al. 2012). Curcumin inhibit multiple intracellular signaling pathways. Proteins of these pathways that target the chromatin include NF-κB, AP-1 COX-2, MMPs, EGFR, β-catenin and TNF. The anticancer effects of curcumin are related to targeting of COX-2, HER2, TNF, EGFR, Bcr-abl, proteasome PI3K, AKT, Ras and NF-κB proteins (Kasi, Tamilselvam et al. 2016).

The current study was performed in order to explore in vitro antitumor activity of curcumin against human pancreatic carcinoma cell line MIAPaCa2 and DNMT1 gene and CDH1 gene expression was assayed by quantitative real-time PCR. Finally, the effects of curcumin on viability and DNMT1 gene and CDH1 gene expression situation were evaluated.

2.Materials and methods

2.1.Cell culture

The MiaPaca-2 ،cell line was bought from Pasteur Institute of Iran and cultured in RPMI 1640 Gibco completed with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic (100 U/ml of penicillin, 10 mg/ml of streptomycin) at 37°C under an atmosphere of 5% CO2. The cells were cultured in cell culture plates, each well containing 6x105 cells for all concentration and control.

After treatment of the cells with curcumin using a concentration of 99% ethanol we prepared dilutions 2,5, 10, 20, 40 and 80 µM. At 24,48 and 72 hours, cells were harvested and isolated from medium.

Real-time reverse transcription-polymerase chain (real-time RT-PCR)

At a spacing of 24,48 and 72 hours, cells were harvested and isolated from medium.

using the kit iraizol RNA extraction (RNA biotechnology CO, Isfahan, Iran), RNA was extracted, according to the manufacturers. For the quantitative analysis of RNA extraction, we used Nanodrop techniques. For cDNA synthesis, we used the RBcDNA synthesis kit (RNA biotechnology CO, Isfahan, Iran). Real-time RT-PCR was carried out using the Rotor-Gene Q appliance (QIAGEN co.) and analyzed by Rest 2009 software.

Primers used for DNMT1 expression were designed by IDT and NCBI

(F) GTGGGGGACTGTGTCTCTGT

(R) TGAAAGCTGCATGTCCTCAC for forward and reverse respectively.

Primers used for CDH1 expression were designed by IDT and NCBI

(F) GGGTTAAGCACAACAGCAAC

(R) ACCTGACCCTTGTACGTGGT

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a RNA integrity control and amplified using primers

5′-CAATGACCCCTTCATTGACC-3′

5′-TGGAAGATGGTGATGGGATT-3′

for forward and reverserespectively. [22]

The cycles were as persued: 95°C for 5 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds, annealing at 60°C for 20 seconds and extension at 72°C for 20 seconds. This was followed by the final extension at 72°C for 10 minutes.

2.2.MTT assay

Cells were cultured in 96-well culture plate (2x103 cell/200µl). 24 hours after the culture, we replaced ambient with 0.1% ethanol as control and concentrations of 2,5, 10, 20, 40, and 80 µM curcumin. Each concentration was accomplished as a quadruplet. After 24, 48, 72 hours of treatment, cells were laundering with PBS and 50 µl MTT solution (Sigma) was added at a concentration of 0.05 mg/ml diluted in PBS Cells were incubated at 37°C for 4 hours to permit the formation of purple formazan crystals due to mitochondrial dehydrogenase activity. Then 200 µl of filtered DMSO was added to each well pursued by 25 µl glycine buffer. The absorbance of the cell suspension was measured by spectrophotometric at 570 nm using an ELISA reader (BioTek ELX800, USA). Data were analysed by one-way analysis of variance ANOVA confirmed by Dunnett's multiple comparison test using Graphpad prism 8 software where $P< 0.05$ was considered to be statistically substantial.

3. Results and discussions

3.1.Evaluation of Cytotoxic Effect Using MTT Test

The MTT test using to estimate Metabolic acting that can be measuring the activity of a mitochondrial enzyme succinate dehydrogenase. MTT is planned for the description of a cytotoxic indicator in a cell population using a 96 well plate format.

In the present study, we applied the MTT test to evaluate the anticancer activity of Curcumin in cell line MIAPaCa-2 assay in both concentration and time-dependent manner as explained Material and methods section. As illustrated in Figure 1 the anchorage-dependent cell viability of MIAPaCa-2 cells after exposure to curcumin was decreased significantly. These data illustrate that curcumin has antiproliferative activity in pancreatic cancer cell lines.

3.2.Real-time PCR

DNMT1 mRNA expression

Our results indicate that the level of expression DNMT1 mRNA, In 24 hours for 5, 10, 20,40 and 80 µM concentrations was decreased after treatment with curcumin, but not 2 µM. in 48 hours all of concentration was decreased and after 72 hours treatment with curcumin all of concentration was decreased (figure 2).

3.3.CDH1 mRNA expression

Level of expression CDH mRNA, in 24 hours for 40 and 80 µM concentration was increased, but not 5,10,20 µM. in 48 hours for

40 and 80 µM concentration was increased. after 72 hours all of concentration was increased (figure 3).

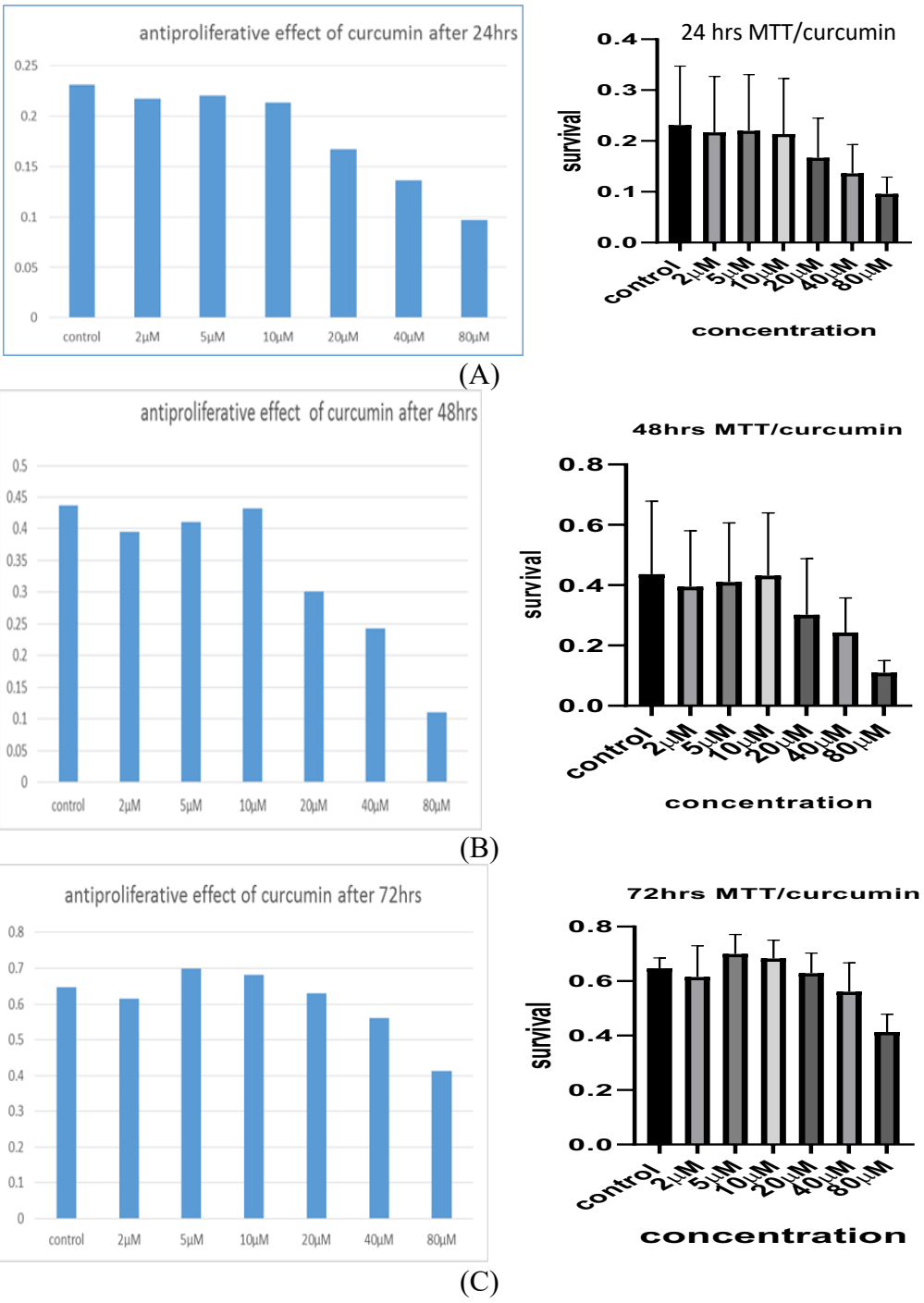
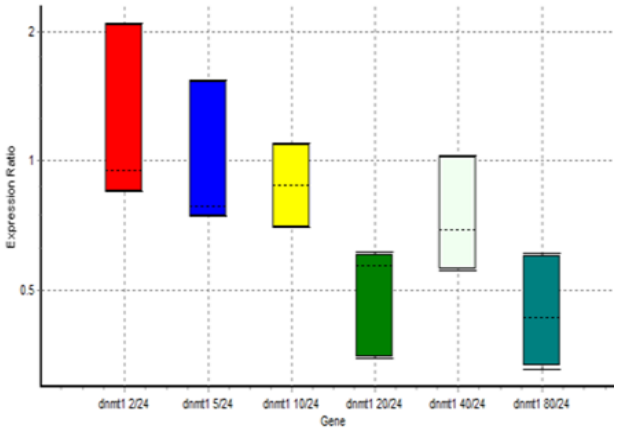
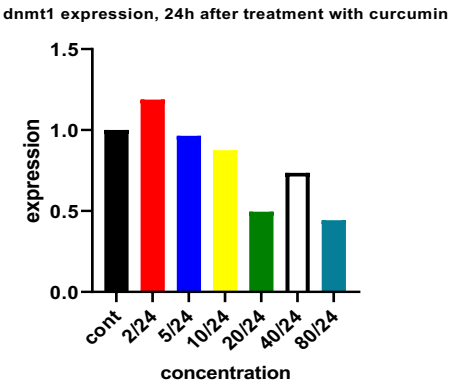
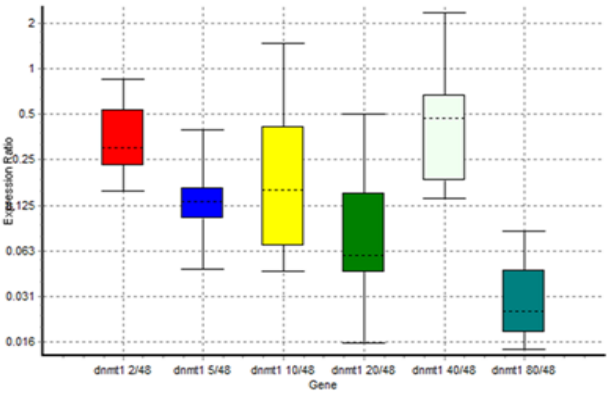


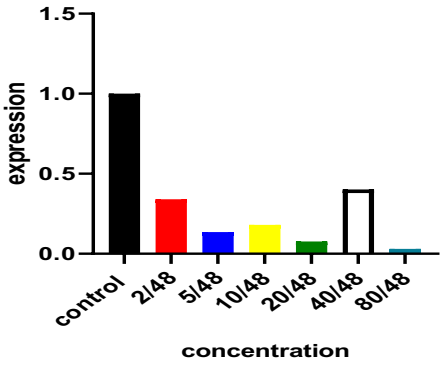
Figure 1. Anchorage-dependent cell viability of pancreatic cancer cell line (MiaPaca-2), (A) 24 hours after treatment with curcumin , (B) 48 hours after treatment with curcumin, (C) 72 hours after treatment with different concentrations (2,5,10,20,40,80µM) of curcumin. Antiproliferative effects were seen at 20 to 80 µM of curcumin with a time- and concentration-dependent manner ($P<0.05$).



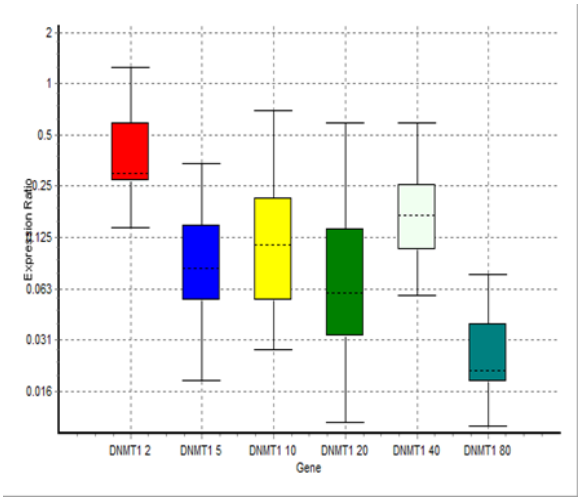


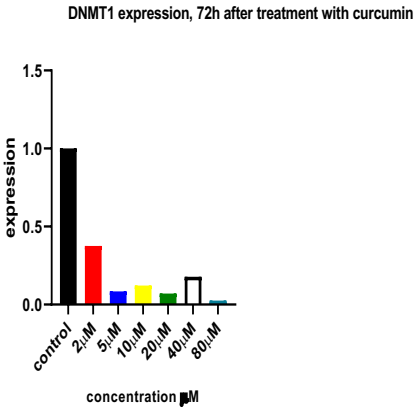
(A)





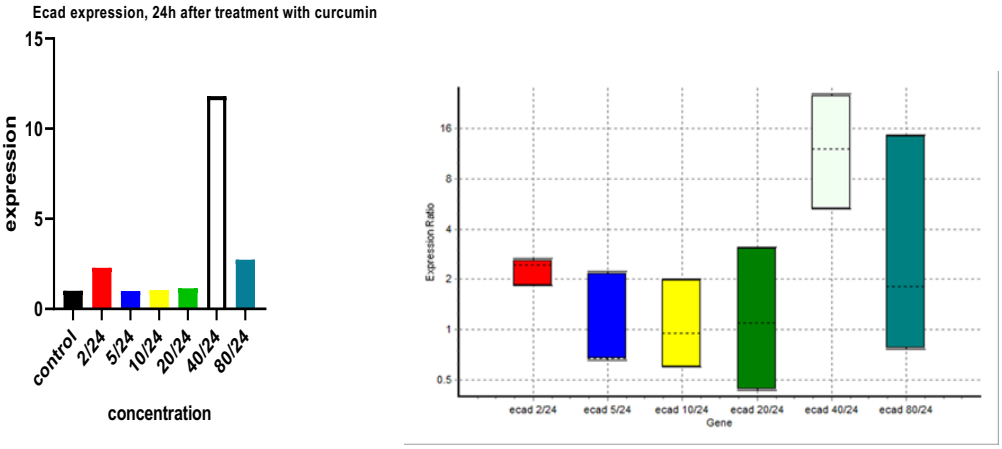
(B)



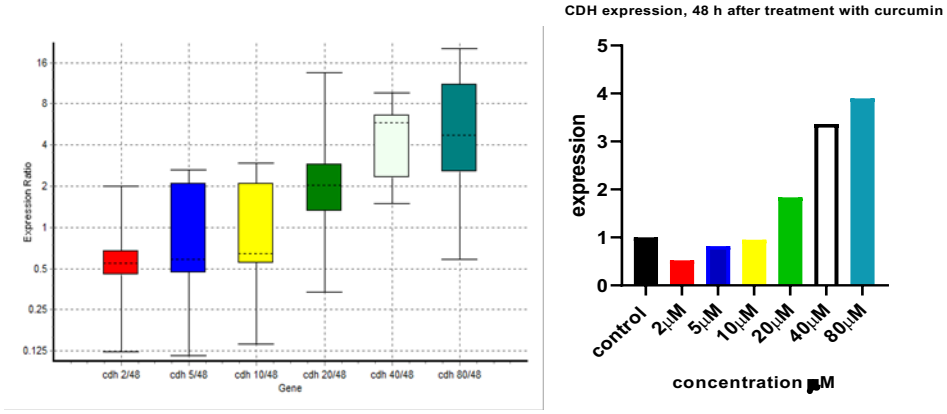


(C)

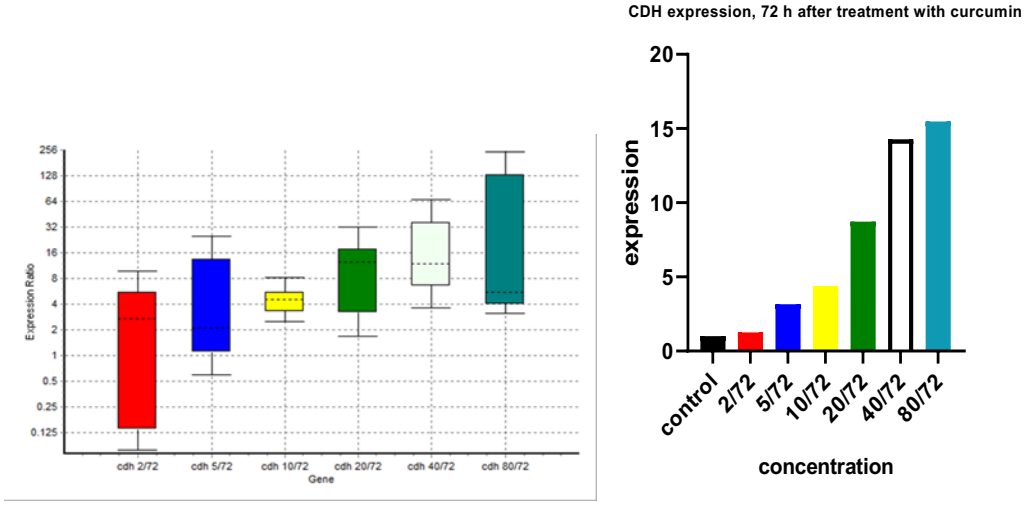
Figure 2. Detecting the transcription of DNMT1 mRNA using real-time RT-PCR. MiaPaca-2 cells were treated with 2,5, 10, 20, 40, and 80 μM curcumin for 24, 48, 72 hours.(A) DNMT1 mRNA expression after 24 hrs treated with curcumin (B) DNMT1 mRNA expression after 48 hrs treated with curcumin. (C) DNMT1 mRNA expression after 72 hrs treated with curcumin. The levels of DNMT1 mRNA were analyzed by REST 2009 software ($P< 0.05$)



(A)



(B)



(C)

Figure 3. (A) CDH mRNA expression after 24 hrs treated with curcumin (B) CDH mRNA expression after 48 hrs treated with curcumin. (C) CDH mRNA expression after 72 hrs treated with curcumin. The levels of CDH mRNA were analyzed by REST 2009 software ($P< 0.05$)

3.4.Discussions

Pancreatic cancer is an aggressive malignancy with an extremely low estimated 5-year survival rate of <2% (Sultana, Smith et al. 2007). In the metastatic stages, pancreatic cancer is never controlled by therapies and chemotherapy drugs, Effectiveness of gemcitabine as a systemic agent in the treatment of advanced stage pancreatic cancer results in a median survival of less than 6 months (Villarroel, Rajeshkumar et al. 2011). This study demonstrated changes in gene expression profiles DNMT1و CDH1 genes in pancreatic cancer cell line in response to exposure to curcumin, that have been reported in previous studies (Shishodia 2013), (Dhillon, Aggarwal et al. 2008).

Antiproliferative effect of curcumin at 20 to 80 μM after 24,48 and 72 hours treatment concentrations of curcumin were seen in pancreatic cancer cell line (MiaPaca-2, respectively ($P<0.05$).

In previous study, has been illustrated inhibitory effects of curcumin observed in G2/M phase cell-cycle arrest and apoptotic cell death in a variety of cancer cells is related to inhibiting NF-κB activation, curcumin has been displayed to suppress the expression of diverse cell survival and proliferative genes, including Bcl-2, Bcl-xL, cyclin-D1and interleukin-6, and accordingly arrest cell cycle, inhibit proliferation, and induce apoptosis (Dhillon, Aggarwal et al. 2008),(Csaki, Mobasheri et al. 2009).

Curcumin substantialy inhibited the proliferation and survival of pancreatic adenocarcinoma, which was related with inhibition of phosphorylation of extracellular receptor kinase (ERK) 1/2, and reduction of protein expression of COX-2 and the EGFR (Zhou, S Beevers et al. 2011).

This study confirms the results of previous research, Our data demonstrated that DNMT1 gene expression is high and CDH1 gene expression is low in cell line MiaPaCa2 that was concordant with prior research. Treatment of Miapaca2 cells with curcumin, leading to downregulation of DNMT1 gene and upregulation of CDH1 gene in this cell line.

According to previous research, Improper CpG island hyper methylation, having been investigated in the early stages of pancreatic cancer and DNMTs protein expression, correlated with advanced stages of the tumor. In addition, high DNMTs protein expression level of patients have been seen to have rather poor survival (Huang, Chou et al. 2019), In most pancreatic cancers, DNMT1 gene expression is increased. DNMT1 gene expression contributes to cell viability, whereas, DNA hyper methylation and over expression DNMT1often inactivates tumor suppressor genes, DNMT1 gene inhibitors have been tested as a treatment for this cancer (Li, Omura et al. 2010).

CDH1 gene expression contributes to maintain an epithelial phenotype, E-cadherin was illustrated to act as an inhibitor of invasion in tumor cell lines and in in vivo tumor models. In humans, There is a strong correlation between CDH1 gene expression and survival of patients (Schmalhofer, Brabletz et al. 2009).

It has been seen in previous studies, CDH1 Gene expression has been decreased in patients with pancreatic cancer by methylation promotor , Silencing or mutation (Ottenhof, De Wilde et al. 2012). Upregulation of DNMT1gene and downregulation CDH1 genes provides conditions for inactivation tumor suppressor genes by methylation and metastasis cancer cells respectively. Based on our in vitro work displayed the activity of curcumin in cell lines of pancreatic cancer,The therapeutic agent of curcumin probably is mediated in part through the antioxidant and anti-inflammatory function of curcumin, Most studies indicate its role in inhibiting cancer tumor growth and metastasis, Curcumin is a safe and non-toxic compound even at a dose of 500-12000 mg and can be used with other anticancer compounds. (von Burstin, Eser et al. 2009, Hamzehzadeh, Atkin et al. 2018). In the future targeting specific gene for identifying people susceptible to cancer and treatment of patient with certain biomarkers, curcumin with its effect on methylation and metastasis can be used as an impressive compound in the prevention and treatment of all types of cancer.

4.Conclusions

This study showed that curcumin by reducing the expression of DNMT1 can reduce methylation and also curcumin by increasing the expression of CDH1 can be used as a preventive agent and in the treatment of many cancers.

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