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Total Polyphenols, Flavonoids Content and Antioxidant Activity of Crude Methanolic and Aqueous Extracts for Some Medicinal Plant Flowers.

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Abstract

The total polyphenols and flavonoids contents of C. cardunculus, A. millefolium, C. officinalis, and M. chamomilla flowers, C. officinalis flowers have the highest of total polyphenols and flavonoids contents, which were 86.11 mg GAE/g and 11.59 mg GAE/g, respectively. Methanolic and aqueous extracts of plant flowers was antioxidant activity by used (FRAP, ABTS and DPPH) The methanolic and aqueous extracts of C. officinalis flowers have the highest reducing power which was ranged from 0.5054 to 1.8019 and 0.4215 to 1.3153 at the concentrations of 10 and 80 mg/ml, respectively. Also, by used (ABTS) it could be percentage were 90.17and 79.31%, While, by used (DPPH) it could be percentage of inhibition (IC50) which were 0.004 and 0.006 for methanolic and aqueous extracts respectively.

Keywords*: polyphenols, flavonoids, plant extracts, antioxidant activity (FRAP-ABTS-DPPH).*

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Introduction

Total polyphenols include several classes of phenolic compounds that are secondary plant metabolites and integral part of human and animal diets. Flavonoids are large group of the phenolic compounds consisting mainly of flavonols, flavanols and anthocyanins. Phenolic compounds can play an important role in preventing body cells and organs from injuries by hydrogen peroxide, damaging by lipid peroxides and scavenging or neutralizing free radicals **(Sroka and Cisowski, 2003)**. It has been reported that free radical scavenging and antioxidant activity of many medicinal plants are responsible for their therapeutic effect against cancer, diabetes, tissue inflammatory and cardiovascular diseases **(Cai *et al.,* 2004)**.

Pereira *et al,* 2013), who found that reducing power and DPPH activity of *Artichoke*. Also, **Dias *et al,* (2013)**, who studied that reducing power and DPPH activity of *Achillea millefolium*. Moreover, **Rigane *et al,* (2013)**, who found that the highest antioxidant activities using (FRAP-ABTS-DPPH) assays were obtained with aqueous-methanol flower extract from *C. officinalis*. Likewise, **Guimarães *et al,* (2013)**, who mentioned that the reducing power of wild *german chamomile* for methanolic extract.

Materials and methods

Plant materials

Flowers sample from each species were air dried in the shade and ground into a fine powder. The powdered air dried flowers were divided into two extracts: First extract: Powdered air dried flowers (2 Kg) from each plant were extracted by soaking at room temperature for six times with methanol (30 L), then the methanolic extracts were concentrated to nearly dryness under reduced pressure using the rotary evaporator at 45°C to achieve the crude methanol extract which kept for further investigation **(El-Khateeb *et al.,* 2014)**. The yields of extracts were 17.9%, 16.5%, 19.8% and 18.3%, for *C. cardunculus, A. millefolium, C. officinalis,* and *M. chamomilla*, respectively. Second extract: Powdered air dried Flowers (2 Kg) of dried samples were extracted with distilled water by boiling at temperature from 80 to 100°C in reflux for 3 h to achieve an initial extract. All extracts were filtered after cooling to room temperature. Finally, the extracts were lyophilized and preserved at −20°C until further use **(Kim *et al.,* 2011)**. The yield of aqueous extracts were 7.7%, 6.2%, 9.4% and 8.6%, for *C. cardunculus, A. millefolium, C. officinalis,* and *M. chamomilla*, respectively,

Total polyphenols content of investigated Flowers:

Total phenolic contents of air dried flowers were determined by using Folin–Ciocalteu reagent method according to **Lin and Tang (2007)**, the following experiment has been achieved at Chemistry department Faculty of Agriculture, Mansoura University. About 0.1g of air dried Flowers was dissolved, separately in 1 ml distilled water. Aliquots of 0.1 ml from previous solution was taken and mixed with exactly 2.8 ml of distilled water, 2.0 ml of (2% w/v) sodium carbonate and finally 0.1 ml of 50% (v/v) of Folin–Ciocalteu reagent was added. Mixture was incubated for 30 minutes at room temperature and the absorbance of the resulting color was measured at 750 nm against distilled water as blank, using a Spekol 11 (Carl Zeiss-Jena) spectrophotometer. For quantitatively determination a standard curve of gallic acid (0-200mg/l) was prepared in the same manner. Total phenol contents were expressed as milligram gallic acid equivalent (GAE)/g based on dry weight.

Total flavonoids content of investigated Flowers: Total flavonoids content of air dried Flowers were determined calorimetrically using aluminum chloride as described by **Chang *et al.,* (2002)**, the following experiment has been achieved at Chemistry department Faculty of Agriculture, Mansoura University. About of 0.1g of air dried Flowers were dissolved in 1ml of distilled water. Resulting solution (0.5 ml) was mixed with 1.5 ml of 95% ethyl alcohol, 0.1 ml of 10% aluminum chloride (AlCl3), 0.1ml of 1M potassium acetate (CH3COOK) and 2.8 ml of distilled water. After incubation at room temperature for 40 min, the reaction mixture absorbance was measured at 415nm against distilled water as blank, using a Spekol 11 (Carl Zeiss-Jena) spectrophotometer. Quercetin was chosen as a standard of flavonoids for making the standard curve (0–50mg/l). The concentration of total flavonoids contents was expressed as milligram quercetin equivalent (QE)/g based on dry weight.

Determination of Reducing power, (FRAP) radical scavenging activity:

Reducing power of methanolic flowers extracts was determined according to the method of **Oyaizu (1986)**, The following experiment has been achieved at Chemistry department Faculty of Agriculture, Mansoura University.: Extract (0–100mg) from each sample in 0.20mol phosphate buffer, pH 6.6 (2.5ml) was added to 2.5ml potassium ferricyanide (10mg/ml), mixture was incubated at 50^{o}C for 20min. Trichloroacetic acid (TCA) (2.5ml, 100mg/ml), was added to the mixture then centrifuged at 650g for 10 minutes. The supernatant (2.5ml) was mixed with distilled water (2.5ml) and 0.5ml ferric chloride solution (1mg/ml) was added and the absorbance of the resultant color was measured using a Spekol 11 (Carl Zeiss-Jena) spectrophotometer at 700nm. Higher absorbance of the reaction mixture indicated greater reducing power.

Determination of ABTS radical scavenging activity: ABTS (2,2'-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) assay was based on the method of **Re *et al.,* (1999)** with slight modifications. 2ml of ABTS solution (1mg/1ml 0.1M phosphate buffer, pH 7.0) were

added to 3ml of MnO2 (25mg/ml in previous phosphate buffer). Mixture was shaken and centrifuged for 10 minutes, clear supernatant was separated. Exactly 1mg of crude methanolic flowers was dissolved in a mixture solvent (1ml) of methanol and previous phosphate buffer in the ratio of 1:1. Resultant extract solution (20µl) was added to the ABTS solution mixture, as previously described. Positive control sample was prepared exactly in the same manner but differ only in the addition of 20µl of 2mM ascorbic acid, instead of extract solution. Blank sample was prepared exactly in the same manner but differ only in the addition of 20µl of distilled water, instead of extract solution. Absorbance of the resulting greenish-blue solution was recorded at wave length 734nm, using a Spekol 11 (Carl Zeiss-Jena) spectrophotometer. The decrease in absorbance is expressed as a percentage of inhibition which was calculated from the following equation:

$$\% \text{ Inhibition} = \frac{\text{A Blank} - \text{A Test}}{\text{A Blank}} \times 100$$

Determination of (DPPH) radical scavenging activity:

The DPPH free radical scavenging activity of Asteraceae (Family compositae) flowers extracts at different concentrations were measured from bleaching of the purple colour of (2.2 Diphenyl -1-picryl hydrazyl) was based on the method of **Pratap *et al.,* (2013)**. Exactly 0.1 ml solution of different concentration of extract was added to 1.4 ml of DPPH and kept in dark for 30 min. The absorbance was measured at 517 nm, using a Spekol 11 (Carl Zeiss-Jena) spectrophotometer. And the percentage inhibition was calculated by using the following Equation.

$$\text{Percentage inhibition (\%)} = (\text{A Blank} - \text{A Test}) / \text{A Blank}) \times 100$$

Statistical analysis: Statistical analyses of all experimental data were done using the statistical software package **(CoStat, 2005)**. All comparisons were first subjected to one way analysis of variance (ANOVA) and significant differences between treatment means were determined using Duncan's multiple range test at $P<0.05$ as the level of the significance **(Duncan, 1955)**.

Results and discussion

Total poly phenols and flavonoids contents of investigated flowers:

Table (1) showed the total polyphenols (mgGAE/g) and total flavonoids (mgQE/g) contents of *C. cardunculus, A. millefolium, C. officinalis* and *M. chamomilla* flowers. Data in Table (1) illustrated that, *C. cardunculus, A. millefolium, C. Officinalis,* and *M. chamomilla* flowers contained average values of 81.77, 65.44, 86.11, 76.22, mgGAE/g dry weight for total polyphenols, respectively. Finally, *Calendula officinalis* flowers have the highest concentration of total polyphenols, that was 86.11 mg GAE/g. While, *A. millefolium* flowers have the lowest concentration of total polyphenols, was 65.44 mg GAE/g. Also, *C. cardunculus* and *M. chamomilla* containing moderate values of total polyphenols which were 81.77 and 76.22mg GAE/g, respectively.

Total flavonoids as shown in Table (1) ranged from 5.70 to 11.59mgQE/g dry weight for *Matricaria chamomilla* and *Calendula officinalis* flowers, respectively. It can be observed that *C. officinalis* flowers contained the highest amount of total flavonoids. Whereas, *C. cardunculus, A. millefolium* and *M. chamomilla,* have convergent values, (6.82, 6.77 and 5.70 mgQE/g) of total flavonoids, respectively.

The data of *C. cardunculus* extracts for total phenolic and total flavonoids content were 81.77 mg GAE/g and 6.82 mg QE/g, were not in the same line with those reported by **Soumaya *et al.,* (2013)**, who initiated that total phenolic and flavonoids content in seeds artichoke which were 23.25 and 8.93 mg/g. While, for stems contain 12.75 and 8.93 mg/g, respectively. The findings were higher than of *A. millefolium* extracts for total phenolic and

total flavonoids content which were 65.44 mg GAE/g and 6.77mg QE/g, those recorded by **Eghdami and Sadeghi (2010)**, who found that the total polyphenols was 48.4 mgGAE/g of *A. millefolium* flowers aqueous extract. Previous data for *A. millefolium* flowers contain which were 65.44 and 6.77mg/g of total phenolic and flavonoids content, respectively. These results were lower than those reported by **Eghdami (2010)**, who quantified that total flavonoids content was 13.15 mgQE/g of *A. millefolium* flowers aqueous extract. While, the total phenolic, flavonoids contents in methanolic extract 123.9 mgGAE/g and 41.2 mgQE/g, respectively. Total phenolic and total flavonoids contents for *Calendula officinalis* flower were 11.59 mg GAE/g and 86.11 mg QE/g, respectively. The data were not in the same line with those reported by **Rigane *et al.,* (2013)**, who stated that total phenolic and total flavonoids contents which were 109.27 mg GAE/g and 76.44 mg QE/g dry weight in *C. officinalis* flower extract, respectively. The findings were higher than the results of *M. chamomilla* extracts for total phenolic and total flavonoids content which were 76.22 mg GAE/g and 5.70 mg QE/g, recorded by **Guimarães *et al.,* (2013)**, who found that the total polyphenols and total flavonoids contents in *M. chamomilla* which were 5 and 1.56 g/100g on dry weight basis, respectively.

Table 1. Total polyphenols (mgGAE/g) and total flavonoids (mgQE/g) contents of investigated Flowers on dry weight base.

Plant Flowers	Total polyphenols (mgGAE/g)	Total flavonoids (mgQE/g)
Cynara cardunculus	81.77	6.82
Achillea Millefolium	65.44	6.77
Calendula officinalis	86.11	11.59
Matricaria chamomilla	76.22	5.70

Reducing power of crude methanolic and aqueous extracts of flowers :

The results were not in the same line with those reported by Haghi *et al.,* (2014), who stated that total phenolic contents ranged between 1.77 to 50.75 mg GAE/g dry weight base in *M. chamomilla*, While the results were higher in total flavonoids contents ranged between 0.82 to 36.75 (mg QE/g) dry weight in *M. chamomilla* extracts. **Ana *et al.,* (2013)**, found that total phenolic content in aqueous and ethanolic extracts obtained 43.35 and 47.78 mgGAE/100g, respectively. Whereas, total flavonoid content in aqueous and ethanolic extracts obtained 41.32 and 24.18 mgCE/100g, respectively. The obtained data are presented in table (2) the absorbance showed the reducing power for different concentrations of crude methanolic and aqueous extracts of *Cynara cardunculus, Achillea millefolium, Calendula officinalis,* and *Matricaria chamomilla* flowers. Data expressed as absorbance at 700nm for producing color as a result for using four concentrations (10, 20, 40, and 80 mg/ml) for each sample. From table (2), some points could be deduced:

The reducing power capacity increased with increasing the methanolic and aqueous extracts concentrations for all samples. *Calendula officinalis* flowers have the highest reducing power which was ranged from 0.5054 to 1.8019 for methanolic extract at the concentrations of 10 and 80 mg/ml, respectively. While, *Calendula officinalis* flowers have the highest reducing power which was ranged from 0.4215 to 1.3153 for aqueous extract at the concentrations of 10 and 80 mg/ml, respectively. The reducing power of *Cynara cardunculus, Achillea millefolium* and *Matricaria chamomilla* flowers, which were 0.7991, 0.9409 and 0.9965 for methanolic extract at concentration of 80 mg/ml, respectively. While, the reducing power of *Cynara cardunculus, Achillea millefolium,* and *Matricaria chamomilla* flowers, for aqueous extract at the concentrations of 10 and 80 mg/ml, which were 0.7721, 0.8312 and 0.8946 respectively. High levels of reducing power indicated the presence of some compounds which could be consedered electron donors and could react with free radicals to convert them into more stable products **(Arabshahi and Urooj, 2007)**.

The results of *Cynara cardunculus* flowers in methanolic extract for reducing power was 0.7991at Concentration 80 mg/ml, These findings was not in the same line with those reported by **Pereira *et al.,* (2013)**, who found that antioxidant activity concentration, for reducing power, (1.81/ mg/ml), of artichoke. Previous data of *Achillea millefolium* flowers for reducing power activity which ranged from (0.15 and 0.32) at Concentration 10 and 20 mg/ml, consecutively. which agreed with those obtained by **Dias *et al.,* (2013)**, who found reducing power activity ranged from (0.17 to 0.25 mg/ml), in *Achillea millefolium*. **Anita *et al.,* (2011)**, found antioxidant activity of *C. officinalis* leaves, flowers, after drying and fresh using FRAB, were 62.18, 27.69, 42.1 and 28.0µg/g on dry weight basis, respectively. While, **Rigane et al., (2013)**, found that the highest antioxidant activities using FRAP assays were obtained with aqueous-methanol flower extract from *C. officinalis* was 28.37 mmol of trolox). Present data agreed with those obtained by **Guimarães et al., (2013)**, who mentioned that the reducing power of wild German chamomile for methanolic extract, was 232.49 µg/ml.

Table 2. Reducing power of crude methanolic and aqueous extracts of flowers determined as absorbency at 700nm.

Extract	Concentration mg/ml	Optical density at 700nm			
		Cynara cardunculus	*Achillea millefolium*	*Calendula officinalis*	*Matricaria chamomilla*
Methanolic	10	0.0925	0.1570	0.5054	0.1596
	20	0.1921	0.3219	0.9219	0.3209
	40	0.3902	0.6518	1.2502	0.6488
	80	0.7991	0.9409	1.8019	0.9965
Aqueous	10	0.0881	0.1229	0.4215	0.1088
	20	0.1741	0.3009	0.6913	0.2974
	40	0.3255	0.6058	0.9632	0.5798
	80	0.7721	0.8312	1.3153	0.8946

Antioxidant capacity of flowers methanolic and aqueous extracts determined by ($ABTS^{+}$) cation radical:

The capacity of *Cynara cardunculus, Achillea millefolium, Calendula officinalis,* and *Matricaria chamomilla* flowers. methanolic and aqueous extracts to scavenge the ABTS radical was determined separately and compared with the reduction of ascorbic acid as a control sample which is known as a strong reducing agent. From table (3), it could be seen that all extracts showed different degrees of inhibition capacity, but their capacities were inferior than ascorbic acid which have the maximum inhibition (91.41%). From the same table, it is clear that methanolic extract of *C. officinalis* flowers have the lowest absorbance value (0.062) with the highest value of inhibition percentage (90.17) followed by *M. chamomilla* flowers which have 88.83% as inhibition capacity. Moreover, the capacity of *C. cardunculus* and *A. millefolium* methanolic flowers extracts to scavenge the ABTS radical was 83.10 and 72.44%, respectively. From the same table, it is clear that aqueous extract of *C. officinalis* flowers have the lowest absorbance value (0.059) with the highest value of inhibition percentage 79.31% followed by *M. chamomilla* flowers which have 76.15% as inhibition capacity. Moreover, the capacity of *C. cardunculus* and *A. millefolium* methanolic flowers extracts to scavenge the ABTS radical was 74.81 and 68.02%, respectively. Different studies indicated that the electron donation capacity which reflecting the reducing power of bioactive compounds was associated with high antioxidant activity **(Siddhuraju et al., 2002)**.

From tables (2 and 3), it was observed that methanolic extract of Calendula flowers have the highest reducing power and antioxidant activity which containing the highest amounts of total polyphenols and total flavonoids comparing with other investigated flowers. This may be due to the presence of some components such as quercetin-3-O-glucoside **Rigane *et al.,* (2013)**, with suitable concentrations in Calendula flowers which have highly reducing power and antioxidant activity. **Soumaya *et al.,* (2013)**, who found that antioxidant

activity of Artichoke stems showed the highest capacity to quench superoxide with IC_{50} value of 2.1 µg/ml, compared to seeds (5.6 µg/ml). These results confirmed the beneficial effect of *Cynara cardunculus* organs on human health due to their high antioxidant activity. The present data of *Achillea millefolium* for antioxidant capacity was (72.44%) are agreed with those found by **Ertas *et al.,* (2014)**, who mentioned that methanolic extract of *A. cappadocica*, with highest amount of total phenolic was the most potent antioxidant activity (70,62%), while water extract with the least amount of phenolic, was the weakest in the activity (92.19 µgPE/mg). While, **Haroon *et al.,* (2014)**, who found that the antioxidant activity IC_{50} and ABTS for methanol and chloroform extracts was average 3 µg/ml. **Preethi *et al.,* (2006)**. presented that *C. officinalis* extract was found to be scavenged superoxide, Hydroxyl radical, inhibition of lipid peroxidation, ABTS and nitric oxide in averages of 500, 480, 2000, 6.5 and 575 mg/mL, respectively. Also, **Anita *et al.,* (2011)**, who found that antioxidant activity of *C. officinalis* leaves using ABTS assays was 18.01µg/g, on dry weight basis. The present data of *Matricaria chamomilla* for antioxidant capacity was 88.83% are agreed with those found by **Munir *et al.,* (2014)**, who evaluated the antioxidant potential of *Matricaria chamomilla* ethanolic extract antioxidant potential IC_{50} on some microbial against using concentrations of 2.5, 5, 10 and 20 µg/ml which were 72.15, 75.86, 80.23 and 82.8%, respectively. While evaluating the antioxidant capability by **Proestos *et al.,* (2013)**, who found that inhibition measured by the ABTS assay for *Matricaria chamomilla* extracts was 12.4 %, respectively.

Table 3. Antioxidant capacity of methanolic extracts of flowers determined by ($ABTS^{+}$) cation radical.

Plants	Methanolic		Aqueous	
	Absorbance	% Inhibition	Absorbance	% Inhibition
C. cardunculus	**0.099**	**83.10**	**0.092**	**74.81**
A. millefolium	**0.092**	**72.44**	**0.089**	**68.02**
C. officinalis	**0.062**	**90.17**	**0.059**	**79.31**
M. chamomilla	**0.069**	**88.83**	**0.064**	**76.15**
+Ve Control (Ascorbic acid)	**0.045**	**91.41**	**0.045**	**91.41**
-Ve Control	**0.524**	**0**	**0.524**	**0**

Determination of Antioxidant Activity Using the 2,2 Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method:

The capacity of *Cynara Cardunculus, Achillea Millefolium*, *Calendula Officinalis*, and *Matricaria Chamomilla* methanolic and aqueous flowers extracts, radical scavenging ability using the stable radical DPPH. The antioxidant activity of methanolic and aqueous extracts prepared from the studied plant species are reported in Table (4). The concentration of an antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter widely adapted to measure the antioxidant activity **(Sanchez *et. al*. 1998)**. The lower EC_{50} pointed to the higher antioxidant activity. The antioxidant activity of the tested extracts were measured using DPPH radical scavenging activity. The antioxidants scavenging activities of DPPH are attributed to their hydrogen-donating abilities (Biswas *et al.,* 2010). Vitamin C was used as the reference compound. From table (10), it is clear that the scavenging effect (IC_{50}) of methanolic and aqueous extracts for *Calendula officinalis* flowers have the most effective of inhibition percentage (0.004 and 0.006), respectively. Followed in activity *Matricaria chamomilla* which were (0.006 and 0.009), respectively. Finally the scavenging effect of *Cynara Cardunculus* and *Achillea millefolium* flowers for methanolic extract which were (0.032 and 0.049), respectively. While, the scavenging effect of *Cynara Cardunculus* and *Achillea millefolium* flowers for aqueous extract were 0.041 and 0.056, respectively. Different studies indicated that the electron donation capacity which reflecting the reducing power of bioactive compounds was associated with antioxidant activity (Siddhuraju *et al.,* 2002). From table (10), it was observed that methanolic and aqueous extracts of *Cynara*

Cardunculus, and *Calendula officinalis* flowers have the highest reducing power and antioxidant activity which containing the highest amounts of total polyphenols and total flavonoids comparing with other investigated flowers **Khaldi and Jaime, 2012 and Rigane *et al.,* 2013**.

The data were parallel with that recorded by **Pereira *et al.,* (2013)**, who determined the antioxidant capacity of artichoke flowers when used as feed supplements. The antioxidant activity for DPPH scavenging were 2.13mg/ml. **Vitalini *et al.,* (2011)**, who calculated the IC_{50} of antioxidant activity for methanolic extract of *A. millefolium* using DPPH scavenging which was 1.18 µM. **Dias *et al.,* (2013)**, who found that antioxidant activity using DPPH assay for methanolic extract was 0.50mg/ml. **Haroon *et al.,* (2014)**, who found that the antioxidant activity IC_{50} determined using DPPH for methanol and chloroform extracts were average of 27µg/ml. **Rigane *et al.,* (2013)**, The highest antioxidant activities using DPPH assay were obtained with aqueous-methanol flower extract from *C. officinalis* (0.35 mg). **Anita, *et al.,* (2011)**, who found that antioxidant activity (DPPH) of *Calendula officinalis,* ranged from (0.1 and 0.5 µg/g), respectively. **Guimarães *et al.,* (2013)**, who found the antioxidant activity using DPPH of wild german chamomile methanolic extract, was 800.36µg/ml.

Table 4. Antioxidant capacity of methanolic and aqueous flower extracts determined by ($DPPH^+$) cation radical.

Plants	Extracts	
	Methanolic	Aqueous
C. cardunculus	**0.032**	**0.041**
A. millefolium	**0.049**	**0.056**
C. officinalis	**0.004**	**0.006**
M. chamomilla	**0.006**	**0.009**

Conclusion

The medicinal plant extract containing high percentage of active compounds such as Polyphenols and Flavonoids for *C. officinalis, M. chamomilla, C. cardunculus* and *A. millefolium* flowers respectively. The impact of natural extracts as antioxidant tested using (DPPH-ABTS-FRAB assays) showed high ability of these plants to scavenging the free radicals in laboratory.

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