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Evaluation of *in vitro* Antioxidant Activities of Lemon Juice for Safety Assessment

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ABSTRACT

Safety assessments of branded lemon juice are currently evaluated with parameters such as the formalin index, acidity, Brix, pH and dry content. In this study, other parameters such as vitamin C, antioxidant and phenolic compounds were evaluated to introduce some new and reliable indexes for safety assessments of lemon juice. Two groups of lemon juice were evaluated in the study. The two groups consisted of branded and natural products and were tested for total phenolic compounds (Folin-Ciocalteu), antioxidant capacity (2,2-diphenyl-1-picrylhydrazyl) and ascorbic acid content (2-4-dinitrophenylhydrazine). Results demonstrated that total phenolic contents in both groups showed non-significant difference but the group of natural lemon juice samples had the better antioxidant capacity (795.61 mg VEE L⁻¹) and higher ascorbic acid content (187.52 mg L⁻¹). Levels of antioxidant capacity ranged from 476.60 to 1378.12 and from 235.47 to 888.59 (mg VEE L⁻¹) in branded and natural sample groups, respectively. Amounts of ascorbic acid in branded lemon juice samples ranged from 23.53 to 492.91 and in the natural lemon juice samples amounts ranged from 99.32 to 196.49 mg L⁻¹. Analysis of interrelations between these three measured parameters indicated that phenolic compound and ascorbic acid content both had significant correlations with antioxidant capacity. In summary, ascorbic acid content and antioxidant capacity are parameters suitable for safety assessments of lemon juice, but further investigation such as flavonoid profile may also be helpful.

Key words: Ascorbic acid content, DPPH-HPLC assay, Folin-Ciocalteu, lemon juice antioxidant

INTRODUCTION

Lemon (*Citrus limon* L.) Burm. f.) is the third most important species of citrus fruit after orange and mandarin, with production totaling more than 4,400,000 tons during the 2001/2002 season. Citrus fruits are a universally well-liked raw material for the production of fruit juices. The appetizing flavors of citrus fruit drinks are enjoyed worldwide. The presence of bioactive compounds, such as hydrocinnamic acid, ferulic acid, cyaniding glucoside, flavonoid, vitamin C, carotenoid, hesperidin and naringin content contribute to the value of lemon in terms of it being associated with promoting good health (Xu *et al.*, 2008). Natural antioxidants found in fruit vegetables are considered to be beneficial to human health (Souri *et al.*, 2008; Hajimahmoodi *et al.*,

2008a). Phenolic compounds exist in plants and are known to have high antioxidant ability and free radical scavenging capacity, as they inhibit enzyme activity (Kahkonen *et al.*, 2001; Hajimahmoodi *et al.*, 2008b). Most research shows that ascorbic acid and carotenoids are abundant in citrus fruit (Dhuique-Mayer *et al.*, 2005). Ascorbic acid has numerous biological functions, including the synthesis of collagen, hormones and neurotransmitters (Iqbal *et al.*, 2004). Ascorbic acid demonstrates an antioxidant effect that under certain conditions can protect against oxidative induced DNA damage (Sweetman *et al.*, 1997). Citrus fruits are delicious and have the benefit of antioxidant properties (Morton *et al.*, 2000). It is therefore imperative to evaluate the amount of antioxidant compounds in commonly consumed fruit. Hence, the main goal of this study was to compare levels of total phenolic contents, antioxidant capacity and ascorbic acid contents between branded lemon juice and natural lemon juice.

MATERIALS AND METHODS

Chemicals and reagents: All standards were of analytical grade and purchased from Merck (Darmstadt, Germany).

Sample preparation: The lemon juice samples were divided into two groups (26 natural lemon juices from domestic collections and 74 branded lemon juices collected from supermarkets). Lemons in the first group were washed, peeled and squeezed to extract the juice and then clarified with Whatman No. 4 filter paper. All samples were stored as recommended on the labels and were analyzed before expiry dates. This study was done during the fall and winter months of 2011 (October-March).

Antioxidant capacity based on DPPH-HPLC method: Fresh methanolic DPPH (1,1-diphenyl-2-picrylhydrazyl) stock solution at a concentration of 0.1 mmol L⁻¹ was prepared, diluted to 2 mL methanol. Fifty microliter of each sample was added to 2 mL DPPH solution. Mixtures were shaken for a few seconds and then kept in the dark for 40 min at room temperature. After filtration through a 0.2 µm Minisart RC 4 membrane filter (Sartorius, Germany) 20 µL of each sample was injected in to the HPLC. A blank was prepared by adding 50 µL of distilled water to 2 mL of DPPH solution. The effluent was monitored at 517 nm. The difference between the blank and each sample in the reduction of DPPH Peak Area (PA) was taken to determine the percentage of radical-scavenging activity for each sample. Figure 1 shows the level of DPPH absorbance and the blank peak in one randomly selected juice sample (Hajimahmoodi *et al.*, 2010).

Liquid chromatography and separation condition: These experiments were done using an analytical HPLC system consisting of a pump (Maxi-Star K-1000, Knauer, Germany), a UV spectrophotometer detector (Knauer, Germany), controlled by software (EuroChrom 2000, Version 1.6, Knauer Co., Germany). The applied stationary phase was Eurospher 100 C8 Column (4.6 mm×25 cm, 5 µm; Knauer, Germany) eluted isocratically at the mobile phase (Methanol: deionized water; 80:20) at a flow rate of 1 mL min⁻¹ (Hajimahmoodi *et al.*, 2010).

Determination of total phenol content: Total phenolic contents were determined by the Folin-Ciocalteu method. Two hundred microliter of paper filtered lemon juice was added to 1.5 mL of 5% methanolic solution Folin-Ciocalteu then mixed and shaken for 5 min. After 5 min, 1.5 mL of 5% Na₂CO₃ solution was added, mixed and allowed to stand for 90 min, then the level of

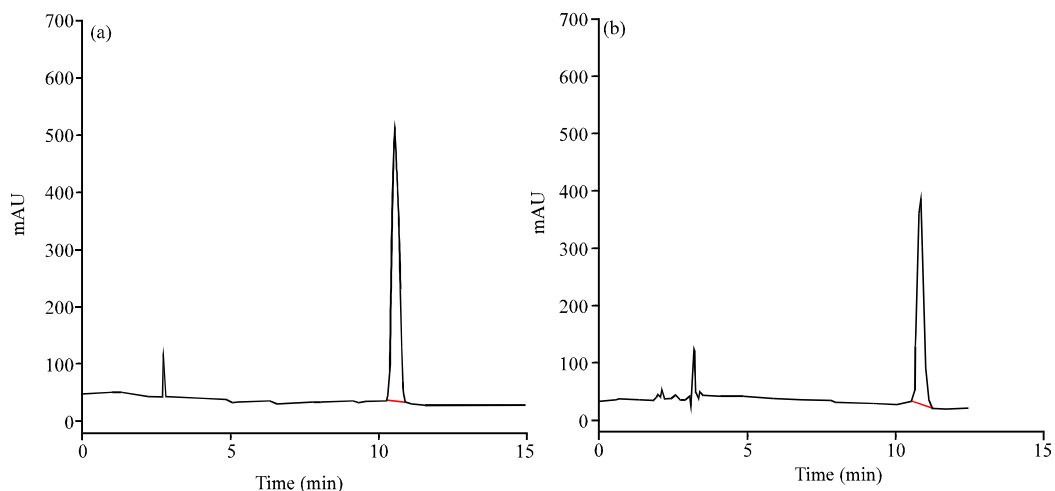


Fig. 1(a-b): DPPH chromatogram of (a) Sample and (b) Blank

absorbance was measured at 750 nm. Total phenolic contents were quantified according to a calibration curve of Gallic acid standard solution ($25\text{-}150\ \mu\text{g mL}^{-1}$ in 50% methanol). The total phenolic content for each sample was expressed as mg L^{-1} of Gallic acid equivalent (Hajimahmoodi *et al.*, 2008b; Ardekani *et al.*, 2010).

Evaluation of ascorbic acid: To determine total ascorbic acid content for each sample, the well-established method using 2,4-dinitrophenyl hydrazine was applied, as cited in Mc Comick and Wright (1979). One hundred microliter of paper filtered lemon juice was added to 80 μL DTC (2,4-dinitrophenylhydrazine thiourea copper (II) sulfate solution). Test tubes were put into a water bath at 37°C for 3 h, 600 μL of 65% sulfuric acid was added to test tubes, shaken and allowed to remain at room temperature. Levels of absorbance were measured at 520 nm with a spectrophotometer.

Statistical analysis: All analyses were done in triplicate ($n = 3$). Results were reported as Mean \pm SD. Statistical analysis was carried out using the software package SPSS v17.0 (SPSS Inc., Chicago, USA) and comparison of averages was based on the analysis of variance (One-Way ANOVA) at significance level $p\text{-value} < 0.05$.

RESULTS

Total phenolic content: The phenolic content in fruit and vegetables has received considerable attention due to its potential for antioxidant activity. Phenolic compounds act as important antioxidants because of their ability to donate a hydrogen atom or an electron in order to form stable radical intermediates. Total phenolic contents were determined for 100 different samples of Iranian lemon juice, as shown in Table 1 as $\text{mg Gallic acid equivalent (mg GAE L}^{-1}\text{)}$. Related phenolic contents ranged from 114.27 to 278.55 (mg GAE L^{-1}) in natural fruit juice samples and ranged from 84.58 to 316.58 (mg GAE L^{-1}) in branded lemon juice samples. The average of total phenolic contents in natural lemon juice samples was 196.81 ± 37.98 (mg GAE L^{-1}) and in branded lemon juice the average was 190 ± 93.48 (mg GAE L^{-1}). Statistical analysis of these two groups

Table 1: Chemical analysis of branded and natural lemon juices

Samples	Total phenolic content (mg GAE L ⁻¹)	Ascorbic acid (mg L ⁻¹)	Antioxidant capacity (mg VEE L ⁻¹)
Natural	196.81±37.98	187.52± 30.80	795.61±122.00
Range	114.27-278.55	136.27-229.05	235.47-888.59
Branded			
1	125.92±40.71	53.27±31.20	637.31±112.45
2	91.89±19.57	63.22±29.00	350.81± 40.70
3	296.57±98.42	320.03±100.00	352.02± 27.13
4	316.58±45.78	96.95±34.30	665.09±125.00
5	84.58±13.19	56.83±5.00	443.29±36.90
6	228.97±44.63	217.87±79.23	701.30±29.18
7	137.13±43.63	58.58±17.70	208.91±37.14
8	108.51±37.00	23.53±2.00	608.87±34.59
9	183.01±71.00	59.56±18.91	620.60±93.89
Total mean	190.00±93.48	109.01±114.85	520.22± 50.19

Values are Mean±SD

showed no significant difference. The ANOVA test determined significant difference between natural samples in the first group and brand No. 1, 2, 3, 4, 5, 7 and 8 in the second group (p-value <0.05).

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay: DPPH is one of the known stable free radical species applied for the assessment of the radical-scavenging potential of various antioxidants (Chandrasekar *et al.*, 2006; Iranshahi *et al.*, 2009). The DPPH scavenging activities of lemon juices were expressed as mg vitamin E equivalent (mg VEE L⁻¹). The various antioxidant capacities of lemon juice samples are summarized in Table 1. This ranged from 208.91-701.30 to 235.47-888.59 (mg VEE L⁻¹) between the branded and natural samples respectively. The average antioxidant capacity in the natural samples (795.61±122 mg VEE L⁻¹) was higher than that in the branded samples (520.22±50.19 mg VEE L⁻¹). In the branded samples the maximum amount of antioxidant capacity related to No 6 (701.30±29.18 mg VEE L⁻¹) and No. 2 recorded the minimum (305.81±40.7 mg VEE L⁻¹). Statistical analysis showed that the antioxidant capacity of the natural group was significantly different than that of the branded group.

Ascorbic acid content: In the tested branded lemon juices, ascorbic acid contents ranged from 23.53 to 320.03 mg L⁻¹. As demonstrated in Table 1 the ascorbic acid content of the natural (187.52±30.8 mg L⁻¹) was higher than the content recorded in the branded lemon juice samples (109.01±114.85 mg L⁻¹). Significant difference of ascorbic acid content was observed between the two groups under evaluation (p-value <0.05).

DISCUSSION

Several studies have highlighted lemons as an important health-promoting fruit, rich in phenolic compounds as well as vitamins, minerals, dietary fiber, essential oils and carotenoids. Xu *et al.* (2008) total phenolic content of a natural Chinese citrus cultivar was 751.82 mg GAE L⁻¹ an amount more than that recorded in this study. This difference may be attributed to the variety of cultivar. In another study lemon peel polar fractions revealed the highest phenolic contents (87.77±1.4 mg GAE g⁻¹), but the juice polar fraction (8.43±0.002 mg g⁻¹ GAE) and crude juice (11.17±0.05 mg GAE g⁻¹) had lower amounts (Guimaraes *et al.*, 2010). Research by Fu *et al.*

(2011) recorded an amount of 61.47 ± 0.57 mg GAE/100 g in whole lemons. In another study by Prasan and Ruthaichanok (2008) amounts of total phenolic compound were evaluated for 20 samples of vegetable juice, of which total phenolic content for *Citrus aurantifolia* attained 296 ± 18.3 mg GAE L⁻¹. The samples evaluated in this research can be compared with Prasan and Ruthaichanok (2008) as the Iranian cultivated lemon variety is also aurantifolia. It is noteworthy that all samples in this study had lower amounts of total phenolic content. Trough tests on natural orange juice (755 ± 18 mg GAE L⁻¹), Gardner *et al.* (2000) determined that the fruit was richer than other samples.

It should be noted that fruit peel is sometimes mixed with juice in the commercial production of fruit juice and this may be the main cause for the high amounts of total phenolic content in some commercial branded lemon juices.

The model of scavenging the stable DPPH radical model that is commonly used to evaluate the free radical scavenging ability of various samples. DPPH is a commercial oxidizing radical that can be reduced by antioxidants. In this assay, the violet colour of DPPH changed to pale yellow due to the abstraction of hydrogen atoms from the antioxidant compound. When there are more antioxidants in an extract, more DPPH is reduced. A water-soluble derivative of vitamin E is Trolox a synthetic branded product that is the commonly used method used to induce antioxidant activity (Du Toit *et al.*, 2001). Antioxidant activity in lemon juice from Fino and Verna varieties was evaluated by Marin *et al.* (2002); tests showed levels of 808 ± 20 and 781 ± 20 mg VCE L⁻¹ in Fino and Verna lemon juice samples, respectively. In another research with FRAP assay levels were 307.43 ± 14.37 mg VCE L⁻¹ (Xu *et al.*, 2008). The antioxidant capacity of American lemon, determined by the current method was 101.2 ± 2.0 mg VCE/100 g in whole and 41.8 ± 0.1 mg VCE/100 g in juice (Floegel *et al.*, 2011).

Antioxidant activity of freshly prepared orange juice in a report by Evaggelia and Theodore, (2008) was 777 ± 55 and this amount in commercial Orange juice was 376 ± 24 (mmol Trolox/100 mL). Ascorbic acid is highly bioavailable and is consequently the most important water-soluble antioxidant vitamin in cells, effectively scavenging Reactive Oxygen Species (ROS). When relating the antioxidant activities of fruit juices to health and risk of disease, it is important to consider the contribution of ascorbic acid in addition to that of phenolic compounds with antioxidant activity (Gardner *et al.*, 2000). Ascorbic acid content in lemon juice decreases in extended storage time. According to previous studies the amount of ascorbic acid in citrus species was 233.44 ± 2.52 mg L⁻¹ (Xu *et al.*, 2008). Ascorbic acid content from squeezed juice samples in Verna species was 262 ± 18.8 mg L⁻¹ and in Fino species was 532 ± 20.2 mg L⁻¹ (Marin *et al.*, 2002). Ascorbic acid assay in samples in this study demonstrated that the mean level of ascorbic acid in natural lemon juice was more than that in the branded lemon juices in Iran. Due to the lower amount of total phenolic content in Iranian lemon juices compared with other studies, the related Persian standard needs to be revised. These revisions should consider more effective use of approved current techniques, to employ new technology and method of production that would allow for better control.

Pisoschi *et al.* (2008) used the cyclic voltammetry method to determine the amount of ascorbic acid on juices. Reported ascorbic acid content of juices ranged from 0.83 to 1.67 for Orange, 0.58-1.93 or 102.15-339.91 mg L⁻¹ in lemon and 0.46-1.84 mg L⁻¹ in grapefruit (Pisoschi *et al.*, 2008). In another study on vegetable juice Prasan and Ruthaichanok (2008) showed that ascorbic acid concentration in lemon was 201.1 ± 2.9 mg L⁻¹.

Results of this research show that antioxidant capacity was positively correlated to the total phenolic content ($r = 0.139$, p -value < 0.05). Antioxidant capacity and measured ascorbic acid also had a positive correlation ($r = 0.228$, p -value < 0.05).

CONCLUSION

This review focused on the analytical aspects of lemon compounds as well as on implications for the food industry and the relevance of lemon for nutrition and health. The natural lemon group had the greater amount of antioxidant capacity and also higher ascorbic acid content than the branded group. To summarize, it should be stressed that the recommended daily serving of fruit should consist of natural lemon juice, as it provides nutritional antioxidants with specific a flavor that could be beneficial as an antioxidant protection system but further *in vivo* tests on humans are suggested to support this research.

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