Estimation of Total Phenols and Flavonoids in Extracts of *Actaea spicata* Roots and Antioxidant Activity Studies

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***Actaea spicata* Linn. (Ranunculaceae) has been traditionally used for the treatment of various ailments such as rheumatism, inflammation, nerve diseases, lumbago, scrofula and chorea, but no systematic phytochemical and pharmacological work has ever been carried out on this potential plant. Preliminary phytochemical screening showed presence of phenols and flavonoids in *A. spicata*. Thus, the present investigation was undertaken to estimate total phenols and flavonoids in methanol extract of *A. spicata* roots, and its ethyl acetate fraction. *In vitro* antioxidant activity was also evaluated in the methanol extract and ethyl acetate fraction using DPPH method. Ethyl acetate fraction was found to contain twice the content of flavonoids and phenols in comparison to methanolic extract, whereas phenolic content in methanol extract was approximately similar to ethyl acetate fraction. A significant antioxidant activity, i.e., mean percentage inhibition of DPPH radical was observed in methanol extract and ethyl acetate fraction at the concentration of 10 µg/ml and 5 µg/ml respectively. Finally, it was suggested that polyphenols are responsible for antioxidant activity of *A. spicata*.**

Key words: *Actaea spicata*, antioxidant activity, flavonoids, phenols

Actaea spicata Linn., commonly known as Baneberry, belongs to family Ranunculaceae. A survey of ethnopharmacologic records reveals that the plant has been traditionally used in the treatment of rheumatism, inflammation, rheumatic fever, lumbago, scrofula, nervous disorders, chorea, and as emetic, expectorant, laxative, stomachic and purgative[1-3]. The plant has also been used in traditional systems of medicines of various countries for the treatment of snake bite, asthma, and externally for skin complaints. In some parts of Europe the powdered leaves, stems and flowers are used as an insecticide[4].

A. spicata has been reported to contain isoquinoline alkaloids magnoflorine, corytubrine; triterpene glycosides including actein and trans-aconitic acid[5]. A thorough survey of literature revealed no pharmacological report on *A. spicata*.

Despite a long tradition of use for the treatment of various ailments, no systematic phytochemical and pharmacological work has ever been carried out on this potentially useful plant. Thus, the present investigations were planned with an objective to estimate total phenols and flavonoids in methanol extract of *A. spicata* roots, and its ethyl acetate fraction. Further, antioxidant activity was evaluated in methanol extract and ethyl acetate fraction of the plant using DPPH method.

Dried roots of *A. spicata* were procured from K. R. Indo German American Trading company, Kurukshetra (Haryana), India in the month of November 2008. Identity of the plant was confirmed through Dr. H. B. Singh, Scientist F, Head of Raw material Herbarium and Museum (RHMD), National Institute of Science and Information Resources (NISCAIR), New Delhi, India (Ref. No, NISCAIR/RHMD/ Consult/-2008-09/1192/224 dated 09-04-2009). Gallic acid and Rutin (Hi-media Laboratories Pvt Ltd., Chandigarh) were used as standard drugs. All the solvents used in the present investigation were, of LR grade, procured from Central Drug House Pvt. Ltd., Mumbai. Folin Ciocalteu's reagent (S.D. Fine Chemicals, Mumbai), Sodium carbonate (Central Drug House Pvt. Ltd., Mumbai), Aluminium chloride (S.D. Fine Chemicals, Mumbai), Potassium acetate (S.D. Fine Chemicals, Mumbai), 1, 1-diphenyl-2-

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picryl hydrazine (DPPH) (SIGMA, USA) used in the present investigation were of LR grade.

Dried, coarsely powdered roots of *A. spicata* (500 g) were extracted with petroleum ether using a Soxhlet apparatus. The marc was air dried and extracted with methanol using a Soxhlet apparatus. The methanol extract was dried under reduced pressure using rotary vacuum evaporator (Perfit, Ambala), and screened for different classes of phytoconstituents[6].

The methanol extract (25 g) of *A. spicata* roots was suspended uniformly in water, placed in three-necked round bottom flask connected with teflon stirrer, and partitioned with ethyl acetate by heating for 30 min at 50° with continuous stirring. This procedure was repeated five more times. All the shakings of ethyl acetate were pooled and concentrated under reduced pressure. The ethyl acetate fraction (6.982 g) obtained was rich in polyphenols.

Ten mg of gallic acid was dissolved in 100 ml of 50% methanol (100 µg/ml) and then further diluted to 6.25, 12.5, 25 or 50 µg/ml[7]. One ml aliquot of each dilution was taken in a test tube and diluted with 10 ml of distilled water. Then 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. Four ml of 20% (w/w) Na_2CO_3 was added in each test tube, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of the standard was measured at 765 nm using UV/VIS spectrophotometer (Schimadzu, Japan) against blank, i.e., distilled water.

One gram of methanol extract was added to 15 ml of methanol (50%) and extracted for three times by maceration for 2h, then filtered and make up the volume with methanol (50%) in volumetric flask upto 100 ml. One ml aliquot of the sample was taken in a test tube and diluted with 10 ml of distilled water. Then 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. Four ml of 20% (w/w) Na_2CO_3 was added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of the sample was measured at 765 nm against blank, i.e., distilled water. Similar procedure was adopted to prepare test sample of ethyl acetate fraction of methanol extract, and absorbance of the sample was measured at 765 nm using UV/VIS spectrophotometer nm against blank, i.e., distilled water.

Quantification was done on the basis of a standard curve of gallic acid. A standard curve of extinction against gallic acid concentration was prepared (fig. 1). Results were expressed percentage w/w and calculated using following formula, Total phenolic content (% w/w) = $GAE \times V \times D \times 10^{-6} \times 100/W$, GAE - Gallic acid equivalent (µg/ml), V - Total volume of sample (ml), D - Dilution factor, W - Sample weight (g).

Ten milligram of rutin was dissolved in 100 ml of methanol (80%) (100 µg/ml) and then further diluted to 10, 20, 40, 80 or 160 µg/ml[7]. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of methanol (95%), 0.1 ml of aluminium chloride (10%), 0.1 ml of 1 M potassium acetate and 2.8 ml of diluted water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with UV/VIS spectrophotometer. The amount of aluminium chloride (10%) was substituted by the same amount of distilled water in blank.

About 1 g of methanol extract and its ethyl acetate fraction were dissolved separately in 25 ml of methanol (80%). Similarly, 0.5 ml of methanol extract and its ethyl acetate fraction were reacted with aluminium chloride for determination of flavonoids content as described above. These parallel determinations were recorded. The amount of aluminium chloride (10%) was substituted by the same amount of distilled water in blank.

Quantification was done on the basis of a standard curve of rutin. A standard curve of extinction against rutin concentration was prepared (fig. 2). Results were expressed as percentage w/w. Flavonoids content (% w/w) = $RE \times V \times D \times 10^{-6} \times 100/W$, RE - rutin equivalent (µg/ml), V - total volume of sample (ml), D - dilution factor, W - sample weight (g).

The stable DPPH radical was used for determination of free radical scavenging activity of test samples[8]. A solution of DPPH (0.1 mM) in methanol was freshly prepared. Five ml of above solution was added to

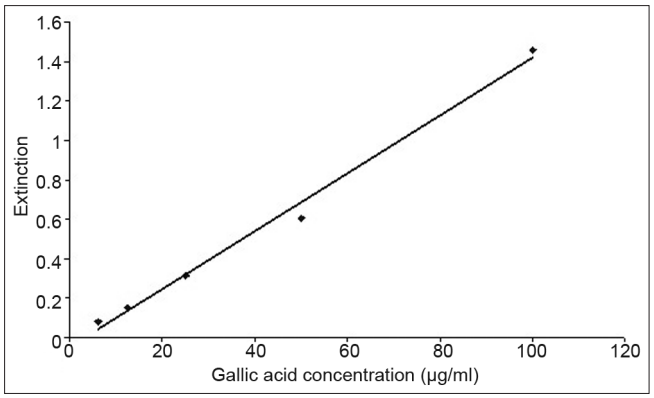

Fig. 1: Standard curve of extinction against gallic acid concentration y = 0.0147x – 0.0465; R^2 = 0.9926

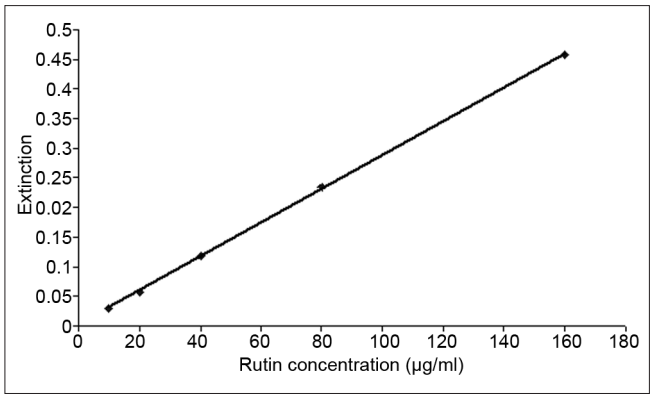

Fig. 2: Standard curve of extinction against rutin concentration y = 0.0029x – 0.0022; R^2 = 0.9998

5 ml of methanol, and kept in dark for 30 min at room temperature. After 30 min, the absorbance was recorded at 517 nm using UV/Vis spectrophotometer against methanol as blank.

Rutin was weighed (10 mg) and dissolved in 100 ml of methanol to get 100 µg/ml stock solution. Lower concentrations (2, 4, 6, 8 or 10 µg/ml) for rutin were prepared by diluting serially with methanol. Equal volume of different concentrations of standard were added to methanolic solution of DPPH, and kept in dark for 30 min at room temperature. After 30 min, the absorbance was recorded at 517 nm using UV/Vis spectrophotometer against methanol as blank.

Fifty milligram of each of methanol extract and ethyl acetate fraction were weighed separately and dissolved in 100 ml of methanol to get 500 µg/ml stock solution. Lower concentrations (10, 20, 40, 80 or 160 µg/ml) were prepared by diluting serially with methanol. Equal volume of different concentrations of methanol extract and ethyl acetate fraction were added to methanolic solution of DPPH, and kept in dark for 30 min at room temperature. After 30 min, the absorbance was recorded at 517 nm using UV/Vis spectrophotometer against methanol as blank.

Percentage radical scavenging activity was calculated by the following formula: % Radical Scavenging Power = [Ac–(As–Ao)]/Ac×100, Ac = Absorbance of control (DPPH); As = Absorbance of sample/standard+DPPH, Where, Ao = Absorbance of sample / standard without DPPH interaction. The measurements were taken thrice, and scavenging effect was calculated based on the percentage of DPPH scavenged.

An exhausted literature survey on *A. spicata* revealed that sporadic phytochemical and pharmacological reports are available on this plant. As *A. spicata* has been used traditionally for the treatment of various ailments, this plant holds great potential for detailed phytochemical and pharmacological evaluations.

A. spicata roots were defatted by extracting with petroleum ether (60-80°) in a soxhlet apparatus. The marc was air dried and extracted for 18 h with methanol using Soxhlet apparatus. The methanol extract was dried and employed for present investigations. Preliminary phytochemical screening of methanol extract of *A. spicata* roots showed presence of phenols and flavonoids. Thus, the present investigation was undertaken to estimate total phenols and flavonoids in methanol extract of *A. spicata* roots, and its ethyl acetate fraction. *In vitro* antioxidant activity was also evaluated in the methanol extract and ethyl acetate fraction using DPPH method.

Quantitative determination of total phenols was done on the basis of a standard curve of gallic acid and linearity of the calibration curve was achieved between 6.25 to 100 mg/ml concentration for gallic acid (r^2=0.9926; fig. 1). Quantitative determination of total flavonoids was done on the basis of a standard curve of rutin and linearity of the calibration curve was achieved between 10 to 160 mg/ml concentration for rutin (r^2=0.9998; fig. 2). Ethyl acetate fraction was found to contain twice the content of flavonoids and phenols in comparison to methanolic extract, whereas phenolic content in methanol extract was approximately similar to ethyl acetate fraction (Table 1). This

TABLE 1: PERCENTAGE CONTENT OF TOTAL PHENOLS AND FLAVONOIDS IN THE METHANOL EXTRACT OF *A. SPICATA* ROOTS, AND ITS ETHYL ACETATE FRACTION

A. spicata roots	Total phenols content (% w/w)	Total flavonoids content (% w/w)
	Mean^n±SD	Mean^n±SD
Methanol extract	16.00±0.30	2.65±0.05
Ethyl acetate fraction	17.60±0.25	5.85±0.08

n = 3

TABLE 2: ANTIOXIDANT ACTIVITY OF METHANOL EXTRACT OF *A. SPICATA* ROOTS, AND ITS ETHYL ACETATE FRACTION

Treatment	Concentration (mg/ml)	Mean^n percentage inhibition of DPPH radical±SD
Rutin	2.0	41.00±0.825
	4.0	74.77±1.055
	6.0	76.64±0.571
	8.0	77.19±0.381
	10.0	77.52±0.190
Methanol extract	5.0	42.80±0.431
	10.0	84.09±0.375
	20.0	85.96±0.190
	40.0	87.38±0.375
	80.0	88.26±0.190
Ethyl acetate fraction	5.0	84.53±0.325
	10.0	88.48±0.330
	20.0	89.25±0.381
	40.0	89.58±0.190
	80.0	89.80±0.330

n= 3; The values represent mean percentage of DPPH radical inhibited by standard (Rutin) and test samples, i.e., methanol extract of *A. spicata* roots, and its ethyl acetate fraction.

observation suggests that most of flavonoids have been taken up by ethyl acetate fraction from the methanol extract.

Keeping in view the fact that the plants or foodstuffs such as fruits and vegetables containing phenols possess excellent antioxidant activity[9-12], the methanol extract of *A. spicata* roots and its ethyl acetate fraction were also evaluated for antioxidant activity. Antioxidant activity was assessed by determining percentage inhibition of DPPH radical. Rutin was used as standard for present investigation. A significant antioxidant activity, i.e., mean inhibition of DPPH radical was observed in methanol extract and ethyl acetate fraction at the concentration of 10 µg/ml and 5 µg/ml respectively (Table 2). A slight increase in antioxidant activity was observed in methanol extract as well as ethyl acetate fraction in concentration dependent manner. Maximum percentage inhibition of DPPH (88.26%) achieved by methanol extract whereas ethyl acetate fraction achieved 89.80% at the concentration of 80 µg/ml.

Finally, it can be concluded that *A. spicata* justifies its role in traditional claims due to presence of polyphenols. Authors are now involved in isolating polyphenols from the plant by using different chromatographic techniques.

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