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Research Article
Using the Essential Oil of *Micromeria barbata* Plant as Natural Preservative to Extend the Shelf Life of Lebanese Yogurt

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Abstract

Background and Objective: Yogurt is a traditional food that has been considered for a long time as a part of the human meal for thousands of years and even worldwide. However, this fermented food could be spoiled by molds and yeast flora and thus the shelf life will be reduced. This study was conducted to extend the shelf life of Lebanese yogurt by a natural preservative, *Micromeria Barbata* Essential Oil (EO) without changing of organoleptic properties as well as without LAB flora's eradication. **Materials and Methods:** The *M. barbata* EO was assessed with two non-cytotoxic concentration (0.125 and 0.25 µL/100 mL). Furthermore, several cultures have been realized in order to quantify the LAB flora as well as molds and yeast in different storage periods. **Results:** The *M. barbata* EO used at 0.125 µL/100 mL can be used in order to increase the shelf life of yogurt for up to 70 days for sealed samples and 21 days for opening samples. The study also revealed that titratable acidity values after 70 days storage have increased from 0.9% (day 1) to 1.35±1% (day 70). It was observed that the EOs used at 0.125 µL/100 mL did not affect the growth of starter LAB in yogurt samples, but it showed the strongest antifungal activity comparing with the control free oil samples. **Conclusion:** The sensorial analysis proved that yogurt containing 0.125 µL/100 mL EO was organoleptically acceptable and it had a good body and texture that was similar to the untreated one.

Key words: *Micromeria barbata*, essential oil, shelf life, bio-preservative, yeasts, molds

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Yogurt is a kind of fermented milk product consumed in middle east countries without any additive such as sugar, taste or any preservative. Furthermore, the consumption of yogurt, like other dairy fermented product, represents a great opportunity in maintaining the human health[1,2]. Indeed, yogurt is a rich source of protein, potassium, calcium, phosphorus and vitamins[3] B_2 and B_{12} as well as a source of Lactic Acid Bacteria (LAB) which are considered as beneficial microorganisms and could have probiotics properties[4]. Because of these known health benefits of yogurt, its consumption has been increased and became the main dairy product in the global market[5]. Yogurts are now being manufactured in a numerous styles and varieties with different fat contents, flavors and textures suitable for different meal occasions and plates as a snack, dessert, sweet or savory food. In middle east countries, crude yogurt (as fermented milk) is consumed every day and sometimes several times daily. Although, the acidic properties of yogurt, it remains a good environment to many kinds of spoilage micro-organisms such as yeast and molds[6]. Besides yeast and molds, the uncontrollable concentration of LAB could also lead to a modification of some organoleptic properties such as acidity leading to the reduction of yogurt shelf life[7]. Growth of these micro-organisms can cause negative sensory attributes, gas production, changes in color, breaking of emulsions and decreases in pH during the storage[7]. Indeed, due to the high consumption of yogurt, the need of shelf life extension remains critical for dairy products industries as well as for consumers[8]. In general, the ideal shelf life once refrigerated is one month, after which there is a discernible reduction in sensory and microbiological characteristics which can cause measurable economic losses to the producer[8]. Therefore, manufacturers overcome this problem by treating the yogurt with chemical preservatives which are not acceptable by consumers due to their undesirable effects[9]. For example, chemical preservatives can present some problems like benzene, one of the most carcinogenic substances can be formed from benzoïc acid in foods by decarboxylating action of some spoilage micro-organisms[10]. In addition, *Saccharomyces cerevisiae* and *Pichia anomala* are able to decarboxylate sorbic acid to 1,3-pentadiène causing a kerosene-like off-odor[11]. Furthermore, the World Health Organization has recently called for a worldwide reduction in the consumption of salt in order to reduce the incidence of cardiovascular disease[12]. In this context, bio-conservation of food remains the solution to replace all chemical preservative. Natural preservatives such as antimicrobial peptides (bacteriocins) and plant essential oils could be alternative to chemical preservative in food sector[13,14].

Essential oils are plant-derived volatiles extracted from different parts of plants, for example, leaves, peels, barks, flowers, seeds and roots by several methods, such as; steam distillation, hydrodistillation and others[15]. Some essential oils are classified as generally recognized as safe (GRAS) by the US Food and Drug Administration[16]. They are known to possess antioxidative, antibacterial and antifungal properties[17]. Such biological activities are ascribed to the chemical complexity of these products, the resultant activity may be the consequence of the synergistic effect of two or more components acting on different cellular targets[18].

This study was designed to use a novel essential oil extracted from *Micromeria Barbata* regional plant, at 2 different concentration 0.125 and 0.25 µL/100 mL as natural preservative to extend the shelf life of yogurt without causing any organoleptic alteration to the product. Therefore, titratable acidity, sensorial characteristics as well as microbiological analysis (LAB, yeast and molds) were evaluated in this current study.

MATERIALS AND METHODS

This study was conducted at Quality Control Center (QCC) laboratories, Chamber of Commerce, Industry and Agriculture of Tripoli and North Lebanon from March-August, 2019.

Essential oil extraction: Essential oil was extracted by hydrodistillation technique from a *M. barbata* plant that live in the rocky mountain area of North Lebanon in 1700 m above sea level. The dried plant was powdered and used for extraction by using a hydrodistillation technique during 3-4 h in an all-glass Clevenger type apparatus (VWR, USA) that separates water from oil. The extracted oil was stored in a dark glass tube and kept under refrigeration between 4-8°C until analysis as previously described by Elyemni *et al.*[19].

Extract Oil (EO) cytotoxicity test: The cytotoxicity test was conducted as described by Kumar *et al.*[20]. The cell lines used in this test was kidney tubular cells and hepatocytes tubular cells (ATCC, USA). Several dilutions (10, 20, 50 and 100 µg mL^{-1}) of *M. barbata* EOs with complete medium were added to different cell lines. Plates were incubated at 37°C for 48 h in CO_2 incubator. The MTT assay was performed by using 96-well plates (Corning Costar, USA) at 37°C with 5% CO_2, for

72 h. The cytotoxicity of *M. barbata* oils on different cell lines at different dilutions was evaluated. The cell survival was identified by MTT assay and the color intensity was quantified by absorbance measurement at 570 nm.

Manufacture of Lebanese yogurt: Yogurt was manufactured according to Tamime and Robinson[21]. Briefly, fresh cow milk (full fat) was pasteurized at 90°C for 5 min and then cooled to 40-45°C. Instead the commercial LAB starter, 3% of previously made yogurt was added to the pasteurized milk and mixed followed by an aerobic incubation for 8 h till texture modification. Finally, the plastic containers were cooled and stored at 5±1°C for 75 days.

The pasteurized pre-yogurt was divided into three equal portions. The first portion was served as control (untreated yogurt), second and third portions were supplemented with 0.125 and 0.25 µL/100 mL of *M. barbata* essential oil, respectively called EO-treated yogurt.

Microbial analysis: Ten grams from yogurt samples were pooled in 90 mL of sterile peptone water (Bio-RAD, France) in sterile 500 mL stomacher bags (Interscience, France). Samples were blended in a stomacher (Interscience, France) for 3 min.

The LAB counts were determined on MRS agar (Scharlau, Spain) after incubation at 37°C under anaerobic conditions for 3 days as previously described with some modification[22]. In addition, yeast and molds (Y and M) counts were carried out on Yeast Glucose Chloramphenicol agar (Bio-RAD, France) after incubation at 25°C for 5 days[23].

The number of colonies (LAB or Y and M) that appeared on the plates was counted and expressed as Colony Forming Units (CFU mL^{-1}) by using plate counter reader (Selecta, Spain).

Titratable acidity in stored yogurt: The acidity of yogurt was assessed in all storage duration by using the method of titratable acidity as described before by LibNOR organization (http://www.libnor.gov.lb/). One gram of yogurt sample was added with 10 mL of distilled water followed by the addition of 2 mL of phenolphthalein reagent (Sigma-Aldrich, Germany) as a pH indicator. Titrations were performed with 0.1 N of NaOH (Sigma-Aldrich, Germany) solution. According to Lebanese Standards (http://www.libnor.gov.lb/), Titratable Acidity (TA) of yogurt shouldn't be more than 1.5% expressed as lactic acid concentration.

Sensory evaluation test: The sensory effects after EO treatment were assessed using an acceptance test as previously described with some modification[24]. Yogurt samples with 3 different concentrations of EO: C_1 = 0 µL/100 mL oil-free sample used as control, C_2 = 0.125 µL/100 mL and C_3 = 0.25 µL/100 mL were equally pooled in plastic containers coded with three-digit random numbers. The sensory evaluation was performed by 100 volunteers of both sexes, aged 18-75 years old. Each volunteer evaluated the samples by rating them using a five-point scale where, 5 = Like, a lot and 1 = Dislike, a lot for various characteristics such as; color, odor and flavor[25].

Extraction of *M. barbata* EOs: The Clevenger hydro distillation of *M. barbata* plant gave 2% yield essential oil that was stored immediately at 4°C. The composition of the essential oil was already determined by using GC-MS method[26].

Statistical analysis: No statistical analysis was done unless the calculation of SD and create Graphs by using Microsoft Excel.

RESULTS

***M. barbata* essential oil cytotoxicity:** The MTT assay was used to evaluate the cytotoxicity of *M. barbata* EO against human kidney and hepatocytes tubular cells at the tested concentrations. As shown in Fig. 1, the rate of cell survival was marked apoptosis in a dose-dependent manner was recorded in the treated cell lines with different dilutions of EO. Results showed that at the concentrations of 10^4 µL/100 mL (1/10) and 2 µL/100 mL (1/500), the cytotoxicity rates were 60 and 10%, respectively.

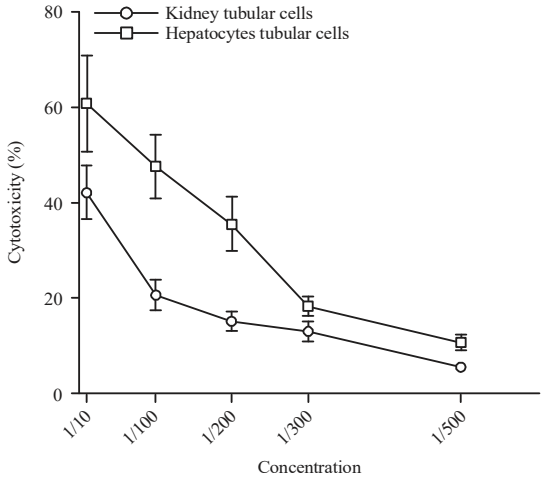

Fig. 1: Cytotoxicity of *M. barbata* EO by using MTT method

Table 1: Effect of *M. barbata* plant EO on the counts of yeast and molds in yogurt after 25 days of opening at 5±1°C

		Yeasts and molds	
Days	Control	0.125 µL/100 mL	0.25 µL/100 mL
1	-	-	-
4	-	-	-
7	-	-	-
11	-	-	-
14	More than 1000 CFU mL^{-1}	-	-
18	More than 1000 CFU mL^{-1}	-	-
21	More than 1000 CFU mL^{-1}	100±10 CFU mL^{-1}	100±10 CFU mL^{-1}
25	More than 1000 CFU mL^{-1}	More than 1000 CFU mL^{-1}	More than 1000 CFU mL^{-1}

EO: Essential oil

Table 2: Results of yeast, molds and LAB counts in treated and untreated yogurt stored at refrigerator (5±1°C) for 70 days

	Control		0.125 µL/100 mL		0.25 µL/100 mL	
Days	Y and M	LAB	Y and M	LAB	Y and M	LAB
1	NCD	5.64×10^7	NCD	5.66×10^7	-	5.97×10^7
15	NCD	2.38×10^7	NCD	3.45×10^7	-	$2,55\times10^7$
30	NCD	3.35×10^7	NCD	2.92×10^7	-	3.15×10^7
37	NCD	3.39×10^7	NCD	3.14×10^7	-	3.80×10^7
42	More than 1000 CFU mL^{-1}	3.28×10^7	NCD	3.10×10^7	600 CFU mL^{-1}	3.57×10^7
47	More than 1000 CFU mL^{-1}	NCD	NCD	NCD	1400 CFU mL^{-1}	NCD
49	More than 1000 CFU mL^{-1}	NCD	NCD	NCD	25000 CFU mL^{-1}	NCD
54	More than 1000 CFU mL^{-1}	NCD	NCD	NCD	27000 CFU mL^{-1}	NCD
61	More than 1000 CFU mL^{-1}	NCD	NCD	NCD	13000 CFU mL^{-1}	NCD
70	More than 1000 CFU mL^{-1}	2.6×10^6	NCD	2×10^6	5800 CFU mL^{-1}	4×10^5
75	More than 1000 CFU mL^{-1}	NCD	1200 CFU mL^{-1}	NCD	1900 CFU mL^{-1}	NCD

NCD: No-culture detectable

Effect of EO on microbial growth after 25 days of refrigerated yogurt after opening: Microbiological analysis of treated yogurt (with 0.125 and 0.25 µL/100 mL of *M. barbata* EO) and untreated yogurt stored at 5±1°C (cold storage conditions) were conducted for 25 days.

The results showed that starter bacteria count was slightly decreased in treated as well as in untreated yogurt during the 25 days (Fig. 2). As Fig. 2 shown, starter LAB counts indicated that in untreated yogurt and after one day of opening, a count of 5.64×10^7 CFU mL^{-1} was observed and then it was changed to 3.04×10^7 CFU mL^{-1} after 21 days of opening.

Similarly, the LAB counts in yogurt supplemented with 0.125 and 0.25 µL/100 mL decreased from $\approx5.97\times10^7$-$\approx3.46\times10^7$ CFU mL^{-1}, respectively after 21 days of opening.

In addition, Table 1 showed that yeast and mold counts were less than 1000 CFU mL^{-1} (100 CFU mL^{-1}) in yogurt treated with both EOs concentrations after 21 days of opening. However, in untreated yogurt, yeast and mold count became above 1000 CFU mL^{-1} after 14 days of opening.

Effect of EO on microbial growth after 70 days of refrigerated sealed yogurt (s-yogurt): The LAB count was determined in s-yogurt samples after 1, 15, 30, 37, 42 and 70 days of cold storage. The results showed that in EO-treated and untreated yogurt, LAB counts decreased gradually during 75 days of storage to be no detectable on the 47th day (Table 2). As shown by Table 2, on the 70th day, the LAB was regrew and then disappeared in the 75th day of storage.

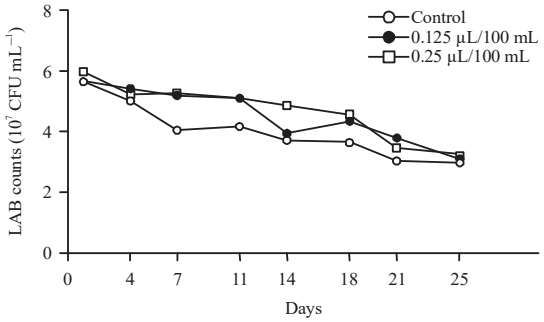

Fig. 2: Effect of *M. barbata* plant EO on the count of LAB culture bacteria in yogurt after 25 days of opening

On the other hand, yeast and mold which are considered as indicators of the yogurt spoilage were measured during 75 days of cold storage. In this regard, fungal colonies were not detected in s-yogurt containing 0.125 µL/100 mL of *M. barbata* EO throughout 70 days of cold storage, whereas their concentration exceeded the acceptable level in the untreated control as well as the 0.25 µL/100 mL EO-treated s-yogurt after 42 and 47 days of storage, respectively (Table 2).

Table 3: Effect of *M. barbata* EO concentrations on sensory evaluation of yogurt

Sensory characteristics	Control	0.125 µL/100 mL	0.25 µL/100 mL
Color	White	White	White
Odor	Pleasant	Pleasant	Pleasant
Taste	Sweet	Sweet	Sour
Total score	4.09/5	4.01/5	3.58/5

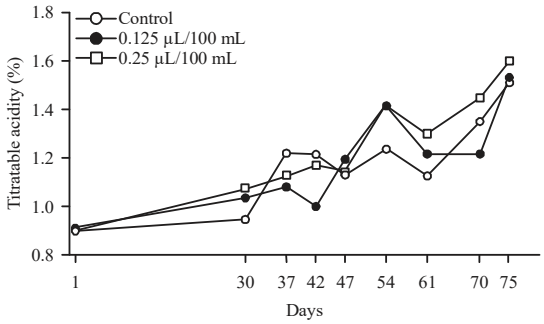

Fig. 3: Determination of titratable acidity in yogurt samples during storage at refrigerator for 75 days

Changes in yogurt acidity during storage: The effect of storage on the Titratable Acidity (TA) values of EO-treated and untreated yogurt samples are presented in Fig. 3. A gradual increase in TA along the storage period was observed. As shown in Fig. 3, the initial TA values for the different yogurt types were at around 0.9% at the first day of storage whereas, TA was changed to 1.35, in untreated yogurt after 70 days of cold storage. However, the EO-treated yogurt showed an increasing of TA reaching 1.53 (0.125 µL/100 mL) and 1.6% (0.25µL/100 mL).

Sensory evaluation of yogurt samples: As shown in Table 3, there were no considerable differences in the color and odor of the EO-treated samples as compared with the untreated yogurt. However, there was noticeable differences in flavor in only EO-treated yogurt with 0.25 µL/100 mL.

DISCUSSION

In the last 10 years, yogurt has been preferred by consumers due to its beneficial effect on their health[27]. Yogurt is a traditional food and has nutritional and sensory properties[28] and a good medium for probiotic culture[29]. The excessive consuming of yogurt forces the industries to find out preservation techniques to improve the quality with boosting durability of yogurt and thus enhancing its shelf life. One of the methods of preservation in dairy products is the use of chemical additives such as; natamycin, sodium benzoate (E211) and potassium sorbate (E202)[9] in order to inhibit Y and M growth and consequently extend the yogurt shelf life. In order to extend food shelf life with natural preservative, scientists have investigated several natural substances as eventual natural preservative including EOs[17]. Although the food industry primarily uses essential oils as flavorings, they represent an interesting source of natural antimicrobials for food preservation[14].

To determine the minimal concentration of *M. barbata* EO to be used in this study, the cytotoxicity test was conducted. Results showed that the concentration of 2 µL/100 mL was non-cytotoxic (10% of cell death) (Fig. 1). Moreover, this concentration is more than ten times the concentration used in yogurt samples.

The addition of EOs to food aroused great interest because of their antagonistic action against pathogenic and spoilage micro-organisms[30]. However, this action may affect the LAB presence in fermented dairy product[31]. In this study, results showed there was a decreasing of LAB count to 3.04×10^{7} CFU mL^{-1} after 21 days of opening in untreated and EO-treated samples (Fig. 2). These results showed that the used EO had no activity on LAB strains during 21 days in cold storage. However, increases in the oil concentrations led to decreases in LAB counts (Fig. 2).

In fact, all commercial yogurt preparations available in the Lebanese market claimed that yogurt expires after four days of opening under cold storage conditions.

These results are very suitable because yogurt must contain a minimum of 10^{6}-10^{7} CFU g^{-1} viable and active starter LAB at the moment of consumption according to regulations in various parts of world (http://www.libnor.gov.lb/). This result agreed with previous study[32] that found refrigerated storage decreased the viable counts of LAB during the storage period.

On the other hand, according to Lebanese standards Institution "LibNOR", yogurt must contain no more than 1000 CFU mL^{-1} of yeasts and molds which are deemed responsible for dairy product spoilage (http://www.libnor.gov.lb/). In order to extend the shelf life of yogurt without exceeding the national limit as well as preserving its organoleptic properties. Yogurt samples were treated with 2 different concentrations of EO and examined during 21 and 75 days in opened and sealed situation.

Results showed that the incorporation of *M. barbata* EO into the opened yogurt at different concentrations (0.125 and

0.25 µL/100 mL) significantly inhibited growth of yeasts and molds and prolonged the microbiological shelf life of yogurt for up to 21 days after opening under cold storage conditions (Table 1). In addition, the results showed that storage at 5°C of 0.125 µL/100 mL EO-treated s-yogurt resulted in a dramatic reduction in fungal population (NCD) and consequently this treatment could extend the shelf life of yogurt up to 70 days (Table 2). In similar study, shelf life of concentrated Labneh yogurt, treated with 0.3% of cinnamon EO was extended from 16-24 days when stored at 6°C, thus, yeast and molds were inhibited while an acceptable flavor was tasted[33].

The organoleptic properties modification during yogurt storage was based on titratable acidity and the sensory evaluation conducted by 100 volunteers. Results showed that TA was slightly increased during the 70 days cold storage of EO-treated yogurt compared to the untreated sample (Table 3). According to these results, different concentrations of *M. barbata* EO did not have any significant influence on TA. However, the increase in TA during the storage period was correlated with the production of lactic acid by the starter LAB strains[34].

Similar results were showed in several studies and authors have reported an increasing in TA of yogurt during cold storage in treated and untreated yogurt[25,35,36]. The sensory evaluation of the yogurt samples based on color, odor and taste. This sensory test was carried out by 100 volunteers using 5 points scales. Sensory attributes are of great importance to measure consumer attitudes and their influence on food choice and acceptability. Several study have reported that after 20 days of yogurt cold storage, the organoleptic properties have not affected in untreated and EO-treated yogurt[36,37].

CONCLUSION

The use of *M. barbata* EO in a very low concentration 0.125 µL/100 mL is effective to extend the shelf life of yogurt. The EO-treated yogurt remains without any modification of organoleptic properties and could be cold stored for 21 days in opening mode and up to 70 days in sealed mode. These findings could serve the dairy product industries to use the *M. barabata* EO as natural preservative with high economical income. This study can be extended and continued in all fermented dairy products such as cheese and unpasteurized dairy product.

SIGNIFICANCE STATEMENT

The use of EO in low concentration could be a good natural method to extend product shelf life without any health hazards.

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