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Antimicrobial, Biochemical, Organoleptic and Stability Properties of Cookies Fortified By Pomegranate Juice during Storage.

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ABSTRACT

In the present work, Pomegranate peels (PMP), Juice (PMJ) extracts were tested for antibacterial and antifungal activities (AA). GC-MS analysis was done in identifying the major phenol contents. Four concentrations of PMJ were used besides control (15%, 18%, 21% and 24%) for supplementation of cookies. Cookies samples were stored even 45 days at room temperature with studying sensory evaluation at zero time and at the end of storage besides fungal count at the end of storage. Using PMP and PMJ, either with water or alcohol showed positive response against tested bacteria and fungi. The aqueous PMJ extract was more effective in controlling the tested pathogenic microbes either bacteria or fungi than PMP extract. PMJ was more effective than PMP, whereas water was more effective than alcohol extract in controlling the microorganisms. Finally, it could be recommended that supplementation with 15% Juice of pomegranate can improve the safety and quality of cookies.

Keywords: *Punica granatum*, antimicrobial activity, toxigenic fungi, pathogenic bacteria.

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INTRODUCTION

Pomegranates (*Punica granatum* L.) are the fruits belonging to the family *Punicaceae*. It is native from Iran and extensively cultivated in the Mediterranean region [1, 2]. Pomegranates are a popular and healthiest fruit on earth. More of studies reported different benefits of its as medicinal properties, anti-inflammatory effects, may help fight prostate and breast cancer, may lower blood pressure and can help fight bacterial and fungal infections [3].

The fruit contains three parts: the seeds, which represent about 3% of the fruit weight, and themselves contain 20% oil; the juice, represent about 30% of the fruit weight; and the peels which also include the interior network membranes. One cup of arils (174 grams) contains: 7 grams fiber, 3 grams protein, as well as 30% vitamin C, 36% vitamin K, 16% Folate, and 12% potassium of the RDA. [3] reported that, one cup of pomegranate arils containing 24 grams of sugar and 144 calories.

Recently, new trends in food processing are using the natural bioactive sources as antioxidants and antimicrobial to support bakery products for improving human health consumption. Arils of Pomegranates are juicy and rich in anthocyanins and phenolic compounds. Also, the fruits are rich in tannins with remarkable antimicrobial activity (AA) [4]. AA has been demonstrating against several highly pathogenic, interestingly, the peel fraction has higher total phenolic content and antioxidant activity than the pulp fraction [5]. It has a substantial amount of polyphenols such as ellagic acid (EC), ellagitannins (ETs) as one example, in Fig.1 and Gallic acids (EG). Also, Pomegranates juice contained anthocyanins, proanthocyanidin compounds (Fig.2), glucose, organic acid, ascorbic acid, EA, ETs, gallic acid, caffeic acid, catechin, quercetin, rutin and minerals [6].

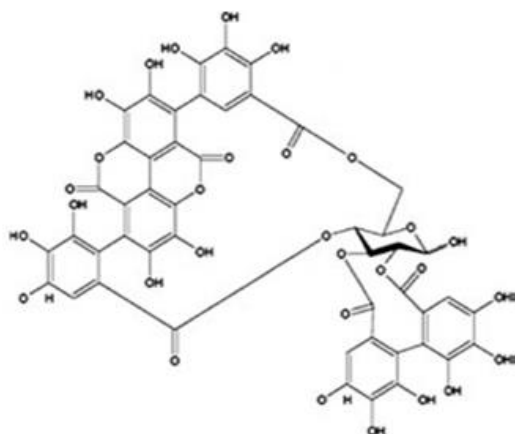


Figure (1). Punicalagin one of ellagitannins (ETs), present in pomegranate peel.

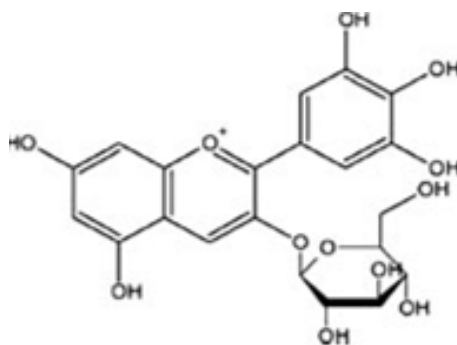


Figure (2). Principal anthocyanins present in pomegranate juice delphinidin-3-oglucoside present in pomegranate juice.

Bakery products, specific cookies are considered as the most viable and acceptable carriers of supplements [7]. Wheat flour being the base material of cookies, although is a good source of carbohydrates,

however, it may lack appreciable concentrations of fiber, minerals and bimolecular like antioxidants to meet the growing nutritional demands of the vulnerable populations.

The present study aims at highlighting the phenolic compounds of PM peel, juice, its antibacterial, antifungal effects and its possible utilization as an ingredient of choice to nutritionally enrich cookies. In addition, the organoleptic characters of fortified cookies were made by panelists at zero time of storage and after 45 days of storage besides study its load of fungi at end of storage.

MATERIALS AND METHODS

Material

Pomegranate fruits (PM, Manfalouty cv) were harvested at a mature stage from the private orchard near Cairo, Egypt. The samples were divided into two portions, first for extraction of water and ethyl alcohol for juice (aril, residue of the seed after extraction), and peel part (white parts-coating arils and the outer red surface of fruit) separately for chemical and microbiological analysis. Whereas, the second portion for preparing juice for supplementation biscuits with juice only then processed cookies were stored to follow the changes of organoleptic characters during storage even 45 days at room temperature (20⁰C, 70-75 % RH).

Extraction of PM juice

Fresh fruits were carefully washed, cut into halves, quarters, then fruits were manually separated from the pomegranate peels (PMP), piths and their juice was extracted using an electric juicer (bifinett model KH 450). To get pure juice, extraction stage was passed again through the juicer for further juice extraction. The red fresh juice was drained and screened throughout the cheese cloth for separation of the seeds (PMJ). Water and ethanol extraction were done, then concentrated using a rotary evaporator at low temperature (40⁰C) and stored in a deep freezer at (-18⁰C). The collected PJ pith (peel inside - outside peel parts plus the residue of the seeds) was dried overnight for several days at room temperature (30-35⁰C). Total Soluble Solids (TSS %) as percentages was 0.19 % and 20 % for ethanol and water extract respectively.

Microbiological analysis

Three clinically isolated pathogenic bacterial strains that included two gram negative bacteria (*E. coli* and *S. typhi*) and one gram positive bacteria (*Staph. aureus*) were used for the present investigation. Besides, four fungi strains (*A. niger*, *A. flavus*, *A. parasiticus* and *P. digitatum*). All the pathogenic bacterial strains were get from the Faculty of Agriculture, Ain - Shams University, Egypt, maintained on slants of nutrient agar for bacterial strains with aeration at (37 °C). Whereas, four fungal strains were obtained from the National Research Centre, Giza, Egypt, then maintained on slants of Potato Dextrose Agar (PDA) for fungal strains with aeration at (27-30 °C). Whereas PDA, Nutrient agars (NA), Yeast Extract Sucrose Broth (YES) were obtained from DIFCO Laboratory, Detroit, MI 48232-7058, U.S.A. De Man Rogosa Sharp Broth (MRS) was purchased from Sigma Chemical Company, St Louis, MO 63103, U.S.A.

Antimicrobial activity of extracts (AA)

AA of juice, peel separately was determined using the well agar diffusion method as follows: Suspensions of microorganisms containing 10⁶ CFU/ml were inoculated onto a plate surface. Test plates (diameter of 10 cm) were prepared with 20 ml potato dextrose agar and nutrient agar, and holes of 5 mm in diameter were punched in the agar plates. One hole was filled with 100 µl ethanol each plant extract and the other with 100 µl water plant extracts separately; also wells were filled with 100 µl 95% ethanol and 100 µl water as a control. The diameter of the inhibition zone (mm) around the holes was measured after 72 hrs at 37°C for pathogenic bacteria and after 5 days at 25 °C for moulds [8]. Tests were performed in duplicates [9].

Total Fungi Counts (CFU/ml)

The fungal counts for samples were isolated according to the method of [10] as follows: samples were ground in a Buhler mill and 10 grams of each ground sample were transferred to sterilize flask containing 90 ml of sterilized saline solution. Serial dilutions i.e. 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ were prepared, then 1 ml was transferred

to a Petri dish containing potato dextrose agar medium and incubated at $25 \pm 2^\circ\text{C}$ for 7 days [11]. The fungal colonies were counted, then picked, purified on potato dextrose agar (PDA) slants and incubated for 5 days at $25 \pm 2^\circ\text{C}$ for identification. The purified fungi colonies were identified according to the methods described by [12].

GC - MS / MS analysis

The analysis was carried out using GC (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5%-phenyl methyl poly siloxane) capillary column (30 m \times 0.25 mm i. d. and 0.25 μm film thickness). The carrier gas was helium with the linear velocity of 1ml/min. Ultra-high purity helium (99.999%) was used as the carrier gas at a constant flow rate of 1.0 ml/min. The injection, transfer line and ion source temperatures were 220,280 250 $^\circ\text{C}$, respectively. The ionizing energy was 70 eV. Electron multiplier voltage was obtained from auto tune. The oven temperature was programmed from 60 $^\circ\text{C}$ (hold for 2 min) to 280 $^\circ\text{C}$ at a rate of 10 $^\circ\text{C}/\text{min}$. The crude samples were diluted with appropriate solvent (1/100, v/v) and filtered. The particle-free diluted crude extracts (1 μl) were taken in a syringe and injected into injector with a split ratio 30:1. All data were obtained by collecting the full-scan mass spectra within the scan range 40-550 amu. The percentage composition of the crude extract constituents was expressed as a percentage of the peak area. The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature .All the solvents and chemicals used were of analytical grade.

Preparation of fortified cookies with juice

All the ingredients used in biscuits were purchased at the local market. Differences in biscuits' composition are shown in Table (1). The dough was sheeted using a rolling pin to a thickness of 3.0 mm. Cookies were shaped with a cutter of 50-mm diameter and baked on aluminum trays at 205 $^\circ\text{C}$ for 8 min, cooled then stored in airtight containers. Then the total fungal count (CFU/ml) and panel tests were performed for all samples.

Table 1. Composition of control and concentrated Pomegranate juice biscuits (percentages are expressed on the basis of the total mass of used flour).

Ingredient	Pomegranate juice concentrations (%)				
	Control	15	18	21	24
White wheat flour	100	100	100	100	100
Vanilla	0.5	0.5	0.5	0.5	0.5
Oil	20	20	20	20	20
Egg	20	20	20	20	20
Sugar	20	20	20	20	20
Baking powder	2.5	2.5	2.5	2.5	2.5
Concentrated Pomegranate juice	0.0	15	18	21	24
Water	As needed				

Sensory Evaluation of fortified cookies

Sensory evaluation for the appearance, color, taste, odor, texture, and overall acceptability were done in order to determine consumer acceptability. The tests were performed after preparation (zero time) and after 45 days of storage at room temperature (20 $^\circ\text{C}$, 70-75% RH). A scale ranging from 1 to 10 (1 is very bad and 10 for excellent) was used for sensory evaluation. Twenty panelists participated in the test. A preliminary experiment was done to choose the suitable PJ – concentrations, as 15, 18, 21 and 24%. The biscuit samples were blind-coded by using numbers and presented to the assessors in random order on paper plates. Water was served to the assessors for cleansing the palate while testing different samples. The control biscuit was introduced in evaluations randomly among other samples. Then stored even 45 days to determine the total count of fungi and the sensory evaluation.

Statistical analysis

Five replicates were performed for each analysis and the values were expressed as mean \pm S.D. One-way analysis of variance (ANOVA) with significant differences between means determined at $p < 0.05$.

RESULTS AND DISCUSSION

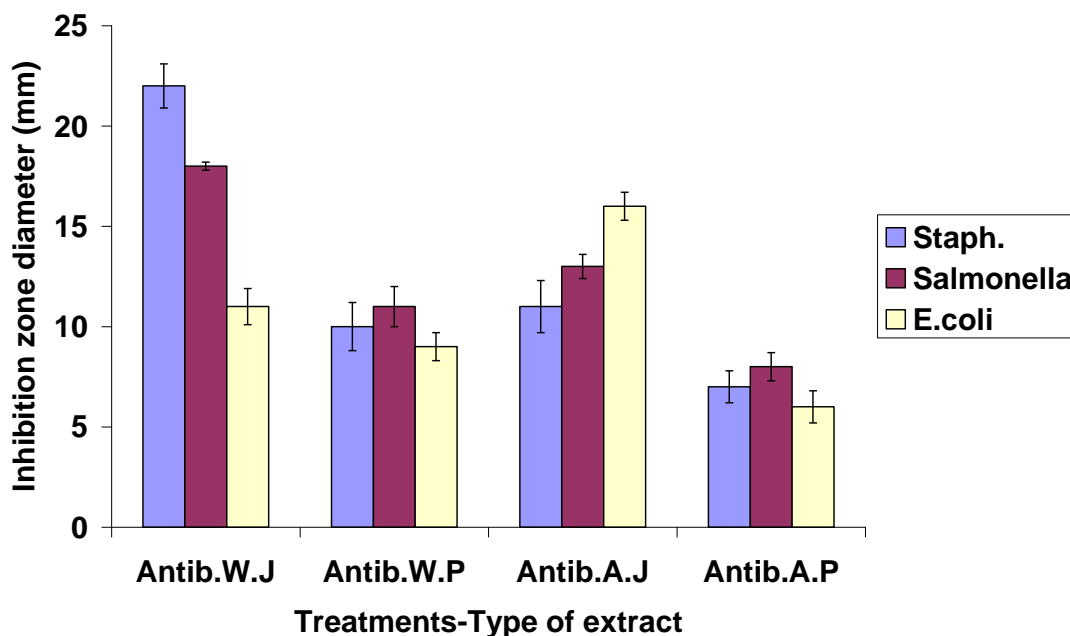
Antimicrobial activities (AA) of PM extracts

The PM is a polyphenol-rich fruit, in the past decade, most studies before using solvent extracts instead of fresh PMJ. We used pomegranate extract by water, alcohol either for juice (PMJw and PMJa) or peel (PMPw and PMPa) to establish in vitro activities against bacteria and fungi (Table 2 and Figs.3&4). Its worth to mention that juice extract contained seeds residue .These may be explained the strong efficacy of juice extract to inhibit the microorganisms due to presence high content of polyphenoles, tannins and flavonoids compounds.

Table 2. Antibacterial and antifungal activity of *Punica granutam* extracts.

Type of extract	Antibacterial activity			Antifungal activity			
	Inhibition zones diameter (mm)*			Inhibition zones diameter (mm)*			
	<i>Staph. aureus</i>	<i>Salm. typh.</i>	<i>E. coli</i>	<i>A. flavus</i>	<i>A. parasiticus</i>	<i>A. niger</i>	<i>P. digitatum</i>
Water juice peel	22 \pm 1.1	18 \pm 0.2	22 \pm 0.9	11 \pm 1.1	15 \pm 0.5	14 \pm 1.0	17 \pm 1.2
Alcohol juice	11 \pm 1.3	13 \pm 0.6	16 \pm 0.7	9.0 \pm 1.4	10 \pm 1.1	11 \pm 1.0	13 \pm 1.2

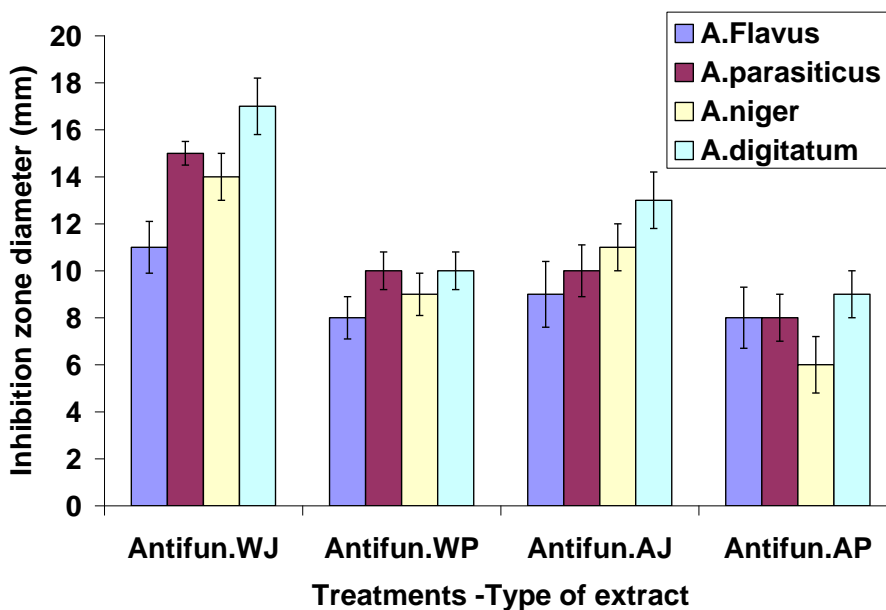
* Inhibition zones diameter (mm) \pm SD



Antib.Wj: water juice extract
Antib.AJ : Antib alcohol juice

Antib.WP: water peel extract
Antib.AP : alcohol peel extract

Figure (3). Antibacterial effect of pomegranate extract.



Antifun.Wj : water juice extract
Antifun.AJ : alcohol juice

Antifun.WP : water peel extract
Antifun..AP : alcohol peel extract

Figure (4). Antifungal impact of pomegranate extract.

AA of PMJ was demonstrated by a clear zone of inhibition in plates inoculated with extract, the highest values of inhibition zone diameter (IZD) were significantly observed against *Staph. aureus* and *E. coli*, which recorded (~22mm) and *S. typh.* which was, 18mm (Table 2 & Fig. 3). The same extract was more effective against fungi, whereas IZD values were highest against *P. digitatum* (17mm), and recorded (15mm) and (14mm) with *A. parasiticus* and *A. niger*, respectively (Table 2 and Fig.4). These results are reasonable due to high concentration of TSS% in PMJw than the PMJa which were 20.0 mm and 0.12 mm, respectively. The obtained results proved that PMJw was more effective in controlling the tested of pathogenic microbes either bacteria or fungi than PMP. Similar findings have been reported by [13, 14, and 15]. The antimicrobial action of the PM extract may be attributed to the presence of tannins compounds [16]. Many workers proved the potentiality of PM extract for inhibition growth of different microorganisms [17, 18].

GC / MS analysis of PM- Phenolic compounds

Polyphenols, including flavonoids, are bioactive compounds that display a number of biological activities which have been reviewed before. It has a substantial amount of polyphenols such as ellagic acid (EC), ellagitannins (EGT) and Gallic acids (EG) [6].

GC-MS analysis proved presence of different derivatives of major phenols, as shown in Tables (3and4). The phenolic compounds only with retention time (Rt) per minutes as obtained in peel, juice extracts of PM besides the mass spectrum of some derivatives are shown in Figs (5 to 9). As shown in Table (3), some identified phenolic compounds are present from PMP extract as observed at Rts (4.6,11.7,14.50 and14.58 minutes) with a high percentage as (37.01%, 23.62%, 5.38% and 30.80%), respectively. The chromatogram of these compounds as in Figs (5 to 7), its identified as (7-Hydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one), 2-[4-(2-Methoxyphenyl)-3H-1,5-benzodiazepin-2-yl]phenol and Phenol, 2,4-di-tert-butyl(peel extract,30.81%). Besides, Zapotin which present in the PMP extract as traces (0.46%) at Rt (11.58). The last one was observed as flavones in PMP by [19]. The obtained other compounds were identified in PMP extracts by workers [20,21].

The phenolic compounds of PMJ extract (juice, seed residue and white peels) contain phenolics, flavonoids, ellagitannins (ETs), and proanthocyanidin compounds. The derivatives of major compounds as Punicalagin, one of ellagitannins (ETs), then produce derivatives, as a glycone-sugars moiety of glycoside as DL-Xylose (11.06 %) and 1-Deoxy-d-arabitol (17.88%)-(Fig. 9), respectively. Also, major derivatives were as

recorded from juice extract, as 2-(3-Hydroxy-3-phenylpropyl) phenol) which occupied 57.36% (Rt=4.9) (Fig.8), besides, the other phenol compounds at Rt 5.24 and 5.29 minutes (Table 5). Similar results were obtained by [22, 23, and 24].

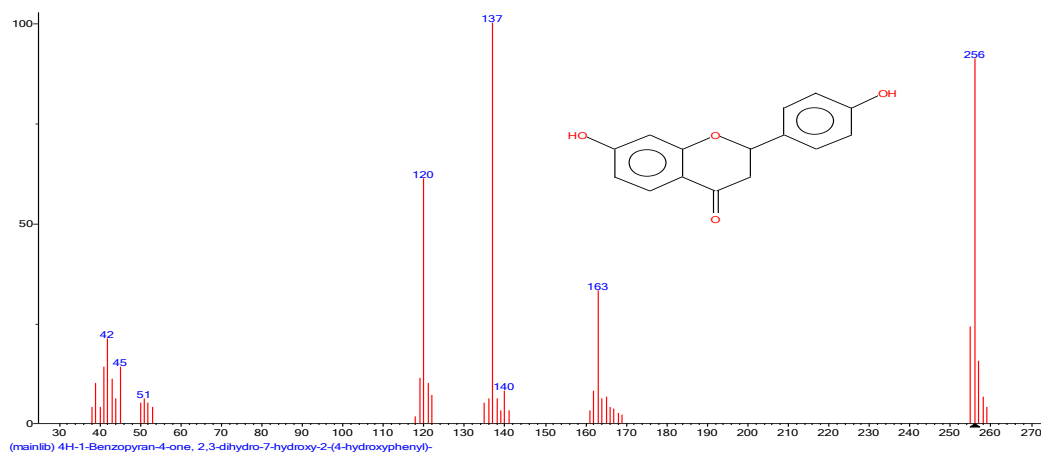


Figure (5).7-Hydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4H-chromen-4-one (peel extract,37.01%).

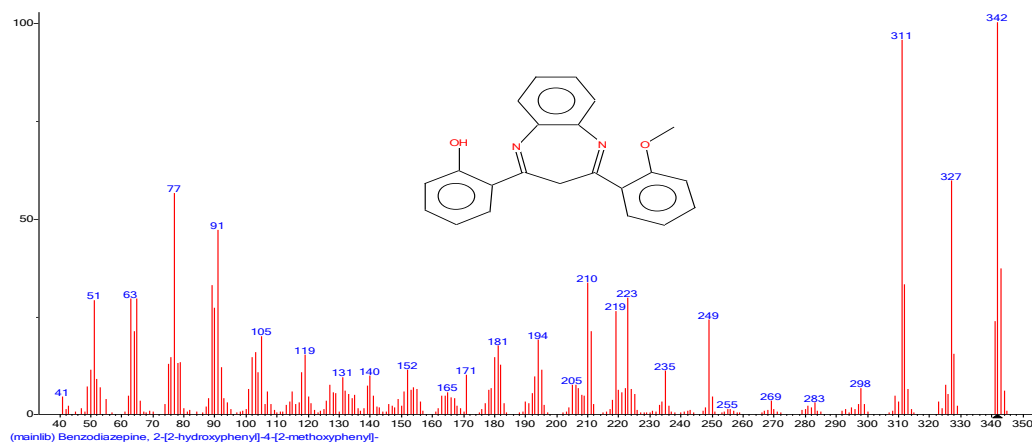


Figure (6). 2-[4-(2-Methoxyphenyl)-3H-1,5-benzodiazepin-2-yl]phenol (peel extract,23.62%).

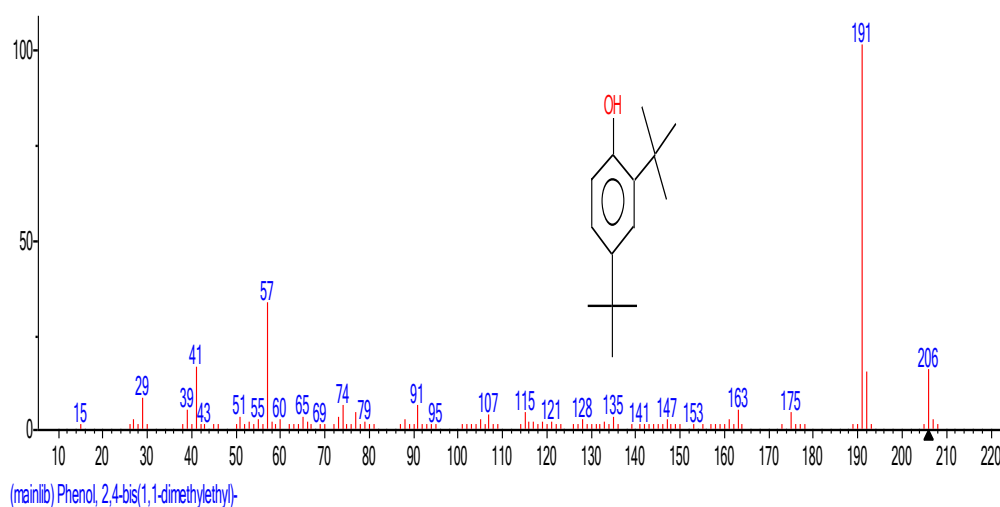


Figure (7). Phenol, 2,4-di-tert-butyl(peel extract,30.81%).

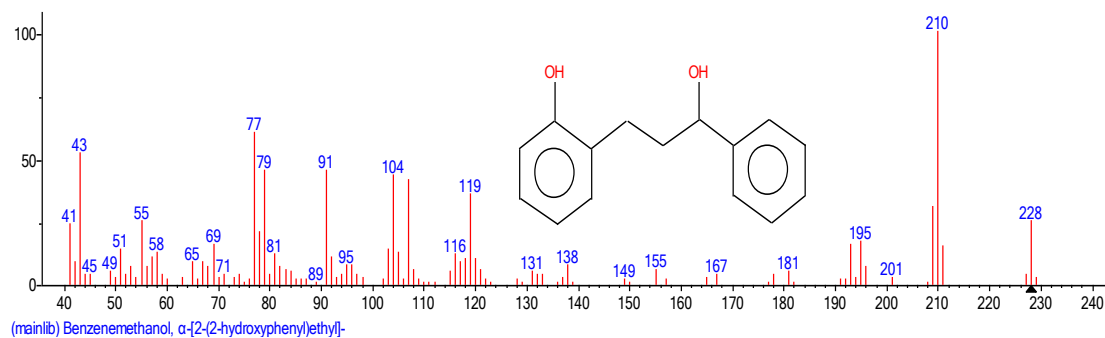


Figure (8). 2-(3-Hydroxy-3-phenylpropyl)phenol (juice extract,57.36%).

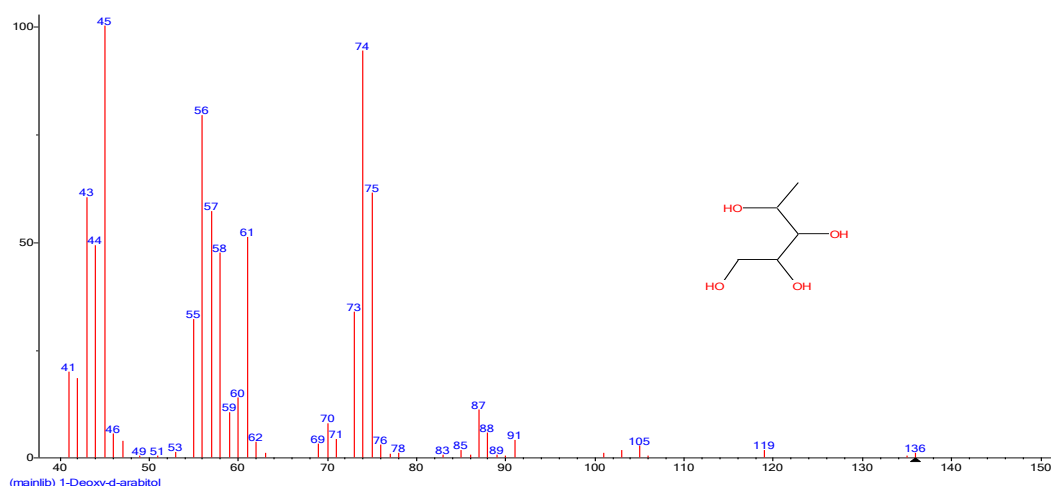


Figure (9) .1-Deoxy-d-arabitol (juice extract, 17.88%).

Table 3. GC-MS analysis of peel compounds of pomegranate.

Retention time (Rt) per minutes	Compound	(%)
4.66	7-Hydroxy-2-(4-hydroxyphenyl)-8-isopentyl-5-methoxy-2,3-dihydro-4H-chromen-4-one	37.01
11.70	2-[4-(2-Methoxyphenyl)-3H-1,5-benzodiazepin-2-yl]phenol	23.62
11.58	Zapotin	0.46
14.50	2,6-Dibutylphenol	5.38
14.55	Phenol, 3,5-bis(t-butyl)	0.86
14.58	Phenol, 2,4-di-tert-butyl-	30.81
17.65	Chromone, 5-hydroxy-6,7,8-trimethoxy-2,3-dimethyl-	1.85

Table 4. GC-MC analysis of juice compounds of pomegranate.

Retention time (Rt) per minutes	Compound	(%)
3.18	DL-Xylose	11.06
3.38	1-Deoxy-d-arabitol	17.88
3.80	o-Acetyl-L-serine	1.44
4.69	Phenol, 4-(2-aminopropyl)-	0.04
4.90	2-(3-Hydroxy-3-phenylpropyl)phenol	57.36
5.24	4-t-Butyl-6-dimethylaminomethyl-2-[4-dimethylaminophenyl]phenol	3.12
5.29	Phenol, 4-[2-(5-nitro-2-benzoxazolyl)ethenyl]-	0.87
7.15	l-Gala-l-ido-octose	7.17
7.33	2-Octanone, 1-phenyl-	1.04
	Total	99.98%

Table 5. Sensory characteristics of fortified cookies with PJ (%.)*

PJ (%) to wheat flour	Mean \pm SD					
	Appearance	Color	Taste	Odor	Texture	Overall acceptability
Control	8.5 ^a \pm 1.1	8.75 ^a \pm 1.3	9.12 ^a \pm 0.83	9.0 ^a \pm 0.8	8.75 ^a \pm 1.3	8.50 ^a \pm 1.0
15%	7.8 ^{ab} \pm 1.1	8.25 ^a \pm 0.9	9.0 ^a \pm 0.9	8.62 ^a \pm 0.9	8.62 ^a \pm 1.1	8.12 ^a \pm 1.0
18%	7.0 ^b \pm 0.8	7.12 ^b \pm 0.8	8.55 ^a \pm 0.5	8.12 ^a \pm 0.9	8.25 ^a \pm 1.0	8.62 ^a \pm 0.7
21%	7.25 ^b \pm 0.7	7.25 ^b \pm 0.7	8.37 ^b \pm 0.9	8.09 ^a \pm 1.1	7.87 ^a \pm 1.0	8.37 ^a \pm 1.1
24%	7.17 ^b \pm 1.7	6.33 ^b \pm 1.6	8.50 ^b \pm 1.4	8.03 ^b \pm 1.5	7.67 ^b \pm 1.0	8.00 ^a \pm 1.1

* No statistical differences at the 5 % level between means with same letter.

Sensory and safety evaluation of fortified cookies with PMJ

Organoleptic analysis of fortified cookies

Sensory analysis was based on appearance, color, taste, odor and overall acceptability. In general, no significant difference at zero time for overall acceptability between samples comparing with control (Table 5). Whereas, control, low PMJ levels (15% and 18%) were distinguished in taste, odor and texture. The scores decreased in both the control and fortified sample, during the storage period even 45 days. Initially, the control and fortified samples were recorded sensory scores less than zero time. After 45 days of storage at room temperature, the obtained results by panelist as shown in (Fig. 10), proved that 15 % was the best one significantly in all characters and near control (values ~9%). The appearance values were scored high values significantly in cookies fortified with PMJ 15%, 18% , and control than other samples. Yellow attractive color was present in control, then 15% samples as in photos (Fig.11). The color change in other samples gradually from light to dark gradually with increasing PMJ content, due to increase tannic and phenols content.

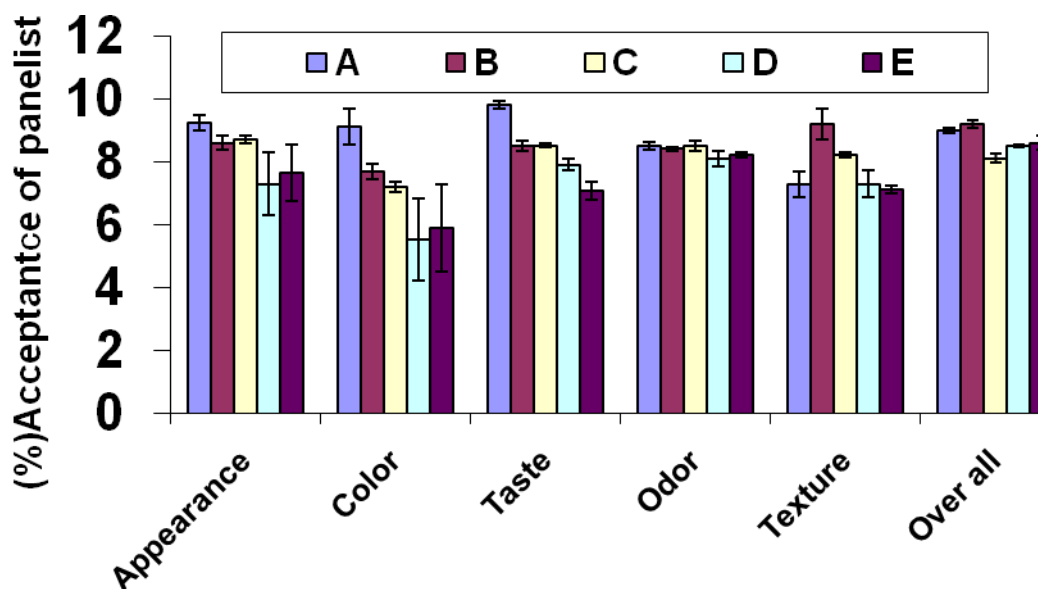


Figure (10). Sensory characteristics evaluation of fortified cookies with PJ (%) at end of storage 45 days at room temperature (20-25°C, 70-75%RH).

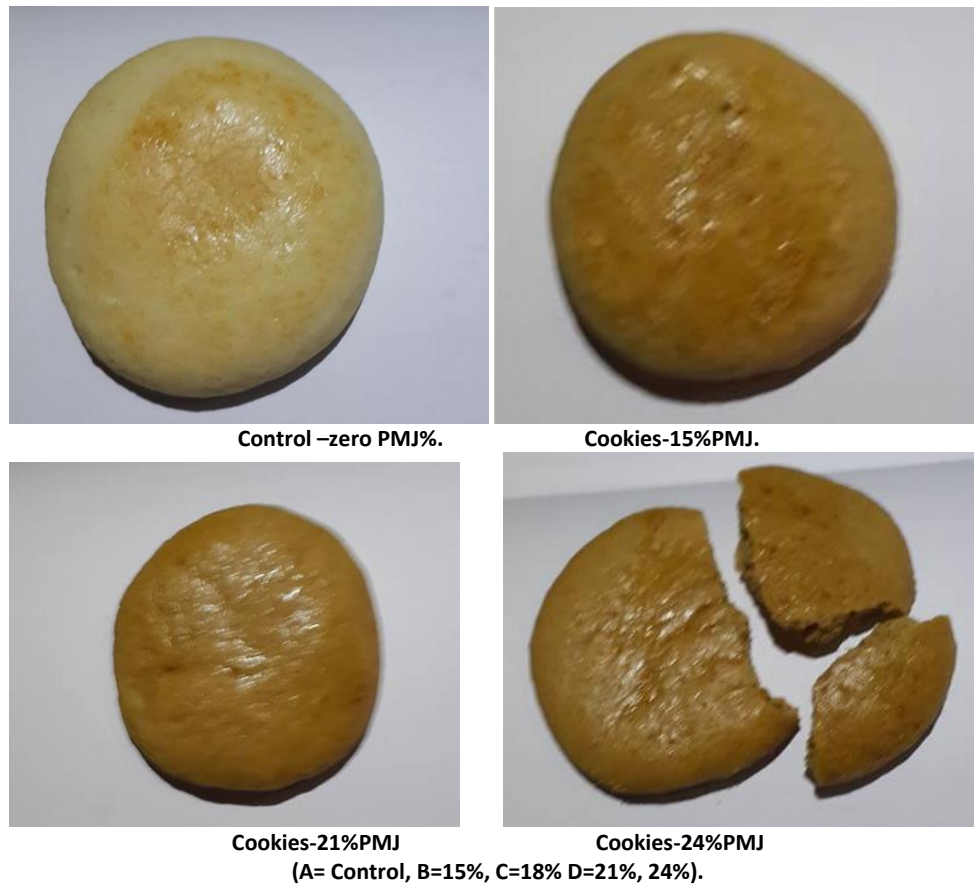


Figure (11). Photos of cookies fortified with PMJ (%) after 45 days of storage.

The same trend was clear in taste, whereas, control-free PMJ was the best accepted taste, but bitterness increased with increasing PMJ concentration, near control values were recorded in 15 % samples. No differences were observed in the odor of cookie samples. Concerning, the texture, whereas, cracking was obviously with 24%, but not presence in other samples. Same trends were observed during storage samples, even 45 days, whereas all tested samples of cookies were without crack, high cohesion in control, all tested samples except 24%. The bitterness may be preventing cohesion of cookies.

These results proved the suitability of fortified PMJ with 15% as critical level. Our results introduced first trial to use juice not peel which usually added, as by-product of juice processing industries, but the fortified products became more bitterness, then rejected by consumers [25]. A similar trend was recorded by workers to use PM extract for supplementation in different foodstuffs [26]. Some reported a significant reduction in color scores of cookies supplemented with B-carotene rich pumpkin peel powder and carrot pomace powder [27].

Total count of fungi (CFU) of cookies

As shown in Fig.12, the total fungi of cookies after storage for 45 days at room temperature increased dramatically, whereas, the supplemented cookies at all concentration were less than control .The low load with fungi was 21% and 24%, then 15%, respectively. Polyphenols content protects the prepared cookies against microorganisms and inhibited growth of microorganisms as proved by workers [15]. Incorporation of natural ingredients bearing antioxidant properties or synthetic antioxidant prevents nutritive losses by retarding or inhibiting oxidation reactions [28]. However, synthetic antioxidants have been reported as controversial with respect to their health safety for utilization in food products [29]. PMJ is a plentiful source of phytochemicals more specifically ellagitannins that impart characteristic free radical scavenging properties.

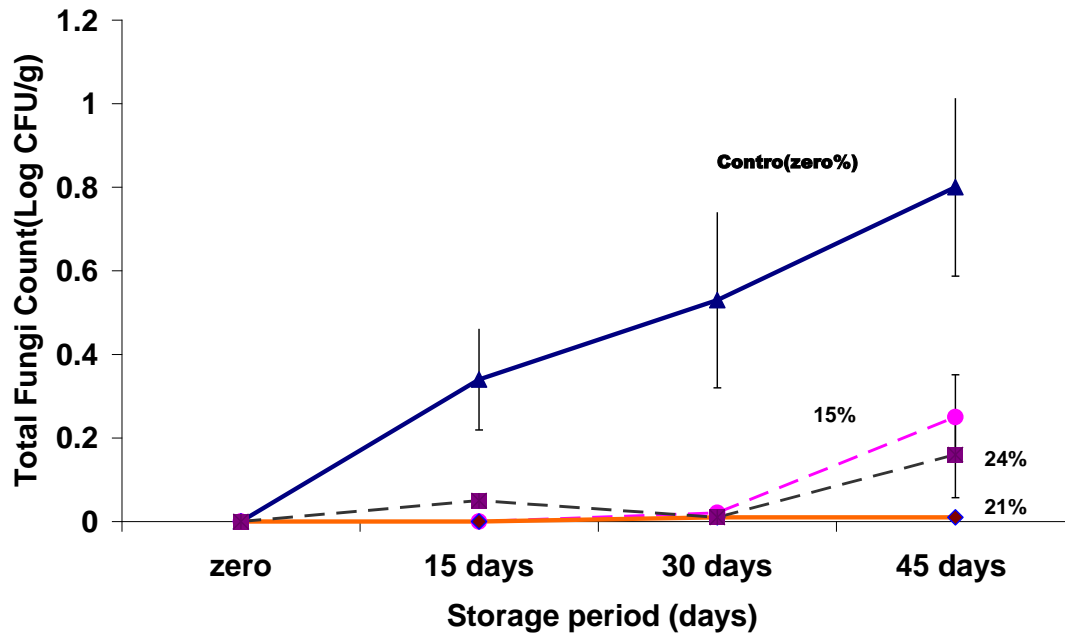


Figure (12). Total fungi load of cookies fortified PMJ during storage period at room temperature for 45 days.

CONCLUSION

Finally, it could be recommended that supplementation with 15% Juice of pomegranate can improve the safety and quality of cookies. Juice as reported in our chemical analysis has the antibacterial activities due to presence different phenols. Also, arils are edible part of PMJ, contains sugars, phenols with less bitterness than peel-inedible part, therefore juice extract has the prefer ability to use in the present work for supplementation of cookies. Workers used pomegranate by products for supplementation of cookies, but consumers rejected samples due to bitterness. Addition that PMJ (15%) improved significantly the sensory attributes (appearance, color, taste, texture and flavor) as compared to the control sample. Increasing the concentration impaired cookies quality due to high bitterness and increase cracking. Therefore, 15% are critical level to use for increasing antibacterial force and accepted overall acceptance by consumers even long storage time.

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