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Evaluation of Antioxidant Activity and Total Phenolic, Flavonoid and Terpenoid Content of Different Parts of *Marrubium vulgare* L.

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A B S T R A C T

Different parts (root, stem, leaves and flower) of *Maruubium vulgare* were evaluated to investigate variation in antioxidant and total phenolic, flavonoid and terpenoid content and their interrelationship. All MeoH extracts as well as the fractions of root, stem, leaf and flower were assessed for their antioxidant activity through DPPH (1,1-diphenyl-2-picrylhydrazyl). Total phenolic content was estimated by using Folin ciocalteu, flavonoid by aluminium chloride colormetric and terpenoid by monoterpene linalool reagent assay. We found a great variation in the concentration of total phenolic and flavonoid content which ranged from 42.83± 2.32 to 314.57± 3.81mg gallic acid equivalent/g DW and 0.067±0.093 to 25.46±1.32 mg quercetin equivalent/g DW among different parts of the herb. Total terpenoid content estimated first time was found highest in flower (692.36±28.36). A significant correlation was observed between antioxidant activity and total phenolic content ($P<0.05$, $R^2 = 0.395$); however, no correlation was observed with total flavonoid and terpenoid content. This first-time study suggested that *M. vulgare* root together with flower could be a potential source of natural antioxidants and may find new horizons in the field of pharmacology.

***Key words*:** Antioxidant, Total terpenoid, DPPH, *Marrubium vulgare*, Total phenol

Throughout the ages, humans relied plant based natural products enriched with antioxidant have been associated with lower incidence of metabolic syndromes like type2diabetes, cardiovascular diseases and cancer [1-3]. So, a relationship has been identified between these diseases and reactive oxygen species (ROS) which damage biological cells, tissues and membranes [4]. This damage or harmful effects can be combated by the intake of many natural antioxidants. Though Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxy-toluene (BHT) are very effective, but they can induce many side effects, which resulted keen interest in the search for plant-derived natural antioxidants.

Marrubium vulgare L., a perennial herb, being the most studied species of genus *Marrubium* due to its relevance in folk medicine to treat various ailments like respiratory and gastrointestinal problems [5]. Researchers turn no stone left to evaluate the herb for its various pharmacological aspects and reported its analgesic, gastroprotective, antispasmodic and antimicrobial activities [6-9]. Studies have shown that there is a relationship between antioxidant potentiality and occurrence of good quantity of secondary metabolites like phenols and flavonoids and in this study we report first time in *Marrubium vulgare* whether there is any such relationship; though the antioxidant activities of this herb were attributed to its different bioactive compounds, such as diterpenoids, phenylethanoidglycosides and essential oils [10-12], but in which plant part (root, stem, leaf and flower) the activity is strong has not been studied till date. Therefore, the main aim of this study was to evaluate interrelationship between antioxidant activity and concentration of phenoilcs, flavonoids and diterpenoids in different plant parts of *Marrubium vulgare*.

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MATERIALS AND METHODS

Plant material

The whole plant *M. vulagre* L. was collected at an altitude of 1743 m from Nowhatta (Srinagar) in the month of May 2018. The plant was identified and authenticated by the taxonomist at Department of Botany, Centre for Biodiversity & Taxonomy, University of Kashmir. The reference material and voucher specimen (NO.2678 KASH) were deposited in the departmental herbarium for future reference. Each part (leaf, flower, stem and root) of the herb was separated and washed thoroughly under running tap water and then rinsed in distilled water; they were allowed to dry for some time. These plant parts were then separately shade dried without any contamination for about 3 to 4 weeks. The dried plant material (each part) was coarsely pulverized to powdered form in a sophisticated instrument, grinder for extraction.

Preparation of extracts

Methanolic extracts of different plant parts (root, stem, leaf and flower) 50 g each were prepared by maceration using methanol (500 mL) as solvent for 4-5 days and then concentrated with intermittent shaking. The extracts were filtered through Whatman filter paper No.1, then concentrated on rotary evaporator at 40°C to get 2.7 g (root), 2.9 g (stem), 6 g (leaf) and 5 g (flower) crude extracts. Each crude extract was then portioned with water and extracted successively with hexane, chloroform and water using 500 mL separating funnel to get their fractions. The solutions of all the fractions were evaporated to dryness to obtain their respective hexane fraction (Hex fr), chloroform fraction (Ch fr) and aqueous fraction (Aq fr) respectively.

Chemicals

Folin-Ciocalteu reagent, sodium carbonate anhydrous, gallic acid, quercetin, sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH) were of Sigma Aldrich (St. Louis, MO, USA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were of TCI company. All solvents used in the experiment (methanol, chloroform, DMSO) were of analytical grade.

Quantitative estimation of phytochemicals

Estimation of total terpenoid content

Total terpenoid assay was determined colorimetry using the procedure followed by [13] with slight modification. 200 µl of extract solution (3 mg/mL in methanol) of each plant part and their fractions were mixed with 1.5 ml of chloroform, and then allowed to rest for 3 min. 100 µl concentrated H_2SO_4 was added to each sample taken in 2.5 mL appendorf, and then incubated for 2 h at room temperature in dark. After 2 hours of incubation terpenoids settled down as dark reddish-brown precipitate. The supernatant was carefully decanted and the precipitate was dissolved in 1.5 ml methanol. 100 µl from each sample in 17 appendrofs were transferred in a 96 well plate for spectrophotometric analyses. Linalool (1.56 – 100 mg/200 µl, $R^2 = 0.999$) was used to prepare a standard curve. The Absorbance was recorded at 538 nm in a spectrophotometer against a methanol. The assay was performed in triplicate and concentration was expressed as equivalent to mg linalool/ g DW.

Estimation of total phenolic content

Total phenolic content was determined by Folin-Ciocalteu method in a 96 well plate following the procedure adopted by [14] with some modifications. Briefly 50 µl of extract solution (5 mg/mL in methanol) of each part and their fractions were mixed with 100 µl of 1:4 diluted Folin–Ciocalteu reagent and shaken for 1 minute in a flat-bottom 96-well microplate. The mixture was left for 5 minutes and then 75 µL of 20% sodium carbonate solution was added and the mixture was shaken at medium continuous speed for 1 min. After 3 h incubation at room temperature, the absorbance of the reaction mixture was measured at 765 nm using the microplate reader Tecan Infinite M Nano Elisa plate Reader (Austria). Serially diluted Gallic acid (0.78, 1.562, 3.125, 6.25, 12.5, 25, 50, 100 µg/ml, $R^2 = 0.991$) was used as standard for calibration. Total phenolic contents were expressed as mg Gallic Acid Equivalents (GAE) per g dry weight of plant extract.

Estimation of total flavonoid content

Total flavonoid content was determined by aluminium chloride colorimetric method adopted by [14] with slight modification. 50 µl of extract solution (5 mg/mL in methanol) was added to 10 µl of 10% aluminum chloride solution and followed by 150 µl of 96% ethanol. 10 µl of 1 M sodium acetate was added to the mixture in a 96 well plate. 96% ethanol was used as blank. Standard solution of Quercetin (0.78, 1.562, 3.125, 6.25, 12.5, 25, 50, 100 µg/ml, $R^2 = 0.989$) in methanol was also prepared with the same procedure. Total flavonoid contents were expressed as mg Quercetin equivalent/g DW.

DPPH radical scavenging assay

Free radical scavenging activity of crude methanolic extract of each plant part (root, stem, leaf and flower) as well as their hexane, chloroform and aqueous fractions were determined with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The assay was conducted in a 96 well plate according to [15] with slight modification. 100 µl of 17 samples (crude MeOH extract of root, stem, leaf and flower, their Hex fr, Ch fr, Aq fractions) at various concentration (1000- 15.62 µg/ml) were added to 100 □L of a methanol solution of DPPH (0.1 mM) in each 96 well. The reaction mixture was incubated for 30 min.at room temperature in dark and absorbance was measured at 517nm using micro-plate reader of Tecan Infinite M Nano Elisa plate Reader (Austria). Ascorbic acid Ascorbic acid was used as positive standard and methanol as positive control. All tests were performed in triplicates and radical scavenging ability (%) was calculated as follows:

$$\% \text{ Scavenging} = [(A_{\text{positive control}} - A_{\text{sample}}) / (A_{\text{positive control}})] \times 100$$

Where, $A_{\text{positive control}}$ is the absorbance of the control reaction (containing all reagents except the test extract or standard), and A_{sample} is the absorbance of the test extract or standard.

Concentration of samples (extracts and standard) resulting in 50% inhibition on DPPH (IC_{50} value) were calculated using GraphPAD Prism Software Version 5.0.

Statistical analysis

Variation in concentration of total phenol, flavonoid and terpenoid content among different plant parts and their antioxidant activities were analyses by both one way and two way ANOVA at 95% level. These analyses were performed in Graph pad prism 5.0.

RESULTS AND DISCUSSION

Extraction yield, total phenol, flavonoid and terpenoid content

The methanoilc extraction yield among different plant parts varied from 5.4 to 12% with decreasing order of Mex L> Mex F>Mex S>Mex>R.

Phenolic compounds which are considered to be important constituents of plant are responsible for various pharmacological activities including antioxidant [16]. The association of redox properties of phenolic compounds allows them to donate hydrogen atoms, electrons and as reducing agent [17]. The total phenolics of 17 samples (MeoH extracts of each part as well as their fractions) and expressed in gallic acid equivalents (GAE) per gram dry extract weight (Fig 1) ranged from 42.83±2.32 to 314.57±3.81and varied significantly. The highest was found in Mex (methanol extract) of root (314.57±3.81). Earlier studies with estimation of total phenolic content was restricted to leaves only showing variation in the phenolic content separately in these studies ranged from 26.8±0.01 to 160 mg GAE/g extract [18]. In our present study leaf also showed highest content of phenolic content (193.04±3.41) compared to previous reports. Another important class of secondary metabolites possessing various biological properties like antibacterial, antiviral and anti-allergic activities is flavonoid [19]. Studied on its quantitative estimation reported 66.3 mg catechin equivalents/g in DW in crude ethyl estate and an amount of 81.21±0.69 and 26.30±0.31 mg RE/g DW in methanol and acetone extract of leaf of the herb [11]. In ethanolic extract of whole plant total flavonoid content was reported to be 37.7±1.66 and 23.25±0.94 mg of RUE/g through percolation and microwave extraction processes [20]. In our present study where we evaluate the total flavonoid content in crude methanolic crude extracts as well as their fractions in leaf, flower, stem and root, significantly ($p<0.001$) varied amount of flavonoid content ranged from 0.067±0.093 to 25.46±1.32 mg QR/ g DW was observed with highest content found in hexane fraction of leaf (25.46±1.32 mg QR/g DW).

Terpenoids which are reported to possess numerous biological activities including inhibition of cholesterol synthesis [21] was never estimated in *M. vulgare* till date. In our study total terpenoid content expressed as mg linalool equivalent (Le)/ g dry extract among plant parts and their methanolic extract as well as their fractions varied significantly ($p<0.001$). The highest terpenoid content was found in hexane fraction of flower (692.36±28.36 mg Le/g dry weight) and lowest in Chloroform fraction of stem (111.81±44.55 mg Le/g DW). Non availability of data related to total terpenoid content in *M. vulgare* made us unable to compare our results, however to our best knowledge; we report total terpenoid quantitatively first time in the herb.

The above phytochemical parameters (total phenol, flavonoid, terpenoid) estimated in the present study showed statistically significant ($p<0.001$) variation in their amounts in different plant parts of *M. vulgare*.

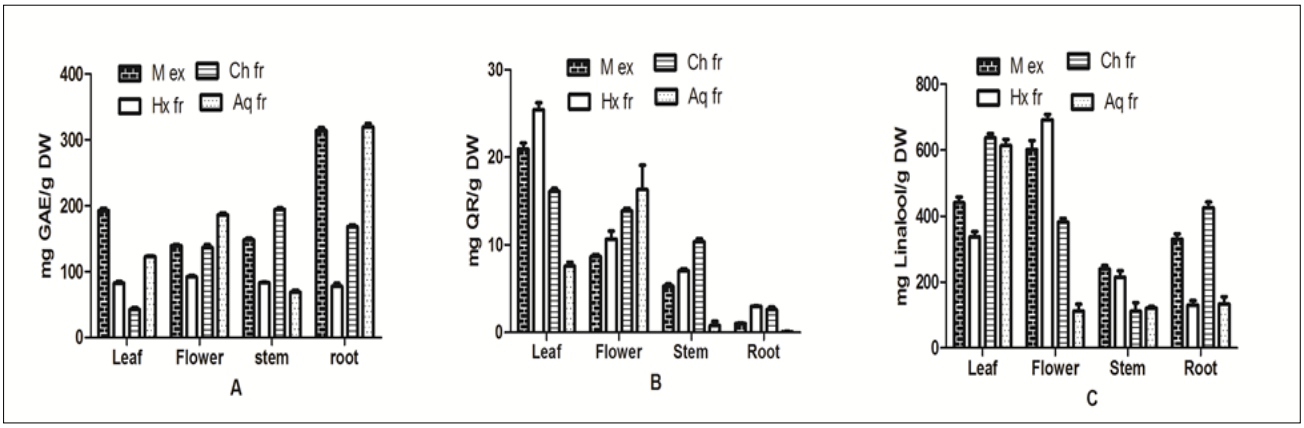

Fig 1 Total Phenol (A), flavonoid (B) and terpenoid (C) content of leaf, flower, stem and root
Results are expressed mean±SD (n=3). Mex: methanol extract; Hx fr: Hexane fraction; Ch fr: chlorofotm fraction; Aq fr: aqueous fraction varied. Means of different fractions showed significant ($p<0.001$) variation in TPC, TFC and TTC among different plant parts

Table 1 Inhibitory concentration 50 (IC_{50} in µg/mL) of different parts of their extracts of *M. vulgare* and Standard

Part	Extract/Fraction	IC_{50} (in µg/mL)
Leaf	Methanol	529.03
	Hexane	>1000
	Chloroform	308.20
	Aqueous	890.90
Flower	Methanol	437.13
	Hexane	348.03
	Chloroform	>1000
	Aqueous	292.13
Stem	Methanol	370.23
	Hexane	>1000
	Chloroform	573.03
	Aqueous	433.10
Root	Methanol	54.84
	Hexane	>1000
	Chloroform	193.20
	Aqueous	>1000
	Ascorbic acid*	15.85

*Ascorbic acid was used as standard

Antioxidant activity

Antioxidant capacity, a widely used parameter by researchers to correlate the free radical scavenging ability and biological activities is based on the ability of natural products to donate free electrons is measured by DPPH assay. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that changes the deep violet colour of DPPH solution to pale yellow upon a reduction reaction [22]. In the present study evaluation of free radical scavenging activity of different plant parts, their methanol extracts and fractions (Hx fr, Ch fr and Aq fr) determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay is represented in (Fig 2).

From the results expressed as % DPPH inhibition, it was observed that metanolic extracts of root possess the

highest % of inhibition capacity (89.01%) followed by Aq fr of flower (87.6%). All the samples showed concentration dependent increase in radical scavenging capacity. The highest IC50 inhibition was recorded in Methanolic root extract and its chloroform fraction (54.84 and 193.2 µg/mL) followed by Aq fr of flower (292.13 µg/mL), while the lowest ones was observed in Chl fr of flower, aq fr of root and Hx fr of leaf, stem and root with an IC_{50} value above 1000 µg/mL (Table 1). Comparing these results with IC_{50} value of ascorbic acid (15.85 µg/mL) it is found that all the fractions as well as crude methanolic extracts though possess promising antioxidant activity but low to that of standard (ascorbic acid). A good correlation ($P<0.05$, $R^2 = 0.395$) was also found between these IC_{50} values and TPC, that is in consistent with the reports of other studies [23]. However, no correlation was found with Ttr and TFC. We could not find any reports on antioxidant activity of the various polarities extracts of different parts (leaf, flower, stem and root) of *M. vulgare*; however, [24] reported variation in antioxidant and total phenolic, flavonoid content in leaves collected from different locations of the herb with IC_{50} of 33.7 µg/mL for crude methanol extract compared to 529.03 µg/mL in MeOH extract of leaf in the present study. This variation can be attributed due to various factors including climate condition and geographical distribution [25].

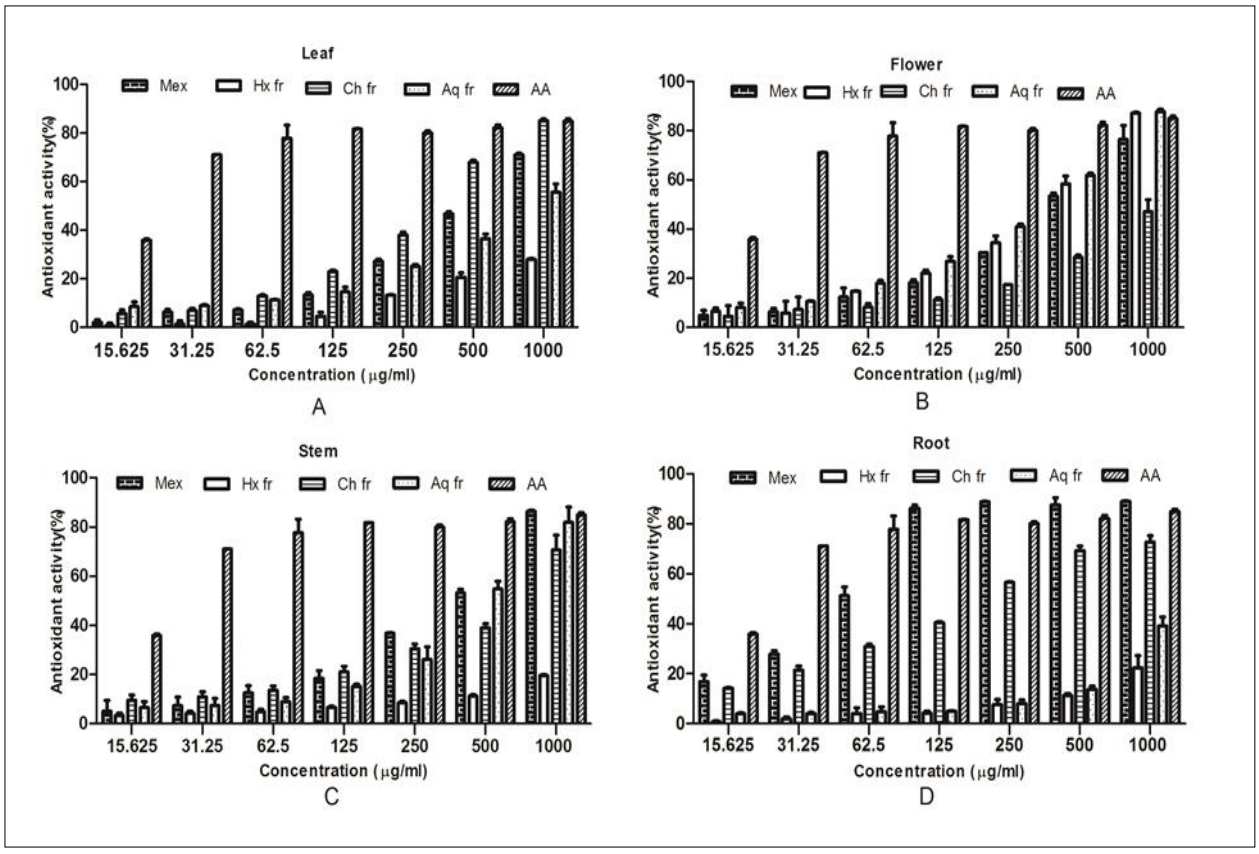

Fig 2 Antioxidant activity (%) of Methanol extract of Leaf (A), Flower (B), Stem (C) and Root (D) and their fractions hexane (Hx fr), chloroform (Ch fr), aqueous (Aq fr). Results are expressed in mean±standard deviation in triplicate measurements

CONCLUSION

In the present study we first time report a significant ($p<0.001$) variation in the amount of phenol, flavonoid and terpenoids contents among different plant parts of *M. vulgare* as well as their various polarity extracts (Methanol, hexane, chloroform and Aqueous). Among these prepared extracts, methanolic crude extract of root followed by aqueous fraction of flower contains highest content of phenol and terpenoid as well antioxidant activity compared to other parts. This comparative study suggested that *M. vulgare* root together with flower could be a potential source of natural antioxidants for the protection of various oxidative stress related diseases. Further investigation into the isolation and identification of responsible antioxidant components and their mechanism of action is necessary to better understand.

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