Journal of Food and Nutrition Research, 2014, Vol. 2, No. 12, 925-930
Available online at http://pubs.sciepub.com/jfnr/2/12/11

DOI:10.12691/jfnr-2-12-11

Effect of Geographical Region and Harvesting Date on Antioxidant Activity, Phenolic and Flavonoid Content of Olive Leaves

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Received September 13, 2014; Revised November 10, 2014; Accepted November 17, 2014

Abstract The effect of geographical region and harvesting date (seasonal change) on antioxidant activities (AA), total phenolic content (TPC) and total flavonoid content (TFC) of olive leaves obtained from different geographical regions of Palestine (north, middle, and south) at different maturation stages (June 2013, October 2013, and January 2014) was investigated in this study. Results revealed that both geographical region and maturation stage affect AA, TPC, and TFC of the olive leaves. Highest AA, TPC, and TFC were obtained for samples collected in June. TPC was found to be highest in north and lowest in south, while the highest AA, and TFC contents were alternating between north, middle, and south. During different maturation stages, TPC, TFC, and AA varied between 21.56 - 47.52 mg (GAE), 19.3 - 32.6 mg catechin equivalents, 318.53 – 1106.43 µmol FRAP equivalents per gram of dry olive leaves, respectively.

Keywords: *Olive leaves, antioxidant activity, phenolic content, flavonoid content, harvesting date, geographical region*

Cite This Article: Fuad Al-Rimawi, Imad Odeh, Abdallah Bisher, Jehad Abbadi, and Mohammad Qabbajeh, "Effect of Geographical Region and Harvesting Date on Antioxidant Activity, Phenolic and Flavonoid Content of Olive Leaves." *Journal of Food and Nutrition Research*, vol. 2, no. 12 (2014): 925-930. doi: 10.12691/jfnr-2-12-11.

1. Introduction

Olive leaves are rich with polyphenolic and flavonoid compounds which exhibit many activities (e.g. antimicrobial, anticancer, antioxidant), and consequently play important role in disease resistance. Natural phenolic compounds in olive leaves are used in pharmaceutical, food, and cosmetic preparations as natural food additives, anti-ageing in cosmetics, antibacterials, antioxidants, preservatives, and dietary supplements instead of synthetic chemicals [1,2,3]. Olive leaves are well-known for their hypotensive effects [4], antimicrobial [5], and anti-viral properties [6].

There are many parameters affecting TPC, TFC, and AA of olive leaves, including, among others, geographical region, maturity stages or harvesting date, olive fruit cultivar, climate, type of soil..etc. Ryan et al. [7] reported that there are differences in amounts and type of phenolic compounds in olive leaves, fruits and seeds from different cultivars from Italy. Briante et al. [8] reported that there are significant changes in the phenolic composition of fruits and leaves during the maturation period of two Italian cultivars of Olea europaea. Hajimahmoodi et al. [9] have found differences in antioxidant activity, phenolic contents and reducing power between six Iranian olive cultivars. Kostas Kiritsakis et al. [10] were also found differences in the total amounts of phenols in the olive leaf extracts from different Greek olive cultivars. Ben Salah et al. [11] has investigated the effect of olive leaves varieties from Tunisia on the total phenol and flavonoid content and for the in vitro antioxidant properties. Similarly, Janina Diogo [12] and Abdel-Sattar et al. [13] determined the total phenolic content of olive leaves from Turkey, and Saudi Arabia, respectively.

To date no systematic study has been performed on antioxidant activity and phenolic content or flavonoid content of olive leaves from Palestine. The objective of the current work is therefore to study the antioxidant activity, phenolic and flavonoid contents of one variety of olive leaves from Palestine, and to study the effect of geographical region and harvesting date on these three parameters of olive leaves. Our investigation involved the use of Folin-ciocalteu, FRAP and the aluminum chloride colorimetric assays in order to determine the total phenolic content, antioxidant activity and total flavonoid content in the collected olive leaf samples, respectively.

2. Material and Methods

2.1. Chemicals

2,4,6-tripyridyl- S-triazine (TPTZ), ferric chloride hexahydrate, catechin, gallic acid, sodium hydroxide,

hydrochloric acid, acetic acid, sodium nitrite, aluminum chloride, iron (II) sulfate hexahydrate, and sodium bicarbonate are from Sigma-Aldrich company.

2.2. Reagents

FRAP reagent was prepared according to Benzie and Strain (1999) by the addition of 2.5 ml of a 10 mM tripydyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3.6H_2O$ and 25 ml of 0.3 M acetate buffer at pH 3.6. Acetate buffer (0.3 M) at pH 3.6 was prepared by dissolving 16.8 g of acetic acid and 0.8 g of sodium hydroxide in 1000 ml of water.

2.3. Olive Leaf Samples

Olive leaves samples (Nabali cultivar) were obtained from three different regions of Palestine (south, middle, and north). Olive leaves samples were collected at different maturity stages in June 2013, October 2013 and January 2014.

2.4. Extraction of Olive Leaf Samples

The olive leaves samples were dried at 30°C, grinded with a blender and extracted with distilled water (pH 7) or acidified distilled water (pH 3). Five grams of the dried powdered olive leaves were macerated with 50 ml of water for 2 hours at 40°C. The extracts were then filtered using suction filtration and stored in refrigerator at 4°C until analysis.

2.5. Total Phenolics Content (Folin–Ciocalteu Assay)

Total phenolics were determined using Folin–Ciocalteu reagents [14]. Olive leave extract (40 µl) were mixed with 1.8 ml of Folin–Ciocalteu reagent (pre-diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min, and then 1.2 ml of sodium bicarbonate (7.5%) was added to the mixture. After standing for 60 min at room temperature, absorbance was measured at 765 nm. Aqueous solutions of known gallic acid concentrations in the range of 100 – 500 ppm were used for calibration. Results were expressed as mg gallic acid equivalents (GAE)/ g sample.

2.6. Total Flavonoids Content

The determination of total flavonoids was performed according to the colorimetric assay of Kim et al. [15]. Distilled water (4 ml) was added to 1 ml of olive leaves extract. Then, 0.3 ml of 5% sodium nitrite solution was added, followed by 0.3 ml of 10% aluminum chloride solution. Test tubes were incubated at ambient temperature (25°C) for 5 min, and then 2 ml of 1 M sodium hydroxide were added to the mixture. Immediately, the volume of reaction mixture was made to 10 ml with distilled water. The mixture was thoroughly vortexed and the absorbance of the pink color developed was determined at 510 nm. Aqueous solutions of known Catechin concentrations in the range of 50 – 100 ppm were used for calibration and the results were expressed as mg catechin equivalents (CEQ)/ g sample.

2.7. Antioxidant Activity by FRAP Assay

The antioxidant activity of olive leave extracts was determined using the method of ferric reducing/antioxidant power (FRAP) of Benzie and Strain [16]. Freshly prepared FRAP reagent (3.0 ml) were warmed at 37°C and mixed with 40 µl of olive leaf extract and the reaction mixtures were later incubated at 37°C. Absorbance at 593 nm was read with reference to a reagent blank containing distilled water which was also incubated at 37°C for up to 1 hour instead of 4 min, which was the original time applied in FRAP assay. Aqueous solutions of known Fe (II) concentrations in the range of 2 - 5 mM (using $FeSO_4.6H_2O$) were used for calibration, and results were expressed as µ mole FRAP /g of dry olive leaf.

2.8. Statistical Analysis

Statistical analyses were performed using SAS statistical program to test the difference between the measured parameters (TPC, TFC, and AA) of the olive leaf samples from north, middle, and south Palestine, as well as those collected with different maturation stages by treating main factors (maturation stage and location) separately using one-way analysis of variance (ANOVA). Additionally Pearson correlation was used to test the correlation between measured parameters (AA and TPC, AA and TFC, and between TPC and TFC).

Different capital letters indicate significant differences within columns, while different small letters indicate significant differences within lines of the same extraction treatments: water (a, b, c) or pH 3 (bold a, b, c), while * indicates significant difference between different extraction treatments at each sampling date of the same sampling area.

3. Results and Discussion

3.1. Total Phenolic Content

Results showed that TPC decrease significantly with shifting from June 2013 through October 2013 to January 2014 for olive leaf samples in the three geographical areas of Palestine (see Figure 1). Table 1 shows statistical analyses of the results showing statistical differences between the TPC of the samples of the three harvesting dates represented by different capital letters (A, B, C) within the same column. The reduction of TPC in January 2014 compared to June 2013 sampling was 45-50% in water extracted samples, while in samples extracted with acidified water at pH 3, samples collected in January 2014 from north and middle contained only half the contents of total phenolic compounds compared to samples collected from the same areas in June 2013, but this reduction was sharper in samples collected from the south (61% reduction). The highest TPC was found to be in olive leaf samples from north obtained in June 2013 that extracted by acidified water (50.10 mg GAE/g dry olive leaves), and the lowest value was for samples obtained from south collected in January, and extracted by acidified water (18.63 mg GAE/g dry olive leaves) as seen in Table 1.

Table 1. Total phenolic content (TPC) (as mg Gallic acid/g of dry olive leaf) of olive leaf samples obtained in June 2013, October 2013, and January 2014, extracted with distilled water (pH ~ 7) and acidified water (pH = 3). (Results are expressed as average ± SD. RSD is relative standard deviation of three samples of olive leaf.)

Geographical region	Harvesting month	Extraction solvent	TPC (mg/g)
North Palestine	June 2013	Water (pH 7)	47.52 ± 0.9 * A, a
		Acidified water (pH 3)	50.10 ± 1.8 A, **a**
	October 2013	Water (pH 7)	31.42 ± 0.5 * B, a
		Acidified water (pH 3)	34.41 ± 0.9 B, **a**
	January 2014	Water (pH 7)	26.14 ± 1.2 * C, a
		Acidified water (pH 3)	24.92 ± 1.0 C, **a**
Middle Palestine	June 2013	Water (pH 7)	46.13 ± 0.8 * A, b
		Acidified water (pH 3)	49.34 ± 0.8 A, **b**
	October 2013	Water (pH 7)	27.14 ± 1.2 * B, b
		Acidified water (pH 3)	26.33 ± 1.3 B, **b**
	January 2014	Water (pH 7)	25.42 ± 0.9 * C, b
		Acidified water (pH 3)	23.93 ± 1.7 C, **b**
South Palestine	June 2013	Water (pH 7)	44.13 ± 1.0 * A, c
		Acidified water (pH 3)	48.30 ± 1.5 A, **c**
	October 2013	Water (pH 7)	23.87 ± 0.8 * B, c
		Acidified water (pH 3)	22.63 ±1.1 B, **c**
	January 2014	Water (pH 7)	21.56 ± 1.4 * C, c
		Acidified water (pH 3)	18.63 ± 1.0 C, **c**

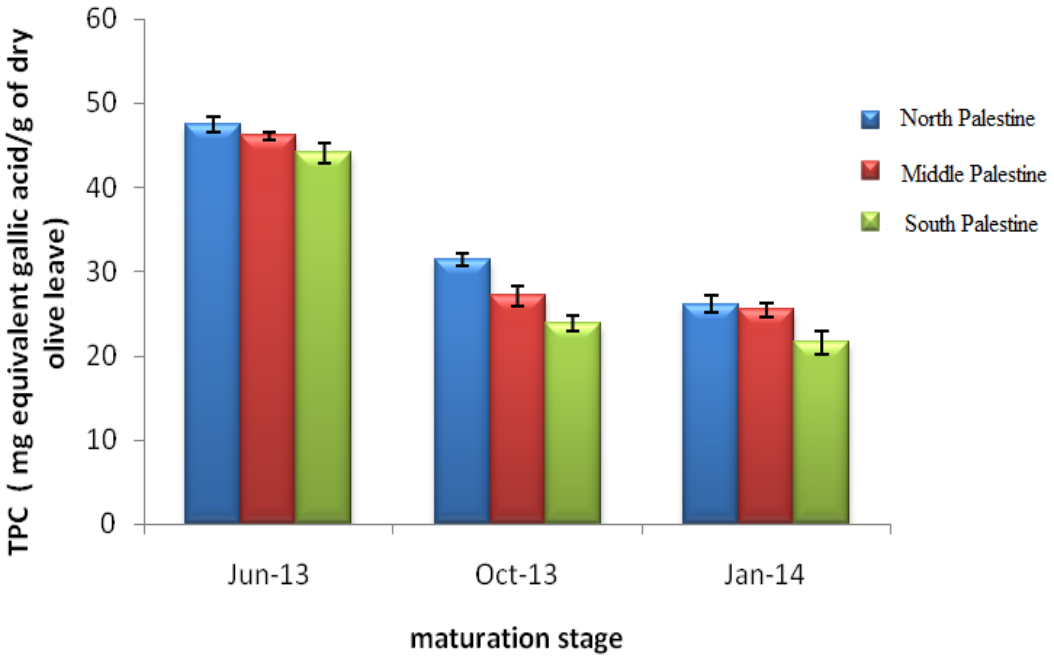

Figure 1. Total phenolic content (TPC) (as mg Gallic acid/g of dry olive leaves) of olive leaf samples obtained from north, middle, and south Palestine in three different maturation stages (June 2013, October 2013, and January 2014). Error bars was added as standard deviation of three samples

Helen [17] evaluated the impact of harvesting date of olive leaves from New Zealand on phenolic composition of olive leaves, in which two cultivars "Frantoio" and "Barnea" were investigated, and leaves were collected from their respective tree in October and November 2010. The leaves collected in October had significantly higher total phenolic (25.17 and 17.17 mg caffeic acid / g dry matter) than those collected in November (17.20 and 15.75 mg caffeic acid / g of dry matter) for the two cultivars. This results support our findings, where higher values of TPC were found to be in June with temperatures ranging from 25°C to 30°C and lowest values were in January with temperatures ranging from 5°C to 12°C.

Regarding the effect of the geographical region of the olive leaves, TPC was found to be greater in samples collected from north followed by middle followed by south, in both extraction solvents (water and acidified water) in all sampling times (June, October and January), Table 1. Statistically it was found that significant differences exist between the three geographical regions (north, south, and middle), indicated by small letters for samples extracted with water, and small bold letters for samples extracted with acidified water.

Comparing both extraction solvents (water and acidified water) in terms of total phenolic content, there was no clear trend because the differences were significant in all samples (represented by * in Table 1), but the higher values alternate between the two extraction solvents in different samples.

3.2. Total Flavonoid Content

The total flavonoids contents was found to be higher when samples were collected in June followed by samples collected in October, and then samples collected in January in all collection areas and in both extraction solvents in agreement with the results obtained for TPC, see Table 2 where this significant difference is represented

by different capital letters (A, B, C) within the same column. The reduction of TFC in January 2014 compared to June 2013 sampling was 7-33% in water extracted samples, while in samples extracted with acidified water, this reduction was from 15-38%, but this reduction was sharper in samples collected from the south and north (38% reduction) compared to middle (15%). The highest TFC were obtained from samples collected from southern Palestine in June when extracted with water (32.6 mg catechin/g of dry olive leaf), followed by northern Palestine when extracted at pH 3 (32.0 mg catechin/g of dry olive leaf), while the lowest value obtained when samples were collected in January (19.3 as mg Catechin/g of dry olive leaf) from middle Palestine and extracted with water.

Table 2. Total flavonoids content (TFC) (as mg catechin/g of dry olive leaf) of olive leaf samples obtained in June 2013, October 2013, and January 2014, extracted with distilled water (pH ~ 7) and acidified water (pH = 3)

Geographical region	Harvesting month	Extraction solvent	TPC (mg/g)
North Palestine	June 2013	Water (pH 7)	26.5± 0. 36 * A, b
		Acidified water (pH 3)	32.0± 0.21 A, **a**
	October 2013	Water (pH 7)	22.4 ± 0.40 *B, b
		Acidified water (pH 3)	25.5± 0.40 B, **a**
	January 2014	Water (pH 7)	19.4± 0.35 C, b
		Acidified water (pH 3)	19.8± 0.06 C, **a**
Middle Palestine	June 2013	Water (pH 7)	21.6± 0.26 * A, c
		Acidified water (pH 3)	22.7± 0.47 A, **b**
	October 2013	Water (pH 7)	20.8± 0.46 B, c
		Acidified water (pH 3)	21.3± 0.96 B, **c**
	January 2014	Water (pH 7)	19.3± 0.21 C, b
		Acidified water (pH 3)	19.4±0.10 C, **b**
South Palestine	June 2013	Water (pH 7)	32.6± 0.06 * A, a
		Acidified water (pH 3)	31.4± 0.45 A, **a**
	October 2013	Water (pH 7)	25.0± 0.99 B, a
		Acidified water (pH 3)	24.6± 0.11 B, **b**
	January 2014	Water (pH 7)	21.6± 0.21 C, a
		Acidified water (pH 3)	19.6± 0.25 C, **b**

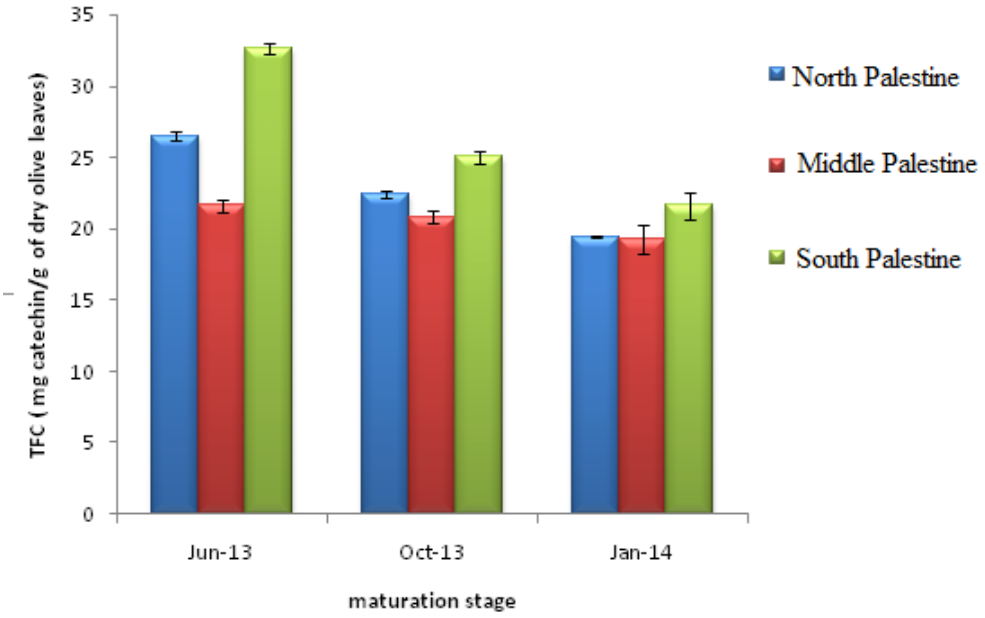

Figure 2. Total flavonoid content (TFC) (as mg catechin/g of dry olive leave) of olive leaf samples obtained from north, middle, and south in three different maturation stages (June 2013, October 2013, and January 2014), extracted with water (~pH=7.0). Error bars was added as standard deviation of three samples

Regarding the effect of geographical region, it was found that there is no clear trend in the TFC in the three geographical regions, for example in June and January, the highest TFC was obtained in south when extracted with water, while in October, the highest TFC was obtained in north when extracted with acidified water.

Comparing both extraction solvents in terms of total flavonoid content, there was no clear trend because the differences were in some samples significant while in other samples the differences are not significant, but the higher values alternate between different extraction solvents in different samples (Table 2, Figure 2).

3.3. Antioxidant Activity (AA)

Similar to TPC and TFC, AA decreased with shifting date of sampling from June through October to January, this was shown in all sampling areas and both extraction solvents (Table 3, Figure 3). As it is seen in Table 3, AA of samples collected in January diminished by about 55-70% compared to those collected in June. The highest AA

values were obtained in samples collected from south in June in both extraction methods (1106.43 and 1187.23 µ mol FRAP/g of dry olive leaf, for samples extracted with water and acidified water, respectively) followed by samples collected from north in the same sampling date, while the lowest value obtained when samples were collected in January (317.37 µ mol FRAP/g of dry olive leave) from middle Palestine when extracted with acidified water. During different maturation stages, antioxidant activity (FRAP assay) measured during different maturation stages was found to be in the range of 422.20 - 936.67, 337.53-747.70, and 318.53 – 1106.3 µmol FRAP equivalents/g of dry of olive leaves (extracted with water) collected from north, middle, and south, respectively.

Results showed that as for TPC and TFC, the extraction solvent did not favor any of the two solvents utilized in these assays. Furthermore, as for TFC, there is no clear trend in the AA of the three geographical regions.

Table 3. Antioxidant activity (AA) (as µ mole FRAP /g of dry olive leaf) of olive leaf samples obtained from north, middle, and south in three different maturation stages (June 2013, October 2013, and January 2014), extracted with distilled water (pH ~ 7) and acidified water (pH = 3)

Geographical region	Harvesting month	Extraction solvent	TPC (mg/g)
North Palestine	June 2013	Water (pH 7)	936.67 ± 5.8 A, b
		Acidified water (pH 3)	935.33±13.2 A, **b**
	October 2013	Water (pH 7)	855.53± 13.6 * B, b
		Acidified water (pH 3)	871.50± 5.1 B, **a**
	January 2014	Water (pH 7)	422.20± 11.0 * C, a
		Acidified water (pH 3)	412.50± 22.9 C, **a**
Middle Palestine	June 2013	Water (pH 7)	747.70± 8.45 *A, c
		Acidified water (pH 3)	643.87± 6.7 A, **c**
	October 2013	Water (pH 7)	656.50± 6.6 *B,c
		Acidified water (pH 3)	616.00± 4.0 B, **c**
	January 2014	Water (pH 7)	337.53± 5.7 *C, b
		Acidified water (pH 3)	317.37± 15.2 C, **c**
South Palestine	June 2013	Water (pH 7)	1106.43± 9.3 *A, a
		Acidified water (pH 3)	1187.23± 13.0 A, **a**
	October 2013	Water (pH 7)	867.23± 11.9 * B, a
		Acidified water (pH 3)	850.63± 12.9 B, **b**
	January 2014	Water (pH 7)	318.53±8.6 *C, c
		Acidified water (pH 3)	355.33± 7.0 C, **b**

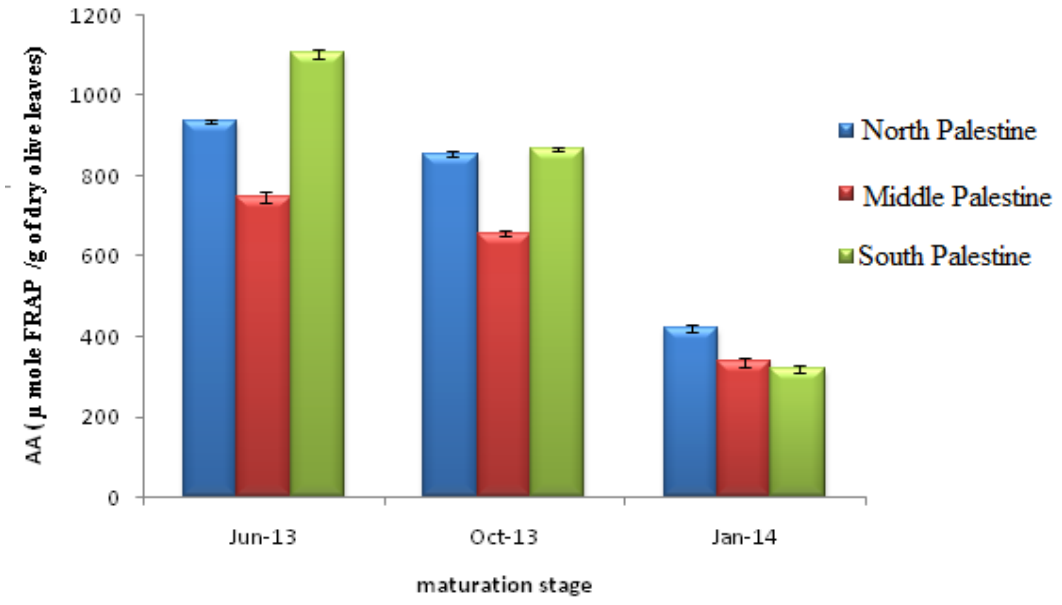

Figure 3. Antioxidant activity (AA) (as µ mole FRAP /g of dry olive leaves) of olive leaf samples obtained from north, middle, and south Palestine in three different maturation stages (June 2013, October 2013, and January 2014), extracted with water (~pH=7.0). Error bars was added as standard deviation of three samples

3.4. Pearson Correlation

Pearson correlation between TPC, TFC, and AA of samples collected from the three regions in Palestine showed that antioxidant activity is significantly correlated with total phenolic content in both extraction treatments, and weakly correlated with total flavonoids. But when all data was pooled, both TPC and TFC were highly and significantly correlated with AA. It was also found no significant correlation between TPC and TFC in any sample under investigation. Similar results were obtained by Abaza, et al. [18] where they got a strong correlation between antioxidant activity and total phenolics content or total flavonoids content. Similar results were obtained also by Bhoyar et al. [19] where a positive correlation was obtained between total phenolics content and FRAP antioxidant activity. This correlation is expected since AA is mainly due to the presence of polyphenolic and/or flavonoid that have many hydroxyl groups which are responsible for the antioxidant activity.

4. Conclusions

TPC, TFC, and AA of olive leaf samples collected from Palestine are affected by harvesting time, and the geographical region. Highest TPC, TFC, and AA were obtained for samples collected in summer (June) compared to those collected in winter (January). TPC was found to be highest in north and lowest in south. The highest and lowest values of TFC and AA of the samples were alternating between north, middle, and south. It was found a positive correlation between AA and TPC as well as between AA and TFC. On the basis of these findings, it is concluded that olive leaves from Palestine is a rich source of phenolics, flavonoid compounds, and more rich compared to those from Iran, Greece, and Italy. Olive leaves therefore constitutes a natural source of potent antioxidants, and could potentially be used in food, pharmaceutical, cosmetic formulations as additives, preservatives, and antioxidants.

Statement of Competing Interests

The authors have no competing interests.

List of Abbreviations

TPC: Total phenolic content
TFC: Total flavonoid content.
AA: Antioxidant activity
FRAP: ferric reducing/antioxidant power
TPTZ: 2,4,6-tripyridyl- S-triazine
GAE: Gallic acid equivalents

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