# Antioxidant potential, total phenolic and flavonoid contents of three culinary medicinal plant species of Lesser Hamalya, Pakistan

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It is well known that oxidative stress due to free radicals can lead to many human illnesses, like cancer. Antioxidants are the agents which scavenge these free radicals and protect the biological system. Food of plant origin is an essential source of reliable antioxidants. These plant-derived antioxidants belong to many biochemical categories but most are phenolics or flavonoids. The Galyat region of Pakistan is rich in plant biodiversity, and local inhabitants frequently use medicinal plants to treat common ailments. Three of these common regional medicinal plants (Dryopteris ramosa, Quercus leucotricophora and Arisaema flavum) were selected and their free radical scavenging potential was investigated using DPPH assay. The crude extract of D. ramosa exhibited the maximum free radical scavenging potential (93.8  $\pm$  0.2 %) while A. *flavum* (56.4  $\pm$  0.4 %) showed the lowest radical scavenging potential at 250 µg/ml. A low SC<sub>50</sub> value of the crude extract of D. ramosa (88.9  $\pm$  0.4 µg/ml) confirmed the relatively high antioxidant potential of this plant species. Among the polarity-based fractions obtained from crude extract of D. ramosa, the ethyl acetate soluble fraction showed maximum free radical scavenging potential. The D. ramosa contained higher amounts of total phenolic and flavonoid compounds than *Quercus* and *Arisaema* species. A significant Pearson correlation at the 0.01 level (2 tailed) was also noticed between  $SC_{50}$  and total phenolic contents of all extracts. A significant difference in percentage scavenging activities of the various extracts was observed. The ethyl acetate soluble phase of D. ramosa may prove to be an especially useful source of natural antioxidants for a variety of medicinal uses. Further studies are in progress.

Keywords: Antioxidant, Total phenolics, Total flavonoids, Dryopteris ramosa, Quercus leucotricophora, Arisaema flavum

# INTRODUCTION

Recent studies have confirmed the detrimental role of free radicals (reactive oxygen species ROS) and their direct role in causing oxidative stress leading to many human disorders. These free radicals are produced in the biological system by a variety of environmental factors, such as a poor diet, exposure to toxins, and emotional stress. Antioxidants are compounds that scavenge ROS and other free radicals and protect living cells. Antioxidants are not only prescribed as preventive measures against ROS to protect human health, but are also used for the treatment of various human diseases [1,2]. Plant-derived antioxidants belong to different biochemical categories, but are typical secondary metabolites concentrated in various plant organs specific to the different species [3-5]. Recently, plant antioxidants belonging to the phenolic and flavonoid classes, including ascorbic acid, tocopherol, various tocotrienols and carotenoids [6] have gained much importance. The

The research presented in this paper evaluates the antioxidant properties of three common and extensively used medicinal plants in the Galyat region, and sheds light on the relationship between the antioxidant potential of these species and their total phenolic and flavonoid contents. The selected plant species include *Dryopteris ramosa* (Hope) C. Chr, *Quercus leucotricophora* A. Camus and *Arisaema flavum* (Forssk.) Schott. The ethnomedicinal uses of these plants are given in Table 1.

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Galyat area ~ 50 km North East of Islamabad, Pakistan, is a moist temperate forest with an area of 1011.714 km<sup>2</sup> [7], lacking modern facilities, especially for health care, and as such the inhabitants are much dependent on indigenous medicinal plant species [8]. The area is rich in medicinal plants [9], and the local people use many plant species to cure various ailments including gastric ulcers, digestive problems [10], fever, as astringents, back ache [11], antidote against snake bite, cattle's mouth and foot diseases, skin diseases [12].

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Botanical name	Local name	Plant's family	Part used	Ethnomedicinal uses
Dryopteris ramosa (Hope) C. Chr.	Pakha	Dryopteridaceae	Frond	Tonic, gastro-intestinal, antimicrobial and anti-cancer (13) gastric ulcer, constipation and aphrodisiac (10)
<i>Quercus leucotricophora</i> A. Camus	Rein	Fagaceae	Leaves, bark	Antitumor (14) diarrhea, indigestion, asthma and gonorrhea (10)
Arisaema flavum (Forssk.) Schott.	Saap Booti	Aeraceae	Rhizome	Antidote (Snake bite) (15) Rhizome juice is applied on earache and skin diseases (16)

Table 1. Selected plant species and their ethnomedicinal uses.

## EXPERIMENTAL

## Plant species

Leaves of the Pakistan wood fern *Dryopteris* ramosa (Hope) C. Chr., the Banj oak *Quercus leucotricophora* A. Camus, and rhizomes of yellow cobra lily *Arisaema flavum* (Forssk.) Schott were collected from the Galyat area, Pakistan. The plants were identified by Dr. Rehmatullah Qureshi at the Department of Botany, PMAS-Arid Agriculture University Rawalpindi, Pakistan. Herbarium specimens were deposited in the Herbarium of Quaid- i- Azam University, Islamabad, Pakistan.

#### Preparation of crude methanolic extract

The crude methanolic extracts were prepared by maceration technique as described in [17] with some modifications and the dried crude methanolic extracts were stored in air – tight containers at  $4 \,^{\circ}$ C.

# Evaluation of free radical scavenging (antioxidant) activity

Each extract was evaluated for its antioxidant potential using a free radical scavenging assay as described in [18, 19] with some modifications. The extract solution was prepared in methanol at a 1:40 ratio (mg/ml) w/v. Ascorbic acid was used as a standard, while methanol was used as a blank. A stock solution (5 mg/ml) of each extract was made in methanol and subsequent dilutions of 25, 50, 100, 150, 200 and 250 µg/ml were prepared. Then, 200 µl from each dilution was mixed with 200 ml DPPH (di(phenyl)-(2,4,6-trinitrophenyl) of iminoazanium) solution and placed in dark at 25-30 °C for 30 min. Next, the contents of each reaction tube were subjected for spectrophometric analysis at 517 nm, and free radical quenching potential was determined using the following equation:

Scavenging (%): [Absorbance <sub>(control)</sub> – Absorbance <sub>(sample)</sub> / Absorbance <sub>(control)</sub>] × 100

The scavenging concentration 50 % (SC<sub>50</sub>) was calculated by regression equation.  $SC_{50}$  is the half maximal scavenging concentration. (SC<sub>50</sub>) is a measure of the effectiveness of a substance in

scavenging the free radicals in specific biological or biochemical function. The terms  $IC_{50}$  and  $SC_{50}$  may be used as synonym by the fact that both have been used to demonstrate 50 % potential concentration. According to the FDA,  $IC_{50}$  represents the concentration of a drug that is required for 50 % inhibition *in vitro*. For competitive binding assays and functional antagonist assays the most common summary measure of the dose-response curve is the  $IC_{50}$ , the concentration of substance that provides 50 % inhibition [20]. *In vitro*  $IC_{50}$  is a very basic starting point in determining the potential efficacy of a developmental drug.

# Estimation of total phenolic contents

For the purpose of estimation of total phenolic contents of the extracts, a standard method was used as described in [21]. For plotting a reference standard calibration curve, different dilutions (25, 50, 100, 150, 200 and 250  $\mu$ g/ml) of gallic acid were used. The reaction mixture contained 500  $\mu$ l from each dilution of gallic acid, 10 × diluted 2.5 ml of Follin-Ciocalteu reagent and 2.5 ml of 7 % (w/v) Na<sub>2</sub>CO<sub>3</sub>. Gallic acid was replaced with 500  $\mu$ l of plant extract (1 mg/ml) to the obtained reaction mixture for the test sample. Each reaction tube was vortexed and incubated at 25-30 °C for half an hour and then spectrophotometric analysis was carried out at 760 nm.

#### Estimation of total flavonoid contents

For the determination of total flavonoid contents of each extract, a standard AlCl<sub>3</sub> method was used as suggested in [22], with slight modifications. For plotting a reference standard calibration curve, different dilutions (25, 50, 100, 150, 200 and 250  $\mu$ g/ml) of quercetin were used. The reaction mixture contained 500  $\mu$ l from each dilution of quercetin, vortexed with 10 % AlCl<sub>3</sub> (100  $\mu$ l), and after one min 100  $\mu$ l of CH<sub>3</sub>COOK (1 M) was added and vortexed again, followed by addition of distilled water (2.8 ml) after one min and once again vortexed. Quercetin was replaced with 500  $\mu$ l of plant extract (1 mg/ml) to the obtained reaction mixture for the test sample. Each of these reaction *M. Ishaque et al.: Antioxidant potential, total phenolic and flavonoid contents of three culinary medicinal plant species* tubes was incubated at room temperature for 30 min and spectrophotometric analysis was carried out at 415 nm. (Table 2). Regression line equations were used to determine the SC<sub>50</sub> of each crude extract and the various fractions obtained from *D. ramosa*.

# Polarity based fractions of D. ramose

The crude methanolic extracts of *D. ramosa* were fractionated into n-hexane, chloroform, ethyl acetate, and the aqueous phase was treated as suggested in [23]. All fractions thus obtained were evaluated for their free radical scavenging potential, total phenolic content and total flavonoid content as described earlier.

#### Statistical analysis

All values are mean of triplicates  $\pm$  standard deviation (SD). Using Statistical Package for Social Science (SPSS) Programme 16.0, univariate analyses of all extracts were performed at  $p \le 0.05$ . Pearson correlation at the 0.01 level (2 tailed) was also determined between SC<sub>50</sub> and total phenolic contents of plant extracts.

## RESULTS

#### Antioxidant potential

The free radical scavenging potential (% scavenging) of crude extracts of D. ramosa, Q. leucotricophora and A. flavum and various fractions of D. ramosa at different concentrations are shown in Table 2. Among the crude extracts, those of D. ramosa exhibited a higher scavenging potential than the other species. For instance, at a concentration of 250  $\mu$ g/ml, the percent free radical scavenging was 93.8  $\pm$  0.2, 86.0  $\pm$  0.2 and 56.4  $\pm$ 0.4 for D. ramosa, Q. leucotricophora and A. flavum species, respectively. Similarly, among the various solvent polarity-based fractions of D. ramosa, the ethyl acetate fractions showed the highest free radical percentage scavenging potential, while the least antioxidant potential was exhibited by the chloroform soluble fraction of D.

wonoid contents of three culinary medicinal plant species ramosa (Table 2). Regression line equations were used to determine the SC<sub>50</sub> of each crude extract and the various fractions obtained from *D. ramosa*. Ascorbic acid was used as a standard and its SC<sub>50</sub> was  $44.5 \pm 0.2 \mu g/ml$ . The crude methanolic extract of *D. ramosa* showed the lowest SC<sub>50</sub> while *A. flavum* had the highest SC<sub>50</sub>. Among the fractions of *D. ramosa*, the ethyl acetate fraction had the lowest SC<sub>50</sub> value (Table 3). The low SC<sub>50</sub> value indicates a higher antioxidant potential and so the ethyl acetate fraction of *D. ramosa* was revealed to have the best antioxidant potential.

# Total phenolic contents

The total phenolic contents of crude extracts and fractions were calculated by a linear regression equation (y = 0.0071x + 0.4332, R<sup>2</sup>= 0.9606) primed with a gallic acid standard calibration curve, and expressed in terms of gallic acid equivalent. Crude methanolic extract of *D. ramosa* had the highest amount of phenolic constituents, followed by *Q. leucotricophora* and *A. flavum* (Table 3). Among the polarity-based solvent soluble fractions of *D. ramosa*, higher phenolic contents were shown by ethyl acetate soluble fractions (55.7 ± 0.5 µg/mg GAE), while the least ones (13.8 ± 0.6 µg/mg GAE) were present in the chloroform fractions of *D. ramosa*.

#### Total flavonoid contents

The total flavonoid contents of crude extracts and fractions were calculated from a linear regression equation (y = 0.0063x + 0.395,  $R^2 =$ 0.9697) primed from a quercetin standard calibration curve. Crude methanolic extract of *D. ramosa* has higher total flavonoids than all other crude extracts in the present study, while the ethyl acetate soluble fraction showed higher total flavonoids than all other fractions obtained from *D. ramosa* (Table 3).

<b>Tuble 2</b> . Di i i i nee fudical seavenging potential of selected plant species.							
Concentrations		25 μg/ml	50 µg/ml	100 µg/ml	150 µg/ml		
Crude extracts (% scavenging)*	D. ramosa	$23.7\pm0.8$	$30.8\pm1.6$	$68.8 \pm 1.8$	$73.7\pm1.6$		
	Q. leucotricophora	$21.5\pm0.6$	$38.1\pm0.1$	$53.3\pm0.2$	$76.3\pm0.4$		
	A. flavum	$05.9\pm0.2$	$24.7\pm0.3$	$32.8 \pm 0.1$	$38.5\pm 0.4$		
Fractions of <i>D. ramosa</i> (% scavenging)*	n-Hexane fraction	$26.6\pm0.5$	$33.3\pm0.4$	$33.2\pm0.2$	$44.7\pm0.9$		
	Chloroform fraction	$17.3\pm0.2$	$19.9\pm0.2$	$21.6\pm0.3$	$26.0\pm0.7$		
	Ethyl acetate fraction	$36.7\pm0.3$	$44.1\pm0.4$	$69.9\pm0.6$	$76.7\pm0.7$		
	Aqueous fraction	$18.3\pm0.2$	$19.1\pm0.1$	$26.9\pm0.5$	$27.7\pm0.5$		
Standard (% scavenging)*	Ascorbic acid	37.1 ± 1.6	$54.3\pm0.5$	$77.8\pm2.9$	$92.0\pm1.4$		

Table 2. DPPH free radical scavenging potential of selected plant species.

\*All values are mean of triplicate  $(n = 3) \pm$  standard deviation.

Table 3. Total phenolics, total flavonoid contents and antioxidant potential of selected plant species.

Extracts (Samples)		Total phenolic	Total flavonoid	Antioxidant potential [SC50 (µg/ml)]		
		contents	contents	Samples	Standard	
		(µg/mg GAE)	(µg/mg QE)	(Extracts)	(Ascorbic acid)	
Crude extracts	D. ramosa	$122.6\pm4.4$	$61.4\pm17.9$	$88.9 \pm 0.4$		
	Q. Leucotricophora	$74.1 \pm 7.7$	$38.7\pm11.0$	$95.6\pm0.4$		
	A. flavum	$19.0\pm6.3$	$4.6 \pm 1.2$	$208.4\pm0.1$		
D. ramosa fractions	n-Hexan fraction	$27.3\pm0.4$	$9.4\pm0.3$	$198.8\pm0.0$	$44.5 \pm 0.2$	
	Chloroform fraction	$13.8\pm0.6$	$5.8 \pm 0.1$	$468.2\pm0.0$		
	Ethyl acetate fraction	$55.7\pm0.5$	$29.7\pm0.4$	$57.8\pm0.1$		
	Aqueous fraction	$18.0\pm0.4$	$7.3 \pm 0.1$	$410.7\pm0.1$		

Legend: GAE = Gallic acid equivalent, QE= Quercetin equivalent, Results are mean of three parallel measurements.  $P \le 0.05$  as significant.  $\pm$  Standard deviation (n = 3).

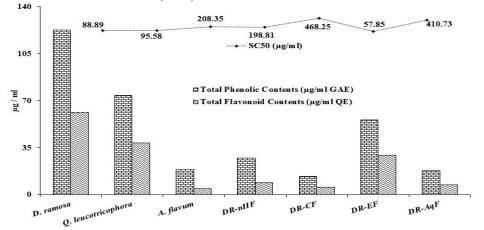


Fig. 1. Relationship between SC<sub>50</sub>, total phenolic content and total flavonoid content of the plant extracts.

## DISCUSSION

It is noteworthy that various chronic health disorders are due to the stress caused by free radicals in the bodies of living organisms [24]. These free radicals can accumulate in the human body, because of various factors including poor diet, environmental factors, and emotional stress. Within living systems therefore, the necessity of antioxidative systems is essential. It is also important to note that substances exhibiting low antioxidant potential in vitro show similar low free radical scavenging abilities in vivo [25]. Human health specialists worldwide are interested in antioxidant substances, especially for their potential use in the treatment of various human diseases. Most of these antioxidants have been identified and isolated from plants, although a few have synthetic origins [26]. It is generally considered that natural antioxidants from plants are much safer than synthetic forms [27].

The Galyat region of Pakistan is mountainous, and distant from modern health facilities. For that reason, local inhabitants rely heavily on medicinal plants species of the area to treat many health problems. The ethnomedicinal knowledge of indigenous people is based on experience and traditions that have been transmitted from generation to generation. This cultural knowledge might provide clues in identifying new therapeutic substances. The findings of this report confirm the medicinal status of three plant species (*D. ramosa*, *Q. leucotricophora* and *A. flavum*) commonly used as medicinal plants in this region (Table 1, Table 2), and is consistent with the findings of previous studies of these species [28-30].

In the DPPH antioxidant assay, the IC<sub>50</sub>, and the exhibited antioxidant potential an inverse relationship. The order of  $SC_{50}$  among the selected crude extract was D. ramosa < Q. leucotricophora < A. flavum, findings that indicate that A. flavum has the least ability to scavenge free radicals. Ethyl acetate soluble fraction of D. ramosa showed better antioxidant potential (SC<sub>50</sub> 57.8  $\pm$  0.1 µg/ml) than crude methanol extracts of the same plant. The reason might be that antioxidant components have polar nature. In a similar study, Lee et al., 2003, isolated two antioxidant compounds from the ethyl acetate soluble fraction of a fern that is closely related to D. ramosa, called thick-stemmed wood fern, Dryopteris crassirhizoma Nakai [30].

Phenolic constituents have recently shown their worth in the food industry through their ability not only to protect lipids from oxidative degradation, but also in their ability to improve the nutritional value and quality of food [31]. The phenolics examined in these studies were primarily derived M. Ishaque et al.: Antioxidant potential, total phenolic and flavonoid contents of three culinary medicinal plant species

from plants, and belonged to several biochemical categories, mainly flavonoids, flavones, and flavonols that exhibited excellent antioxidant properties [2]. It is also important to note that the plant extracts examined in that study had higher total phenolic and flavonoid contents, showed lower IC<sub>50</sub> values, and hence a higher free radical scavenging potential (Fig. 1), consistent with the results of the present study. For instance, the total phenolic contents of the methanolic extracts of D. ramosa were higher compared to the extracts of other species, and similarly, the total phenolic contents of D. ramosa ethyl acetate fractions were higher compared to all other fractions obtained from D. ramosa. Significant Pearson correlation at the 0.01 level (2-tailed) was also noticed between IC<sub>50</sub> and total phenolics contents of all crude extracts and fractions, as reported by others [32-34]. Our results are in accordance with previous reports suggesting that the high antioxidant potential of the ethyl acetate phase of D. ramosa is due to higher contents of phenolics and flavonoids.

# CONCLUSION

Our results show that the antioxidant potential is related to the total phenolics contents of the plants, and also that plant phenolics contents are more concentrated in medium-polarity solvent-soluble fractions, like the ethyl acetate fraction. These findings reveal a significant and potentially useful variation in antioxidant potential for important medicinal plants in the Galyat region of Pakistan, as well as a potential for future research seeking to isolate and identify novel antioxidants from ethnobotanically-associated medicals.

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# АНТИОКСИДАНТЕН ПОТЕНЦИАЛ, ОБЩО СЪДЪРЖАНИЕ НА ФЕНОЛИ И ФЛАВОНОИДИ В ТРИ ВИДА КУЛИНАРНИ РАСТЕНИЯ В ЛЕСЕР ХАМАЛИЯ, ПАКИСТАН

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#### (Резюме)

Добре е известно, че оксидативният стрес, дължащ се на свободни радикали, може да доведе до много болести при човека, например, рак. Антиоксиданти са агентите, които отстраняват свободните радикали и предпазват биологичната система. Храните от растителен произход са съществен източник на антиоксиданти. Антиоксидантите, извлечени от растенията принадлежат на различни биохимични категории, но повечето са феноли или флавоноиди. Районът "Галят" в Пакистан е богат на различни растения и местните жители често използват медицински растения за лечение на различни болести. Избрани са три от тези разпространени в района медицински растения (Dryopteris ramosa, Quercus leucotricophora и Arisaema flavum) и е изучен потенциалът имза отстраняване на свободни радикали с помощта на DPPH анализ. При концентрация 250 µg/ml суровият екстракт на D. ramosa проявява максимален радикал-отстраняващ потенциал (93.8 ± 0.2 %), а А. Flavum – минимален потенциал (56.4 ± 0.4 %). Ниската стойност на SC<sub>50</sub> на суровия екстракт на *D. ramosa* (88.9  $\pm$  0.4 µg/ml) повърди сравнително високия антиоксидантен потенциал на този растителен вид. Между полярните фракции, получени от суровия екстракт на D. ramosa, фракцията, разтвомима в етилацетат показа най-висок потенциал за отстраняване на свободни радикали. Видът D. ramosa съдържа по-големи количества общи фенолни и флавоноидни съединения в сравнение с видовете Quercus и Arisaema. Установена е значима корелация на Pearson на ниво 0.01 (двустранна) между SC<sub>50</sub> и общото фенолно съдържание на всички екстракти. Има съществена разлика в процентната отстраняваща активност на различните екстракти. Фазата от D. ramosa, разтворима в етилацетат може да се окаже полезен източник на природни антиоксиданти с различни медицински приложения. По-нататъшни изследвания са в ход.