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The Effects of Preservation Methods of Grapevine Leaves on Total Phenols, Total Flavonoids and Antioxidant Activity

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ABSTRACT

Preservation methods may affect total phenols, flavonoids contents, and antioxidant capacity of many vegetables and fruits. These effects may cause degradation of antioxidant compounds, formation of new products, or destruction of the active metabolites. This study investigated the effects of different preservation methods, such as canning and freezing, on total flavonoids, total phenols, and antioxidant activity on grapevine leaves, which can be consumed either as medicine or food. Phytochemical screening was performed by using standard analytical methods; antioxidant activity was assayed by using 2,2-diphenyl-1-picryl-hydrazylhydrate reagent method; and total flavonoids and total phenols content were determined by using the rutin reference standard method and by Folin-Ciocalteu's method, respectively. The phytochemical content of all the studied grapevine leaf extracts were same. Fresh leaf extract showed the highest antioxidant capacity as well as total phenols and flavonoids contents. This was followed by the frozen leaf extract, while the canned leaf extract showed lower antioxidant capacity and reduced phenolic and flavonoids contents. Canning and freezing preservation methods of these leaves had no deleterious effects on total antioxidant capacity as well as total phenols and flavonoid contents. Therefore these methods can be used for preparing nutraceutical, cosmeceutical, and pharmaceutical supplements. However, the preservation of *V. vinifera* leaves by canning is economically and environmentally favored over freezing. In addition the storing, handling, and maintenance of canned leaves is easier than fresh and frozen leaves.

Key Words: *Vitis vinifera*; Antioxidant Activity; Phenols; Flavonoids; Preservation methods.

Introduction

Plants are the main source of food, flavors, cosmetics, and medicines. In fact, many edible plants are used as food and medicine. In the last two decades, this issue has attracted the attention of many pharmaceutical firms due to its importance in drug discovery. Phenols with potential antioxidant properties play a very important role in both food and pharmaceutical fields as nutraceutical and pharmaceutical agents. Accordingly, several pharmaceutical companies have invested huge economical efforts in the attempts to find an effective and safe source of phenols (1, 2).

Vitis vinifera L. is a member of the *Vitaceae* family originating in Asia Minor. It is a perennial, climbing, woody plant, and several preparations from different parts of this plant, especially its fruits, are used in folk medicine (3). In many Mediterranean countries, *Vitis vinifera* L. (grapevine) leaves have been used as food and medicine for the treatment of various diseases. Recently, the phenolic and other nonphenolic compounds in various grapevine parts such as berries, stems, petiole, leaves, and shoots have been of great research interest (4).

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The leaves' phytochemical constituents which have been recognized to date are isoprenoids, organic acids, steroids, anthocyanins, sterols, phytoalexin pterostilbene, leucoanthocyanins, rutin, quercitrin, isoquercitroside, kenferol, luteolol, esterifies and free fatty acids, tannins, vitamins, enzymes, and heterocyclic compounds (5-10).

In folk medicine, the leaves' infusions have been used internally to treat hepatitis, hemorrhages, stomachaches and diarrhea, while the fresh leaves externally have been used to heal lance abscesses and wounds (3, 11-13). Additionally, in Palestinian folk medicine it has been used as an antihemorrhage, antiseptic, astringent, tonic, diuretic, antianemia, blood purifying, and antihypercholesterolemic agent (14).

The evidence-based pharmacological studies have been shown that the leaves have antidiabetic, antioxidant (13), antibacterial (15), antileishmanial (16), and neuro-protective potential against peroxide damaging (4).

Grapevine leaves have been used as a popular food material in Palestine and other Mediterranean regions served fresh or with rice and minced meat (13). The leaves used as medicine or as food, both fresh and preserved leaves, are especially available in the autumn, winter, and spring seasons. The leaves are preserved in well-sealed bags in the refrigerator at a temperature not less than -18°C or, after canning them, in well closed plastic bottles at room temperature. This study aims to evaluate the total phenols, flavonoids contents, and antioxidant potential of grapevine leaves preserved using various methods in comparison to fresh leaves.

Material and methods

Chemical Reagents

The reagents that were used for the evaluation of the antioxidant activity included: Methanol (Lobachemie, India), n-hexane (Frutarom LTD, Israel), Trolox ((s)-(-)-6 hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) (Sigma-Aldrich, Denmark), and (DPPH) 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Germany). For phytochemical screening, the utilized reagents included: Millon's reagent (Gadot, Israel), Ninhydrain solution (Alfa Agar, England), Benedict's reagent (Gadot, Israel), Molish's reagent, H_2SO_4, Iodine solution (Alfa-Aesar, England), NaOH (Gadot, Israel), Chloroform, HCl (Sigma-Aldrich, Germany), Magnesium ribbon, Acetic acid (Frutarom LTD, Israel), and $FeCl_3$ (Riedeldehan, Germany).

Folin-Ciocalteu's reagent (Sigma- Aldrich, Germany) was utilized for the evaluation of the total phenol contents. The reagents utilized for evaluation of total flavonoids contents included: rutin hydrate (MP-Biomedical USA), $AlCl_3$, and Potassium Acetate (Sigma Aldrich, Germany). All organic solvents used in this study were of HPLC grade, except methanol (technical quality), which was used as the solvent for extraction.

Collection of V. vinifera leaves

V. vinifera leaves were collected in June 2015 from the Hebron region (Halhol district) in the southern area of the West Bank of Palestine. The voucher specimen was deposited in the Pharmaceutical Chemistry and Technology Division, Laboratory of Pharmacognosy, and identified by the pharmacognosist Dr. Nidal Jaradat. Its voucher specimen code was Pharm-PCT- 2665.

Preparation of fresh V. vinifera leaves

The leaves were washed several times using distilled water and cut in small slices for further use.

Preparation of canned V. vinifera leaves

About 500g of well-washed fresh grapevine leaves were rolled and placed in plastic bottles. These bottles were tightly closed and then stored at room temperature for about two weeks. After that, these canned leaves were cut into small slices for further experimental use.

Preparation of frozen V. vinifera leaves

The leaves were washed several times using distilled water and then placed in well closed plastic package in the freezer for about two weeks at 18°C. After that, these frozen leaves were cut into small slices for further experimental use.

Phytochemical analysis:

Methanolic extract was prepared using the Soxhlet extraction method. About 20g of the dried powder was uniformly packed into a thimble and then extracted using 250 ml of methanol. The extraction process was allowed to continue until the utilized organic solvent, in the siphon tube of the extractor, became colorless. After that each of the obtained extracts was heated to 30-40°C using a water bath until the used solvent completely evaporated. The generated dried crude extracts were stored at 2-8°C until use.

Extraction with water was performed in a beaker by adding 200ml of distilled water to 5g of each of the obtained plant powders. Each mixture was heated to 30-40°C on a hot plate with continuous stirring for 20 minutes. The heated mixtures were then filtered individually using Whatman filter paper.

The filtrated were labeled and stored at 2-8°C till use (17). These two procedures were repeated three times for *V. vinifera* leaves: once for each preservation method and for the fresh leaves.

Preparing of the plant extracts for antioxidant activity, total phenols, and total flavonoids evaluation

Ten grams of each of the ground *V. vinifera* leaf samples were soaked in 1 L methanol (99%), placed in a shaker for 72 hours at room temperature, and stored in a refrigerator for 4 days. Then the reaction mixture was filtered and concentrated under vacuum using a rotator evaporator. These crude extracts were stored in amber dark bottles at -4°C for further use (18).

Determination of antioxidant activity using the DPPH radical scavenging method

A stock solution of 1mg/ml methanolic plant extract was prepared for each sample. The working solutions of different concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 mcg/ml) were prepared by serial dilution with methanol (19, 20).

DPPH was freshly prepared with a concentration of 0.002% w/v. The DPPH solution was mixed with methanol in a working concentration in a 1:1:1 ratio. The spectrophotometer was set to zero using methanol as a blank solution. The first solution of the serial concentration was DPPH with methanol only. The solutions were incubated in dark for 30 min at room temperature before the absorbance readings were recorded at 517 nm.

The percentage of antioxidant activity of both the plants species and the Trolox standard were calculated using the following formula:

Percentage of inhibition of DPPH activity (%) = (A-B)/A ×100%

Where: *A* was the optical density of the blank, *B* was the optical density of the sample.

The antioxidant half maximal inhibitory concentration (IC_{50}) for the plant samples and the standard were calculated using BioDataFit edition 1.02 (data fit for biologist).

Qualitative phytochemical analysis:

Preliminary qualitative phytochemical screening of primary and secondary metabolic compounds such as proteins, starch, phenols, cardiac glycosides, saponin glycosides, flavonoids, alkaloids, steroids, volatile oils, and tannins were carried out according to the standard common phytochemical methods described by Evans, 2009 (21) and Harborne, 1998 (22) for leaf extracts of fresh, frozen, and canned *V. vinifera* leaves.

Determination of total phenolic content

The spectrophotometric method was used to determine the concentrations of phenols in fresh, frozen, and canned grapevine leaf extracts (23). Various methanolic solutions from the extract in the concentration of 1 mg/ml was used for analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated at 45°C for 45 min. The absorbance was determined using spectrophotometer at λ_{max} = 765 nm. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid, and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was calculated (mg/ml) from the calibration line; then the content of phenolics in the extracts was expressed in terms of gallic acid equivalent (mg of GAE/g of extract).

Determination of total flavonoids content

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method (23). The sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained. The absorbance was determined using spectrophotometer at λ_{max} = 415 nm. The same procedure was repeated for the standard solution of rutin, and a dilution series of rutin of concentration 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml was prepared, and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was calculated (mg/ml) from the calibration line; then, the content of flavonoids in the extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

Statistical Package for Social Science (SPSS) version 17.0 was used to analyze the results.

Results

Phytochemical screening

Results of phytochemical screening of fresh, frozen, and canned *V. vinifera* leaf extracts are summarized in Table 1. It is shown that protein, carbohydrates, phenols, flavonoids, and tannins were found in the methanolic extracts. Phenols

Table 1: Phytochemical screening tests for methanolic and aqueous *V. vinifera* leaf extracts

Phytochemical compounds	Fresh *V. vinifera* leaves methanolic extract	Frozen *V. vinifera* leaves methanolic extract	Canned *V. vinifera* leaves methanolic extract	Fresh *V. vinifera* leaves aqueous extract	Frozen *V. vinifera* leaves aqueous extract	Canned *V. vinifera* leaves aqueous extract
Cardiac glycosides	-	-	-	-	-	-
Saponin glycoside	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-
Protein	+	+	+	++	++	+
Carbohydrates	+	+	+	+	+	+
Phenols	+++	+++	+++	++	+	+
Volatile oil	-	-	-	-	-	-
Tannin	+	+	+	++	+	+
Steroids	-	-	-	-	-	-
Flavonoid	+++	+++	+++	+	+	+

(-) the absence of the content, (+) low contents, (++) mild contents (+++) high contents.

and flavonoids were mostly predominant compounds of this methanolic mixture, whereas cardiac glycosides, alkaloids, saponins, and steroids were missing.

Estimation of Antioxidative Capacity of V. vinifera leaves Extracts

DPPH was utilized to estimate the antioxidant activity of the extracts and the antioxidant results of the leaf extract compared with the reference standard (Trolox) which had IC_{50} 2.2±0.31µg/ml. As shown in (Table 2), all *V. vinifera* leaf extracts retain their antioxidant properties against free radicals. The fresh *V. vinifera* leaf extracts possessed maximum antioxidant capacity with IC_{50} value 17.78 ±0.41µg/ml, while the frozen leaves had IC_{50} value 18.45±0.50µg/ml. However, the canned samples possessed the lowest antioxidant capacity with IC_{50} values 19.95 ±0.52 µg/ml.

Table 2: Antioxidant activity for the fresh, frozen and canned *V. vinifera* leaves extracts compared with Trolox (potent antioxidant reference).

Samples	**IC_{50}±SD in µg/ml**
Trolox	2.2±0.31
Fresh *V. vinifera*	17.78±0.50
Frozen *V. vinifera*	18.45 ±0.61
Canned *V. vinifera*	19.95±0.52

Estimation of total phenols

V. vinifera leaves extracts were found to have phenolic contents in high amount. The highest content was found in fresh leaves and followed by frozen leaves (125.45 ±0.66 and 103.33 ±0.82mg GAE/g respectively), while the canned leaves possess lower phenolic content (87.35 ±0.32 GAE/g extract).

Estimation of total flavonoids

Flavonoid contents were found in all studied *V. vinifera* leaves, and the fresh leaves had the highest content followed by the frozen leaves and canned leaves (77.12 ±0.34, 61.3 ±0.74, 45.4 ±0.76mg, RU/g extract) respectively. The results of total phenols and flavonoids contents showed in Fig. 1.

Discussion

The astonishing fact about the storage and preservation of food and edible plants is that it every culture around the world utilizes such practices. In fact, man has tried to harness nature since ancient times by freezing or drying food and plants. Due to their nature, edible plants start to spoil the moment they are harvested. The preservation of food and edible plants enables humans to live in one place and form a community. In fact, each human culture preserves and stores their local folkloric food using the same basic tools of food preservation. These tools include, cooling or freezing, drying, salination, vacuum, and using chemical preservatives such as parabens and vinegar. However, these methods may affect

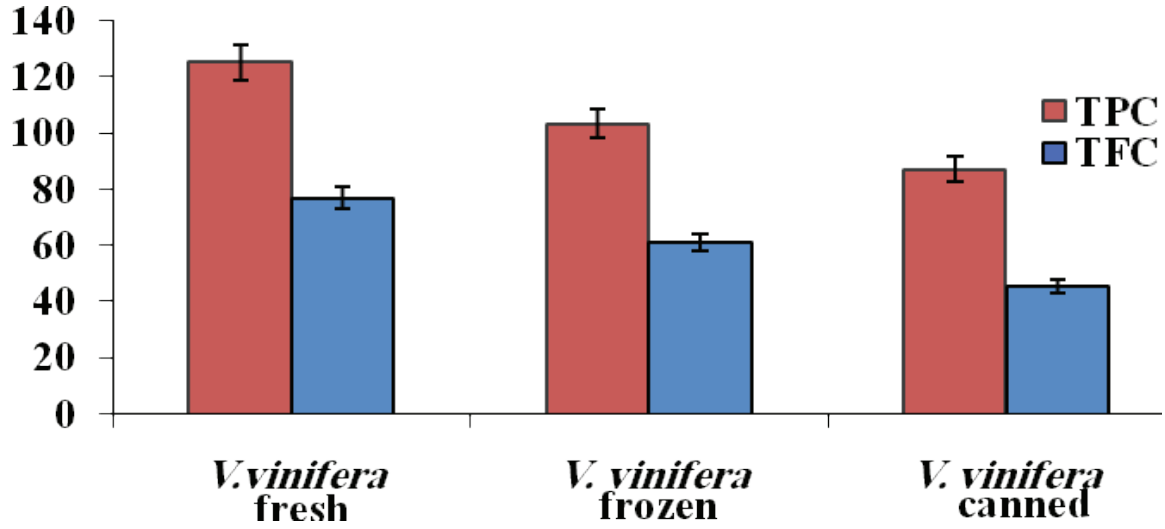

Figure 1: Total phenols and flavonoids contents.

the final organoleptic properties and the medicinal value of these products. In fact, nutraceutical products, including poly-phenols and the antioxidant activity of these products may be strongly affected by storage conditions. Poly-phenols are secondary metabolic phytochemical compounds which play an important role in the plants defense mechanisms, and most of them are known to have medicinal values (24, 25).

Antioxidant constituents in fruits and vegetables are very important components. Nowadays for the best nutraceutical and healthful values, it is very essential to know the changes in antioxidant status as well as changes in total phenols and flavonoids during post-harvest methods of preservation (26).

V. vinifera is one of the most popular edible plants used in the Mediterranean folkloric food. Fresh leaves as well as preserved (frozen or canned) are usually used to prepare this tasty food. Differences in the final organoleptic properties of the cooked leaves could be detected using fresh or preserved leaves. Fresh give the best organoleptic results. Accordingly, changes in the phytochemical content including poly-phenols and their antioxidant activity could be expected.

Previous work conducted by Downey *et al.*, 2007, proved that post-harvest processing can influence phenolic contents of the plant tissue samples (27). Moreover, Eftekhari *et al.*, proved that drying methods of preservation clearly resulted in a considerable increase of quercetin flavonoid content of some kinds of grape leaves and berry skins than freshly harvested material (28).

However, to the best of our knowledge, little information is available in the literature regarding the effects of preservation methods, such as canning and freezing, on the changes of flavonoids, phenolic contents and antioxidant capacities in vegetables and fruits especially in *V. vinifera* leaves. Freezing is considered one of the most effective methods of preservation for the active constituents of the raw plant material for long durations. This method has proved its ability to preserve the active constituents for further pharmaceutical, cosmeceutical, and nutraceutical horizons (29). The results of antioxidant activity of anthocyanin extracts from blueberries showed that there were no significant differences between frozen, dried, and fresh blueberries (30). Even though fresh *V. vinifera* leaves showed the highest antioxidant activity, our results found that only a small difference was observed using the canned and frozen leaves. However, preservation of *V. vinifera* leaves by using canning is economically and environmentally favored over freezing. Canning does not need energy or chemical preservatives and uses recycled plastic bottles. In addition, the storage, handling, and maintenance of canned leaves is easier than fresh and frozen leaves.

Conclusion

Any preservation methods that keep the antioxidant levels will be of interest to the cosmetics, food, and drug industries. In this study there were no notable changes in the total phenolic and flavonoid contents during freezing and canning of grapevine leaves. Additionally, there were no significant differences in antioxidant activity between the fresh, frozen, and canned leaves. Accordingly, these preservation methods can continue to be used in domestic contexts, especially canning which is considered economic and friendly to the environment.

Competing interests

All authors declared that they have no fund and competing interests.

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Authors' contributions

NJ conceived and designed the study, NJ, AZ, IA and FH did the experiments. This paper was drafted by NJ.

Asma Yapraklarını Koruma Yöntemlerinin Toplam Fenol, Toplam Flavonoit ve Antioksidan Kapasite Üzerine Etkileri

ÖZ

Koruma yöntemleri, birçok sebze ve meyvenin toplam fenolik, flavonoit miktarını ve antioksidan kapasitesini etkileyebilir. Bu etkiler antioksidan bileşiklerin bozunmasına, yeni bileşiklerin oluşmasına ya da aktif metabolitlerin yıkımına sebep olabilir. Bu çalışmada ilaç ya da gıda olarak tüketilebilen asma yapraklardaki toplam flavonoit, toplam fenolik ve antioksidan aktivite üzerinde, konserve ve dondurma gibi farklı koruma yöntemleri üzerindeki etkileri incelenmiştir. Fitokimyasal tarama, standart analitik yöntemler kullanılarak yapılmıştır. Antioksidan aktivite 2,2-difenil-1-pikrilhidrazilhidrat reaktif yöntemi kullanılarak değerlendirilmiştir; toplam flavonoit ve fenolik miktarı sırasıyla Folin- Ciocalteu metodu ile rutin referans standart maddesi kullanılarak karar verilmiştir. Tüm çalışılmış asma yaprak ekstrelerinin fitokimyasal içeriği aynıdır. Taze yaprak ekstresi, toplam fenolik ve flavonoit içeriğinin yanı sıra en yüksek antioksidan kapasiteyi göstermiştir. Dondurulmuş yaprak ekstresinde de aynı sonuçlar görülürken; konserve yaprak ekstresi daha düşük antioksidan kapasite göstermiş, toplam fenolik ve flavonoit içerikleri düşmüştür. Bu yaprakların konserve ve dondurulmuş koruma yöntemlerinin toplam fenolik ve flavonoit içeriklerinin yanı sıra toplam antioksidan kapasite üzerinde de zararlı etkisi yoktur. Bu yüzden bu yöntemler nutrasötik, kozmesötik ve farmasötik takviyeleri hazırlamada kullanılabilir. Buna rağmen, konserve edilmiş *V. vinifera* yapraklarının korunması ekonomik ve çevresel olarak dondurulma işleminin tersine bir durumdur. Ayrıca konserve yaprakların yükleme, ambalajlama ve korunması taze ve dondurulmuş yapraklardan daha kolaydır.

Anahtar Kelimeler: *Vitis vinifera*; Antioksidan aktivite; Fenolik; Flavonoitler; Koruma yöntemleri

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