Original paper

**Thymbra capitata** Essential Oil Use to Preserve Physicochemical and Microbiological Qualities of Pomegranate Juice

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This work studied the possible use of *Thymbra capitata* essential oil in the preservation of pomegranate juice qualities. We monitored the effect of the oil on the physicochemical and microbiological qualities of the juice during 245 days of storage at 5 and 20 °C. Treated juices showed slight decrease of pH, total sugars and phenolic contents after 245 days of storage. Additionally, the essential oil had a significant effect on the juice microbiological quality and caused a decrease of bacterial count after the first day of storage. In conclusion, *Thymbra capitata* essential oil may be used as natural preservative in pomegranate juice to preserve its physicochemical and microbiological qualities in a long-term storage.

Keywords: *Thymbra capitata* essential oil, pomegranate juice, physicochemical quality, microbiological quality

Introduction

Pomegranate (*Punica granatum* L.) is a valuable medicinal and nutritional fruit due to its antioxidant, antitumor, anthepatotoxic, anti-lipoperoxidative and antibacterial properties (Jafari et al., 2017). Its juice is rich with natural compounds (polyphenols, flavonoids and mineral nutrients) used to assess juice quality and nutritive value (Bursać Kovačević et al., 2016). Nowadays, consummation of fresh fruit juices increased due to their better organoleptic properties than pasteurized ones. However, unprocessed fruit juices raise a serious health concern due to microbial contamination. The presence of acid-loving or acid-tolerant bacteria and fungi (yeasts and molds) causes the deterioration of nutritional and sensorial properties. Furthermore, the presence of pathogenic bacteria causes the emergence of several food illness outbreaks (Tournas et al., 2006). Pasteurization is the wildest technology used to extend juice shelf-life because it can inactivate both microorganisms and enzymes in juice. However, it can also cause undesirable changes; off-flavors development, polyphenols, nutrients and color losses (Ma et al., 2013).

Essential oils (EOs) and their constituents are an interesting alternative to improve fruit juices safety and shelf-life. They have attracted much interest because of their widespread use in perfumes, pharmaceutical products and as food preservatives and additives (Bakkali et al., 2008). The chemical composition and biological activities of Moroccan *Thymbra capitata* essential oil (TCEO) have been studied in previous reports (Bakhry et al., 2013; El Moussaoui et al., 2013; El Ouariachi et al., 2011), however, to the best of our knowledge, no research on the preservation of juices by TCEO has been carried out. The objective of this work was to study the preservation of pomegranate juice by TCEO; we monitored TCEO effect on juice physicochemical and microbiological qualities during 245 days of storage at 5 and 20 °C.

Material and Methods

**Chemicals and media** All the chemicals used in chemical analysis were purchased from Sigma-Aldrich (Steinheim, Germany). All media used in microbiological analysis were supplied by Biokar Diagnostics (Beauvais, France).

**Essential oil extraction** *Thymbra capitata* (L.) Cav. was collected from north Morocco (Benkarich region). Aerial parts were dried at room temperature and used to obtained essential oil by hydrodistillation.

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Juice preparation  Pomegranate juice was freshly prepared and kept in sterile flasks. Final concentrations of 0.125 % and 0.06 % (v/v) of TCEO were added to the juice. Control samples were prepared without TCEO. Juices were stored at 20 and 5 °C.

pH measurement  The pH variation in tested pomegranate juice was measured using a pH meter (Hanna Instruments, Romania) at the beginning and the end of storage.

Determination of total sugars content (TSC)  To evaluate TSC in pomegranate juice samples, the furfural (2-furancarboxaldehyde formula C₅H₄O₃) reaction method was used (Dubois et al., 1956; Strickland and Parsons, 1972). One mL of juice sample was added to 1 mL of the aqueous solution of phenol (50mg/mL), then, 5 mL of concentrated sulfuric acid was quickly added. The samples were cooled down to room temperature for 30 min and the absorbance was measured at 490nm. Distilled water was used as control. The calibration curve was performed from dilutions of aqueous glucose solution in a concentration range between 0.0125 to 0.125 g/L.

Determination of total phenolic content (TPC)  TPC was determined by Folin-Ciocalteu method (Singleton and Rossi, 1965). 200 μL of pomegranate juice was mixed with 1 mL of 10-fold-diluted Folin– Ciocalteu reagent and 0.8 mL of 7.5 % of sodium carbonate. The mixture was allowed to stand for 2 hours at room temperature. The absorbance was measured at 760 nm using a spectrophotometer. Gallic acid was used as standard. Results were expressed as mg gallic acid equivalent in a liter of juice ± standard deviation (mg GAE/L of juice ± sd). All samples were analyzed in triplicate.

Determination of total flavonoid content (TFC)  TFC of pomegranate juice was determined using the aluminum chloride (AlCl₃) colorimetric method with some modifications (Brighente et al., 2007). One mL of juice was mixed with 1 mL of 2 % AlCl₃ methanolic solution. After 10 min of incubation at room temperature, the absorbance was measured at 430 nm. Quercetin was used as standard. The results were expressed as mg quercetin equivalent in a liter of juice ± standard deviation (mg QE/L of juice ± sd). All samples were analyzed in triplicate.

Microbiological analysis  Microbiological analysis was carried out on natural flora of pomegranate juice; aerobic mesophilic bacteria, Streptococcus thermophilus, yeasts and molds for 245 days of storage at 20 and 5 °C. One mL of the samples was serially diluted in Tryptone Salt Solution (8.5 g/L Sodium chloride, 1 g/L tryptone) and inoculated on appropriate medium. The enumeration of aerobic mesophilic bacteria was evaluated on Plate Count Agar medium (PCA) for 48-72 h at 37°C. M17 medium was used to count S. thermophilus (37 °C for 48 h). Sabouraud Chloramphenicol Agar medium was used to count the molds and yeasts (30 °C for 72 h). Control flasks without TCEO were tested similarly. The mean number of colonies was counted and expressed in log CFU/mL. The error bars in the figures indicate the mean ± standard deviations from the data obtained from three repetitions.

Statistical analysis  All assays were performed in triplicate and the results were expressed as the mean of values ± standard deviation. Statistical analysis was performed to determine significant differences (P < 0.05) using ANOVA followed by Tukey’s test. All statistical analyses were performed using IBM SPSS Statistics (version 20).

Results  Changes of physicochemical qualities during storage  The changes in pH, TSC, TPC and TFC after 245 days of storage of pomegranate juice at 20 and 5 °C are shown in Table 1.

- pH: pomegranate juice initially had an acidic pH ranging between 3.5 and 3.96. After 245 days of storage, we noted that pH decreased with no significant difference between treated and untreated juice. In both storage temperatures, we observed insignificant difference between TCEO concentrations used (P > 0.05).
- TSC: pomegranate juice initially had TSC between 87.18 and 97.48 g/L. After 245 days of storage, TSC in controls decreased significantly at both storage conditions with control at 5 °C showing better quality; from 97.45 to 2.73 g/L at 20 °C and from 87.21 to 5.41 g/L at 5 °C. The decrease was less significant in treated juice at both storage temperatures. At 20 °C, TCEO showed better effect at 0.125 % with a decrease from 97.42 to 96.9 g/L whereas at 0.06 %, it caused a decrease from 97.48 to 81.26 g/L. These values were better than at 5 °C where we observed a slight difference between both TCEO concentrations; from 87.18 to 69.6 and 68.7 g/L for 0.125 and 0.06 % respectively. We also observed insignificant difference between TCEO concentrations (P > 0.05).
- TPC: pomegranate juice initially had TPC between 530 and 535 mg GAE/L. After 245 days of storage, TSC in controls decreased significantly at both storage conditions with control at 5 °C showing better quality; from 533 to 486 mg GAE/L, whereas in treated juice, TPC decreased less significantly (P > 0.05); from 533 to 526 mg GAE/L with 0.125 % TCEO and from 535 to 529 mg GAE/L with 0.06 % TCEO. Thus, a slight difference between both TCEO concentrations was observed. At 5 °C, TPC in control decreased more than at 20 °C; from 533 to 471 mg GAE/L. When treated with TCEO, TPC decreased less significantly (P > 0.05) and showed better values with 0.125 % TCEO; from 535 to 522 mg GAE/L with 0.125 % TCEO and from 530 to 494 mg GAE/L with 0.06 % TCEO.
- TFC: pomegranate juice initially had TFC of 37 mg QE/L. In controls, TFC values decreased after 245 days of storage and showed better quality at 5 °C; from 37 to 20 mg QE/L and 28 mg QE/L at 20 and 5 °C respectively. At 20 °C, the juice showed a decrease of TFC from 37 to 21 mg QE/L and 20 mg QE/L when treated with 0.125 % and 0.06 % TCEO respectively. Moreover, we noted an insignificant difference between both TCEO concentrations (P > 0.05). On the other hand, at 5 °C, TFC of treated juice decreased from 37 to 33 mg
Table 1. Changes of pH, total sugars, phenolic and flavonoid contents of pomegranate juice untreated and treated with *Thymbra capitata* essential oil at the beginning and the end of storage at 5 and 20 °C.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>pH</th>
<th>Total Sugars Content (TSC) (g/L)</th>
<th>Total Phenolic Content (TPC) (mg GAE/L)</th>
<th>Total Flavonoid Content (TFC)(mg QE/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 245</td>
<td>Day 0</td>
<td>Day 245</td>
</tr>
<tr>
<td>Control</td>
<td>3.56 ± 0.15</td>
<td>3.33 ± 0.05</td>
<td>97.45 ± 0.19</td>
<td>2.73 ± 0.012</td>
</tr>
<tr>
<td>TCEO 0.125%</td>
<td>3.5 ± 0.11</td>
<td>3.3 ± 0.1</td>
<td>97.42 ± 0.001</td>
<td>96.9 ± 0.001</td>
</tr>
<tr>
<td>TCEO 0.06%</td>
<td>3.56 ± 0.15</td>
<td>3.3 ± 0.1</td>
<td>97.48 ± 0.016</td>
<td>81.26 ± 0.03</td>
</tr>
<tr>
<td>Control</td>
<td>3.9 ± 0.1</td>
<td>3.23 ± 0.05</td>
<td>87.21 ± 0.085</td>
<td>5.41 ± 0.032</td>
</tr>
<tr>
<td>TCEO 0.125%</td>
<td>3.96 ± 0.05</td>
<td>3.36 ± 0.05</td>
<td>87.18 ± 0.068</td>
<td>69.6 ± 0.001</td>
</tr>
<tr>
<td>TCEO 0.06%</td>
<td>3.9 ± 0.1</td>
<td>3.36 ± 0.15</td>
<td>87.18 ± 0.069</td>
<td>68.7 ± 0.001</td>
</tr>
</tbody>
</table>

QE/L and 28 mg QE/L when 0.125 and 0.06% TCEO were added respectively. Therefore, TCEO at 0.125 % showed slightly better quality than 0.06%.

Changes of microbiological quality The antimicrobial effect of TCEO in pomegranate juice stored for 245 days at 20 and 5 °C was assessed against aerobic mesophilic bacteria (Figure 1), *S. thermophilus* (Figure 2), yeasts and molds (Figure 3). The initial count of microorganisms in juices at 20 and 5 °C were different because the experiments were conducted separately using fresh juice each time.

Generally, we noted significant differences (*P* < 0.05) between the counts of microorganism in treated and untreated pomegranate juice after one day of contact with TCEO. For 245 days of storage, we observed insignificant difference between TCEO concentrations and between storage conditions (*P* > 0.05). The microorganism count of treated juice changed insignificantly (*P* > 0.05) during the storage.

Discussion
Natural compounds prolong the storage stability of food by inhibiting the growth of foodborne spoilage or pathogenic microorganisms and protecting food from oxidative stress damage (Teixeira *et al.*, 2012). For this reason, essential oils (EOs) application in food preservation is of great interest. El Moussaoui *et al.* (2016) observed a significant effect of *Origanum elongatum* essential oil, alone or in combination with heat treatment, on microbiological and chemical properties of pomegranate juice stored at 20 °C for 16 days. This activity depends on EOs composition and concentration. We found that carvacrol is the major component of TCEO (75.52%), followed by *p*-cymene (9.75%) and β-caryophyllene (6.45%). Carvacrol has a broad spectrum of activity. In apple and orange juice, it had the wider antimicrobial activity among the tested EOs constituents (Ait-Ouazzou *et al.*, 2011). The high percentage of carvacrol in our EO can explain its high antimicrobial activity in pomegranate juice. Nevertheless, this activity depends also on the presence of other components interacting with the main component allowing TCEO to have higher activity than the component alone. Against gram-negative bacteria, carvacrol can disintegrate the outer membrane, releasing lipopolysaccharides and increasing cytoplasmatic membrane permeability to ATP. Against Gram-positive bacteria, it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains.

Additionally, the juice composition plays an important role in EOs activity. Sánchez-Rubio *et al.* (2016) reported that the inactivation of *Saccharomyces cerevisiae* in pomegranate and orange juices by *Cinnamomum zeylanicum* leaf essential oil, combined with ultrasound, depended on the composition and physicochemical properties of both juices. In another study, Ait-Ouazzou *et al.* (2011) noted lower synergistic effect between heat and EOs constituents in orange juice compared to apple juice against *Escherichia coli*. This could be due to the abundance of solids in suspension in orange juice compared to apple juice. In food, sugars, fats, salts and other organic or inorganic components may protect bacteria and therefore cause an increase in the bacterial resistance (Burt, 2004).

Pomegranate fruit is extremely nutritious, composed of acids, sugars, vitamins, polysaccharides, polyphenols and minerals (Al-Maiman and Ahmad, 2002). Moroccan pomegranate TSC can vary among cultivars (from 16.1 to...
Fig. 1. Survival of aerobic mesophilic bacteria in pomegranate juice untreated and treated with *Thymbra capitata* essential oil (TCEO) ((a) at 20 °C, (b) at 5 °C). control, □ juice treated with 0.125 % (v/v) TCEO, □ juice treated with 0.06 % (v/v) TCEO (bars represent standard deviations).

Fig. 2. Survival of *Streptococcus thermophilus* in pomegranate juice untreated and treated with *Thymbra capitata* essential oil (TCEO) ((a) at 20 °C, (b) at 5 °C). control, □ juice treated with 0.125 % (v/v) TCEO, □ juice treated with 0.06 % (v/v) TCEO (bars represent standard deviations).

Fig. 3. Survival of yeast and molds in pomegranate juice untreated and treated with *Thymbra capitata* essential oil (TCEO) ((a) at 20 °C, (b) at 5 °C). control, □ juice treated with 0.125 % (v/v) TCEO, □ juice treated with 0.06 % (v/v) TCEO (bars represent standard deviations).
19.3 g/100 g) (Legua et al., 2012). During storage, reducing and non-reducing sugars in juices may change due to various interconversion processes (Singh and Sharma, 2017). Also, storage may cause sugars to break down and produce carboxylic intermediates that will react to form brown polymers (Robertson and Samaniego, 1986). Moreover, spoilage yeasts can produce ethanol and CO₂ from sugar (Bevilacqua et al., 2011). El Moussaoui et al. (2016) reported a decrease in TSC during juice storage that may be explained by fermentation process. We observed low microorganism count in treated juice that could explain the insignificant change in TSC compared to controls. Moreover, TSC decreased more in 5°C compared to 20°C. This could be due to higher rate of solubilization or hydrolysis of acid into sugars at ambient temperature (Singh and Sharma, 2017).

Furthermore, pomegranate is rich in polyphenols, like flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolyzable tannins. These compounds play an important role in the development of characteristic color and flavor of juices (Abid et al., 2014). They also possess several actions: anticancer, antioxidant, antiviral and anti-inflammatory actions (Guerrouj et al., 2016; Karimi et al., 2017). For this reason, it is important to maintain a good polyphenols content of pomegranate juice after a long storage. In pomegranate juice, TPC and TFC were in the range of those observed for Moroccan pomegranate cultivars; between 41.01 and 83.43 mg/100 g for TPC (Legua et al., 2012) and between 14.446 and 56.989 mg RE/L for TFC (Hmid et al., 2017). During storage of many fruits and vegetables, the phenolic compounds can remain stable whereas the flavonoid content can vary differently depending on the studied materials (Kevers et al., 2007). Since EOs contain phenols, TCEO addition conserved TPC and TFC of pomegranate juice. Other researchers noted fluctuations of juices TPC during storage. Piljac-Zegarac et al. (2009) reported that TPC in dark fruit juices (including pomegranate) decreased and finally increased during 29 days of storage at 4°C. On the other hand, Tastan and Baysal (2015) found that TPC of pomegranate juice clarified using chitosan decreased rapidly after 2 months storage at both chosen temperatures (4 and 20°C). They also observed significant differences between storage temperatures at the same month. We also observed a decrease of TPC that was more important in controls than in treated juice with insignificant difference between both storage conditions. Del Caro et al. (2004) found that minimally processed Citrus juices showed a diminution in TFC after storage at 4°C for 15 days. Anthocyanins, group of flavonoids, are also unstable and susceptible to degradation. Vegara et al. (2013b) observed a decrease in anthocyanin content after storage of pasteurized pomegranate juice in 25 and 5°C. This decrease caused a brownish color in the juice during storage. They noted that the decrease was more important in 25°C than in 5°C.

pH is also an important parameter in food preservation. Pomegranate juice had an acidic pH (3.5 - 3.96) that decreased insignificantly during storage. Hmid et al. (2017) also found that pomegranate juice had a pH between 2.85 and 4.22. Moreover, other researchers also reported that pH values of pasteurized pomegranate juice changed insignificantly during storage (Vegara et al., 2013a). Juices acidity allows the growth of lactic acid bacteria, thermo-acidophilic and acid-tolerant bacteria, yeasts and molds. These organisms can cause the degradation of sugars and other nutrients into secondary metabolites and can inhibit, by competition, the growth of other microorganisms (Mosqueda-Melgar et al., 2012). We studied the effect of TCEO on aerobic mesophilic bacteria, lactic bacteria (S. thermophilus), yeast and molds because of their importance in juice quality degradation. We noticed the important degradation of juice quality in controls which showed high microorganism growth compared to treated juice. Acidic pH plays an important role in EOs activity; it increases their hydrophobicity enabling them to dissolve more easily in cell membrane lipids of target bacteria (Burt, 2004). Gutierrez et al. (2009) found that oregano and thyme EOs had very high antibacterial efficacy at low pH values. We also noted a high antimicrobial activity of TCEO in pomegranate juice. However, some studies reported that the activity of EOs or their constituents was unrelated to juice acidity (Friedman et al., 2004; Tserennadmid et al., 2011). Other researchers also found that the studied strain and the inoculum size have a significant influence on the effect of EOs and their components (Nychas et al., 2003; Raybaudi-Massilia et al., 2006). In our study, we noted that all the tested microorganisms exhibited almost the same behavior to TCEO. Also, the preservatives added influence microbial growth along with storage temperature and packaging quality (Singh and Sharma, 2017). Friedman et al. (2004) found that the activity of EOs and their constituents increased with storage temperature and time in apple juice. In our study, we noted that TCEO activity was comparable at both storage temperatures (5 and 20°C).

Conclusion
In this study, Thymbra capitata essential oil was used as a natural alternative in the preservation of pomegranate juice. Monitoring of juice quality parameters showed the ability of the oil to maintain good quality of juice compared to controls during 245 days of storage. Furthermore, the essential oil exhibited significant antimicrobial activity against pomegranate juice natural flora. Thus, this study demonstrates the possible use of the essential oil to maintain good quality of pomegranate juice stored for 245 days at both 20 and 5°C. In conclusion, T. capitata essential oil could offer a great alternative to traditional heat treatments, addition of synthetic agents and to other techniques that may be expensive and can alter the juice quality. Further study of this essential oil effect on juice sensory quality is necessary to optimize its use in pomegranate juice preservation.
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