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Research Article

Determination of the Total Phenolic and Flavonoid in Various Extracts of *Adiantum capillus veneris* Linn, as well as their Radical Scavenging Activity

Naema M El Aali*, Salah N Bugrein, Mohammed F El-Fellah, Maraia F Elmhdwi and Yusra F Layas

Chemistry Department, Faculty of Science, Benghazi University, Libya

***Corresponding Author:** Naema M El Aali, Chemistry Department, Faculty of Science, Benghazi University, Libya.

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Abstract

Adiantum capillus veneris linn is a member of Adiantaceae family which has been used as a folk medicine in management of hyperglycemia. Although there are some reports showing that the aqueous extract of the plant is effective as hypoglycemic agent but there were no scientific reports for its effectiveness.

The powdered dried leaves of *A. capillus veneris* L. was extracted continuously by Soxhelt extractor, five extracts (water, methanol, chloroform, ethyl acetate and acetone) were obtained and evaporated to dryness by rotatory evaporator. The extracts were investigated for antioxidant activity, reducing power and the presence of phenolic and flavonoid compounds.

The antioxidant activity of *A. capillus veneris* L. was confirmed for all extracts with the highest activity for the water extract IC_{50} = 2.7, this assay was also supported with reducing power assay, in which both methanolic extract and water extract exhibited the highest reducing power assay, while the ethyl acetate extract shows the highest total flavonoids content. The highest total phenolic content value was recorded for methanolic extract.

Keywords: Adiantum capillus veneris Linn; Antioxidant; Reducing Power; Flavonoids; Phenolic

Introduction

The knowledge about use of medicinal plants has been accrued through centuries time and such plants are still valued even today, although synthetics, antibiotics etc. have attained greater prominence in modern medicine. It is, however, a fact that these synthetics and antibiotics although they often show miraculous and often instantaneous results, prove harmful in the long run and this is why many synthetics and antibiotics have now gone out of use or suggested to be used under medicinal supervision. In the case of most medicine plants, however, no such cumulative derogatory effect has been recorded and many of medicines obtained from plants are widely used [1].

The World Health Organization (WHO) defines traditional medicine as: "the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being [2].

A. capillus veneris L. has potential importance in medicinal and nutritive purpose. It is used for chest complaints, cough, expectorant and for increasing lactation, colds, kidney function improvement, anti-parasitic and dandruff. Also it is used for depurative, emetic, emollient, febrifuge, galactagogue, Alopecia and tonic [3]. Furthermore this plant reported to be useful as de-toxicant in alcoholism and to expel worms from the body [4], and to has positive modulation of oxidation-linked diseases such as diabetes [5].

Free radicals chemical reactions and several redox reactions of various compounds may cause protein oxidation, DNA damage and lipid per-oxidation in living cells [6]. Therefore, oxidation have been claimed to play an important role in human health implicated in several diseases, including cancer, hypertension, heart attack, aging and diabetes. However, living organisms have developed antioxidant systems to counteract reactive species and to reduce their damage. These complex antioxidant systems include enzymes, such as superoxide dismutase (SOD and catalase (CAT). In addition to enzymatic system, an array of small molecules has been discovered in the past few decades such as glutathione (GSH) macromolecules, ascorbic acid, α-tocopherol, carotenoids, polyphenols, uric acid and bilirubin. Many researches showed that oxidative damage occurs when this system is overwhelmed [5,7].

In a biological system, an antioxidant can be defined as "any substance that when present at low concentrations compared to that of an oxidizable substrate would significantly delay or prevent oxidation of that substrate" [8].

Antioxidants could protect the human body against free radicals that may cause pathological conditions, such as anaemia, arthritis, inflammation, neurodegeneration, aging process and perhaps dementias [5].

Although the folk medicine has been used from long time by populations in treatment of different disease, there is a lack of data regarding the effectiveness of different plants. Therefore, this study was deemed worthwhile to connect folk medicine with scientific prove by antioxidant activity testing to *A. capillus veneris* L.

Exclusion criteria

Plant Collection and Identification

Samples of *A. capillus veneris* L. were collected from the valleys of Derna – Libya during the winter of 2011 - 2012, the botanical identification of *A. capillus veneris* L. was determined with the aid of the description given by the Libyan Flora and was eventually confirmed by comparison with authentic samples obtained from

herbarium of the Department of Botany University of Benghazi. The plant were allowed to dry at room temperature and the leaves were then ground into powder state using a commercial blender, and finally used for the preparation of different extracts.

Plant Extraction

Aqueous Extract: Powdered plant (25g) was extracted with 250 ml of distilled water by Soxhelt extractor (size 29 - 24) for 24 hrs. Then evaporated to dryness at 70 - 80^{0}C by Rotatory evaporator (RE2000).

Methanol, Chloroform, Ethyl acetate and Acetone Extracts: Powdered plants (40g) was divided into four parts (10g for each part), the first part were extracted by methanol for 12 hrs, the second, third and fourth parts was extracted with chloroform, ethyl acetate and acetone respectively for 8 hrs. The extracts were evaporated to dryness by rotatory evaporator at 40^{0}C for the methanolic extract and 50^{0}C, 60^{0}C and 40^{0}C for chloroform, ethyl acetate and acetone extracts respectively.

Chemicals

1,1-Diphenylpicrylhydrazyl (DPPH) was obtained from Sigma Chemicals. Chemicals such as Chloroform, methanol, ethyl acetate, acetone, copper sulfate, ascorbic acid and monobasic dihydrogen phosphate were obtained from Merck company, ferric chloride, sodium nitrite, acetic anhydride, aluminum chloride, potassium iodide, sodium Chloride, methanol and sodium carbonate were obtained from Farmitalia Carlo Erba. Dibasic monohydrogen phosphate, trichloro acetic acid, Ninhydrin reagent and sodium hydroxide were obtained from Redeal De Haennagtca. Potassium ferricyanide was obtained from NICE company. Alloxan monohydrate, iodine, potassium bismuth iodide, magnesium turning and acetone were obtained from BHD.

Sulfuric acid was obtained from Fixanal, ammonia was obtained from PRS Panreac, dichloromethane was obtained from Ferak Berlin, hydrochloric acid was obtained from APS Finechem, lead acetate was obtained from T-Baker lab chemicals.

DPPH free radical scavenging assay (RSA)

Solution of DPPH (0.2 mM) in methanol was prepared by dissolving 0.008 mg of DPPH in 100 ml of methanol. The DPPH radical scavenging activity of *A. capillus veneris* L. were determined according to the method described by Tang., *et al* (2002). Five different extracts of methanol, ionized water, ethyl acetate, chloroform and acetone were dissolved in methanol and different concentration of (100, 200, 300, 400 and 500 mg/L) were prepared and 400 µl of the different concentrations of *A. capillus-veneris* L extracts were added to 2 ml DPPH (0.02 mM). The Mixture was Kept in dark for 30 minutes at room temperature. The absorbance was read at 514.5 nm using spectrophotometer CE 7400, with solvent methanol as blank. The scavenging activity (%) was calculated according to the equation as follows:

$$\%RSA = [(ADPPH-AS)/ ADPPH] \cdot 100$$

where AS is the absorbance of the solution when the sample extract has been added at a particular level, and ADPPH is the absorbance of the DPPH solution [9].

Total Phenolic content

The total phenolic content was determined using colorimetric method and expressed as Pyrogallol equivalents according to the method proposed by Singleton., *et al.* in 1999. To 100 of 100, 200, 300, 400, and 500 mg/L of the different extracts 2 ml of de-ionized water were added and mixed with 600 µl of Folin-Cicalteau reagent, the tube was allowed to stand at room temperature for 5 minutes, and 2 ml of 20% sodium carbonate were add and kept at boiling water bath for 1 minute, after cooling the blue color formed measured at 765 nm by Aquarins (CE700) spectrophotometer Cecil instruments [10].

Reducing power assay

This assay was determined according to the method of Oyaizu (1986). 2.5 ml of 100, 200, 300, 400, and 500 mg/L *A. cappillus veneris* L. extracts were mixed with 2.5 ml of (0.2 M, pH 6.6) sodium phosphate buffer and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$], then the mixture was incubated at 50^{o}C for 20 minutes. 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 1000 rpm for 8 minutes (Centorion K24OR-2003 refrigerated centrifuge). The upper layer (5 ml) was mixed with 5 ml of de-ionised water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. Ascorbic acid was used to produce the calibration curve [11].

Total Flavonoid Content By Spectrophotometer

The determination of total flavonoids was performed according to the colorimetric assay. 4 ml of de-ionized water was added to 1 ml of 100, 200, 300, 400, and 500 mg/L of the different extracts of the sample. 0.3 ml of 5% sodium nitrite solution were added followed by 0.3 ml of 10% aluminum chloride solution. The mixture were incubated at ambient temperature for 5 minutes, and then 2 ml of 1M sodium hydroxide were added to the mixture. Immediately, the volume of reaction mixture was made to 10 ml with de-ionized water. The absorbance of the pink color developed was determined at 510 nm. A calibration curve was prepared with quercetin and the results were expressed as mg quercetin equivalents per gram dry weight of sample [12].

Result and Discussion

Antioxidant activity assay

1,1'-diphenyl-2-picrylhydrazyl (DPPH) reactivity is one popular method for screening the free radical-scavenging ability of compounds or the antioxidant activity of plant extracts, and has been used extensively as a free radical to evaluate reducing substances. The use of DPPH provides an easy and rapid way to evaluate antioxidants. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule DPP-Hydrazine [13]. Tables 1 and 2 show the percentage of inhibition values (%Inh) for DPPH radical-scavenging of Methanol, Water, Chloroform, Ethyl acetate and Acetone extracts of *A. capillus veneris* L. The data were compared to Ascorbic acid which is a synthetic antioxidant standard. Figure 1 shows percentage of inhibition of DPPH radical scavenging of different extracts and ascorbic acid. Standard division (SSD) had been measured.

Concentration "µg/ml"	Ascorbic acid		Methanol		Water	
	SSD	% Inh	SSD	% Inh	SSD	% Inh
100	1.570 ± 0.0212	44.00%	1.558 ± 0.02	28.53%	1.400 ± 0.012	35.77%
200	1.281 ± 0.00308	54.00%	1.392 ± 0.013	36.14%	1.058 ± 0.008	51.46%
300	0.907 ± 0.0040	67.00%	1.010 ± 0.007	53.67%	1.014 ± 0.019	53.48%
400	0.555 ± 0.00406	80.00%	0.792 ± 0.004	63.66%	0.958 ± 0.007	56.05%
500	0.0870 ± 0.03	96.00%	0.686 ± 0.015	68.53%	0.857 ± 0.005	60.68%

Table 1: Antioxidant activity assay for Ascorbic acid, Methanol and Water extracts of *A. capillus veneris* L. leaves

Concentration "µg/ml"	Chloroform		Ethyl acetate		Acetone	
	SSD	% Inh	SSD	% Inh	SSD	% Inh
100	1.614 ± 0.012	25.96%	1.676 ± 0.006	23.11%	1.905 ± 0.03	12.61%
200	1.535 ± 0.014	29.58%	1.587 ± 0.007	27.20%	1.823 ± 0.05	16.38%
300	1.454 ± 0.007	33.30%	1.474 ± 0.012	32.38%	1.792 ± 0.05	17.80%
400	1.349 ± 0.014	38.11%	1.440 ± 0.007	33.94%	1.758 ± 0.03	19.36%
500	1.158 ± 0.008	46.88%	1.324 ± 0.007	39.26%	1.745 ± 0.03	19.95%

Table 2: Antioxidant activity assay for Chloroform, Ethyl acetate and Acetone extracts of *A. capillus veneris* L. leaves

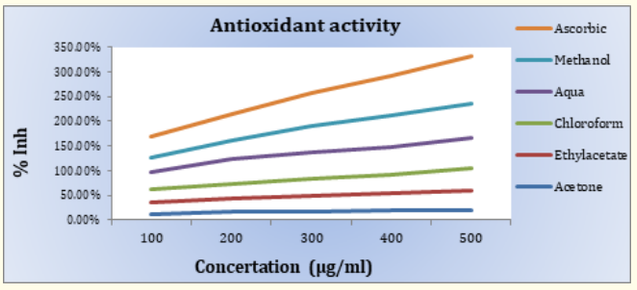

Figure 1: DPPH radical scavenging activity of different extracts of *A. capillus veneris* L. in different solvents. Ascorbic acid used as standard.

Concentration of the sample necessary to decrease initial concentration of DPPH• by 50% (IC_{50}) under the experimental condition was determined. Therefore, the lower value of IC_{50} indicates a higher antioxidant activity [14]. Table 3 shows the IC_{50}% value for the different extracts used in the study.

Solvent	IC_{50}%
Ascorbic acid	1.6
Water	2.7
Methanol	2.99
Chloroform	6.02
Ethyl acetate	7.79
Acetone	21.51

Table 3: The IC_{50} values for *A. capillus veneris* L. leaves in various extracts.

According to the IC_{50}% values the water extract showed particularly high antioxidant activity with IC_{50}% of 2.7 µg/ml which is one time lower than the ascorbic acid with IC_{50}% value of 1.6 followed by methanol, chloroform, ethyl acetate and acetone extracts.

Total Phenolic cotenant

Folin-Ciocalteu (F.C) reagent was used to determine total polyphenol in sample extracts. This reagent oxidises phenolates, re-

sulting in the production of complex molybdenum-tungsten blue which can be detected spectrophotometrically at 765 nm. F.C. reagent is employed routinely in studying phenolic antioxidants [3]. For the *A. capillus veneris* L. leaves extract, the phenolic content was found to be in the order of methanol > acetone > ethyl acetate > chloroform > water as shown in table 4. Methanol is a good solvent for *A. capillus veneris* L. as large amount of phenolics compounds are soluble in methanol when compared with other extracts.

	Mean ± Standard Deviation					
Conc. "µg/ml"	**Pyrogallol**	**Methanol**	**Acetone**	**Ethyl- acetate**	**Chloro-form**	**Water**
100	0.292 ± 0.005	0.016 ± 0.013	0.006 ± 0.005	0.008 ± 0.003	0.005 ± 0.001	0.008 ± 0.0020
200	0.494 ± 0.003	0.026 ± 0.006	0.012 ± 0.003	0.015 ± 0.001	0.012 ± 0.004	0.012 ± 0.001
300	0.797 ± 0.007	0.032 ± 0.006	0.024 ± 0.006	0.028 ± 0.001	0.017 ± 0.002	0.020 ± 0.005
400	0.857 ± 0.002	0.043 ± 0.003	0.030 ± 0.005	0.035 ± 0.003	0.023 ± 0.004	0.026 ± 0.005
500	1.022 ± 0.005	0.054 ± 0.006	0.044 ± 0.011	0.043 ± 0.019	0.043 ± 0.019	0.028 ± 0.001

Table 4: Total phenolic cotenant for pyrogallol, Methanol, Water, chloroform, ethyl acetate leaves extracts of *A. capillus veneris* L.

In general the amount of phenolic content when data were compared to Pyrogallol, which is a highly phenolic natural compound, was significantly low in all extract. Figure 2 shows the difference in total phenolic content for each extract and pyrogallol.

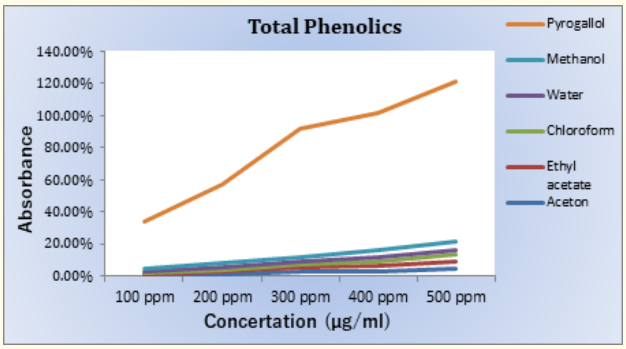

Figure 2: Total phenolic values of the different extracts from *A. capillus veneris* L. in different solvents expressed in µg/ml. Pyrogallol is used as a standard.

Total flavonoids content

Total flavonoids can be determined in the sample extracts by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminum complex using aluminum chloride which can be monitored spectrophotometrically at 510 nm [15]. The results as shown in table 5 showed that the ethyl acetate extract had the highest flavonoids content with a value of 0.263 ± 0.077 (absorbance unit) followed by acetone, chloroform, methanol and finally the water extract which had the lowest flavonoids content with value of 0.082 ± 0.004. All data were compared to Quercetin, which is a flavonoid natural standard. Figure 3 shows the difference in total flavonoids content for each extract and quercetin.

Reducing power

The presence of reducers (i.e. antioxidants) causes the conversion of the Fe+3/ferricyanide complex used in this method to the Fe+2/ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe+2 concen-

	Mean ± Standard Deviation					
Conc. "µg/ml"	**Quercetin**	**Ethyl-acetate**	**Acetone**	**Chloro-form**	**Methanol**	**Water**
100	0.236 ± 0.003	0.095 ± 0.018	0.119 ± 0.025	0.087 ± 0.017	0.139 ± 0.005	0.034 ± 0.010
200	0.337 ± 0.026	0.134 ± 0.020	0.140 ± 0.023	0.116 ± 0.030	0.148 ± 0.001	0.042 ± 0.006
300	0.442 ± 0.087	0.178 ± 0.021	0.167 ± 0.016	0.156 ± 0.035	0.159 ± 0.001	0.066 ± 0.005
400	0.542 ± 0.004	0.205 ± 0.040	0.200 ± 0.014	0.171 ± 0.039	0.164 ± 0.000	0.076 ± 0.004
500	0.588 ± 0.006	0.263 ± 0.077	0.230 ± 0.004	0.222 ± 0.026	0.178 ± 0.005	0.082 ± 0.004

Table 5: Total flavonoids cotenant for Querceten, Methanol, Water, chloroform, ethyl acetate extracts for the leaves of *A. capillus veneris* L

	Mean ± Standard Deviation					
Conc. "µg/ml"	**Ascorbic acid**	**Methanol**	**Water**	**Chloroform**	**Acetone**	**Ethyl acetate**
100	0.293 ± 0.012	0.399 ± 0.007	0.370 ± 0.044	0.216 ± 0.006	0.229 ± 0.024	0.253 ± 0.007
200	0.382 ± 0.032	0.433 ± 0.012	0.407 ± 0.004	0.224 ± 0.002	0.270 ± 0.001	0.255 ± 0.004
300	0.445 ± 0.008	0.435 ± 0.053	0.426 ± 0.004	0.316 ± 0.001	0.284 ± 0.004	0.258 ± 0.001
400	0.693 ± 0.10	0518 ± 0.007	0.470 ± 0.055	0.326 ± 0.003	0.328 ± 0.024	0.263 ± 0.005
500	0.992 ± 0.005	0.535 ± 0.028	0.494 ± 0.044	0.350 ± 0.002	0.346 ± 0.031	0.310 ± 0.000

Table 6: Reducing power for Ascorbic acid, Methanol, Water, chloroform, ethyl acetate and extracts for the leaves of *A. capillus veneris* L.

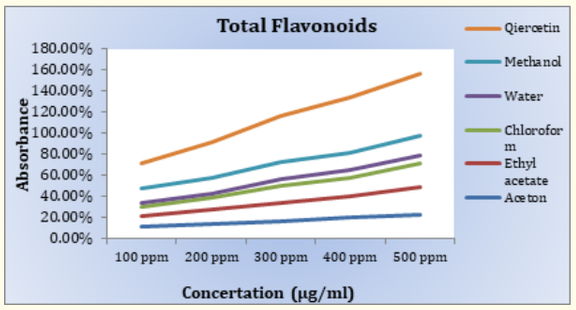

Figure 3: Total flavonoids values of the extract from *A. capillus veneris* L. in different solvents expressed in µg/ml. quercetin was used as standard.

tration; a higher absorbance at 700 nm indicates a higher reducing power [15]. The results as shown in table 6 proved that the methanol extract had the highest reducing power content with a value of 0.535 ± 0.028 (absorbance unit) followed by water, chloroform, acetone and ethyl acetate, which had the lowest reducing power with value of 0.310 ± 0.000.

All data were compared to Ascorbic acid which has high reducing power ability. Figure 4 shows the difference in reducing power assay for each extract and ascorbic acid.

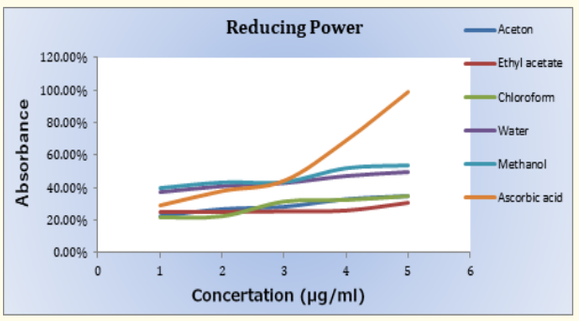

Figure 4: Reducing power assay of the extract from *A. capillus veneris* L. in different solvents expressed in µg/ml. Ascorbic acid was used as standard.

All extract showed strong antioxidant activity with low significant difference, the highest reducing ability was for the methanolic extract with a value of 0.535 ± 0.028 (absorbance unit), which is 1.8 times lower than that for the standard ascorbic acid, while the water extract was 2 times lower than the standard.

Conclusion

The present study gave an insight into the effectiveness of different extracts of the plant *A. capillus veneris* L. as antioxidant in which the antioxidant activity of *A. capillus veneris* L. was confirmed for all extracts with the highest activity for the water extract IC_{50} = 2.7, and the lowest for the acetone extract IC_{50} = 21.51, this assay was also supported with reducing power assay, in which both methanolic extract and water extract exhibited the highest values (0.535 ± 0.028 and 0.494 ± 0.044) respectively at 500 µg/ml, while the ethyl acetate extract gave the lowest value (0.310 ± 0.000) at 500 µg/ml. The measurement of total flavonoids content was the highest value for the ethyl acetate extract (0.263 ± 0.077) at 500 µg/ml, and the lowest value for water extract (0.082 ± 0.004) at 500 µg/ml. The highest total phenolic content value was recorded for methanolic extract (0.054 ± 0.006) at 500 µg/ml and the lowest value for water extract (0.028 ± 0.001) at 500 µg/ml. All of these variations proved the antioxidant activity of all extracts of *A. capillus veneris* L. leaves.

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