

Total Phenolics and Antioxidant Potentials of *Rotula aquatica* Lour

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ABSTRACT

Rotula aquatica Lour is a small branched shrub that grows characteristically among rocks and trailing over gravel in stream beds. It belongs to the family Boraginaceae and widely distributed throughout India. It is commonly called as *Pasanabheda*. The present study was aimed to evaluate the total phenolics and *in vitro* antioxidant activity in root and leaf extracts of *Rotula aquatica* Lour. The dried roots and leaves were subjected to soxhlet extraction utilizing chloroform and methanol followed by preliminary qualitative phytochemical analysis. Total phenolic content was estimated by Folin-Ciocalteu method and *in vitro* antioxidant assays were performed using various radical scavenging activities *viz.* DPPH, superoxide anion, nitric oxide, hydroxyl and iron chelating activities at different concentrations. The results of the total phenolic content estimation was expressed in terms of equivalence with standard and the antioxidant potentiality of various scavenging and chelating activity were expressed in terms of IC₅₀ values. The results showed that both root and leaves possess significant antioxidant activity even though the former was found to be superior than the latter in terms of parameters assessed. Further the methanol extracts were proved to be an ideal for the materials subjected to investigation.

INTRODUCTION

Free radicals and related species have attracted a great deal of attention in recent years. They are mainly derived from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS), and are generated in our body by various endogenous systems, exposure to different physicochemical conditions or pathophysiological states (Devasagayam *et al.*, 2004).

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system, that functions interactively and synergistically to neutralize free radicals. Thus, antioxidants are capable of stabilizing or deactivating free radicals before they attack cells (Beris, 1991). Antioxidants are absolutely critical for maintaining optimal

cellular and systemic health and well-being. The relation between free radicals and disease can be explained by the concept of 'oxidative stress' elaborated by Sies (1986). The recent years have witnessed resurgence of interest in herbal drugs globally as more people are turning to the use of herbal medicinal products in health care (Suvakanta *et al.*, 2005).

Therefore, the need exists for safe, economic, powerful and natural antioxidants to replace the synthetic ones. Obviously, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts (Clements and Decker, 2000; Nahak and Sahu, 2010).

The plant *Rotula aquatica* belongs to the family Boraginaceae reported to contain baunerol, steroids, alkaloids and allantoin. The root extract of *Rotula aquatica* showed antimutagenic activity, diuretic activity due to the presence of allantoin and it is also used as a laxative, in treatment of piles and in venereal diseases. The alcoholic extract of *Rotula aquatica* also reported to be effective against ethylene glycol induced urolithiasis in albino rat (Rastogi and Mehrotra, 1959; Patil *et al.*, 2004). The present research programme was aimed to investigate the total phenolics and free radical scavenging activities in roots and leaves extracts of *Rotula aquatica* Lour.

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MATERIALS AND METHODS

Plant Material

Roots and leaves of *Rotula aquatica* (Fig 1) were collected from rocky beds of river Bhadra, 2Km away from Kuvempu University campus, Shivamogga, Karnataka and authenticated in the Department of Biotechnology, Kuvempu University.



Fig. 1: *Rotula aquatica* plant.

Preparation of extracts

The fresh roots and leaves collected were thoroughly washed with tap water to make it free from soil particles, and then washed with distilled water. After washing, dried at room temperature and reduced to coarse powder. The powdered materials of *Rotula aquatica* were refluxed in a soxhlet extractor with the solvents Chloroform (50– 70°C, Merk Mumbai, India) and Methanol (Merk Mumbai, India) for 48 hrs in batches of 250g each. Every time, before extracting with the next solvent the powder was dried. The yield of crude extracts obtained from solvent were noted, stored in desiccators for maximum of 3 days; later preserved in a deep freezer (-20°C) for further use.

Qualitative phytochemical analysis

The preliminary phytochemical studies were performed for testing the different chemical groups present in chloroform and methanolic extracts of *Rotula aquatica* roots and leaves (Trease and Evans, 1978; Kokate *et al.*, 1990).

Evaluation of *in vitro* antioxidant activities

General chemicals

All chemicals and solvents used in the study were of analytical grade. DPPH (1, 1-diphenyl, 2-picryl hydrazyl), NBT (Nitro blue tetrazolium), NADH (Nicotinamide adenine dinucleotide phosphate reduced), PMS (Phenazinemethosulphate), TCA (Trichloro acetic acid), Ferric chloride and BHT (Butylatedhydroxy toluene), *N*-(1-Naphthyl)ethylenediamine

dihydrochloride were obtained from Sigma chemical co USA. Ascorbic acid, Potassium chloride, sodium nitroprusside, 1,10-phenanthroline and dimethyl sulphoxide were obtained from SD Fine chem. Ltd, Biosar, India. TBA (Thiobarbituric acid) and ferrozine were obtained from Hi media Mumbai, India. EDTA (Ethylene diamine tetra acetic acid), Pottassium ferric cyanide, tannic acid and Hydrogen peroxide (H₂O₂) were obtained from Qualigens Fine chemicals, Mumbai, India.

Total Phenolics

Total polyphenolic compounds were determined according to a protocol similar to that of Chandler and Dodds, 1995. 1ml of the test extract was mixed with 1ml of 95% ethanol, 5ml of distilled water and 0.5ml of 50% Folin Ciocalteu reagent. The mixture was allowed to react for 5 minutes and 1ml of sodium carbonate was added. Thereafter, it was thoroughly mixed and placed in dark for 1hour. After one hour; the absorbance was measured at 725nm using UV-Visible spectrophotometer. Using the standard gallic acid curve the total polyphenolic content was calculated. The concentration of polyphenols was expressed in terms of µg/mg of sample.

Preparation of Stock Solutions of extracts:

The stock solutions of extracts were prepared by dissolving 100 mg of dried extracts in 100 ml of methanol to make a stock solution of 1 mg/ml. Aliquots from this stock solution were further diluted with methanol to get the required concentrations of the extract.

1, 1-Diphenyl 2-Picryl Hydrazyl (DPPH) radical scavenging activity

The hydrogen atom or electron donating abilities of the extracts of *Rotula aquatica* was measured from the bleaching of the purple-coloured methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent (Cuendet *et al.*, 1997). Different concentrations of the extracts in methanol were added to 4 ml of 0.004% methanol solution of DPPH. After 30 min incubation at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I%) was calculated in the following way: % Inhibition = $((A_0 - A_1) / A_0) \times 100$ where, A₀ is the absorbance of the control reaction (containing all reagents except the test compound) and A₁ is the absorbance of the test extract. The values of inhibition were calculated for the various concentrations of extract.

Superoxide anion radical scavenging activity

The scavenging activity of the *Rotula aquatica* extracts towards superoxide anion radicals was measured by the method of Liu *et al.*, 1997. Superoxide anions were generated in a non-enzymatic phenazinemethosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitrobluetetrazolium (NBT). In the experiment the superoxide

anion was generated in 3 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 μ M) solution, 0.75 ml of NADH (936 μ M) solution and different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 μ M) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated as explained previously.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat, 1964. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction (Velavan *et al.*, 2007). 2ml of 10 mM sodium nitroprusside in 0.5ml phosphate buffer saline (pH 7.4) was mixed with various concentrations of the extracts and the mixture was incubated at 25 °C for 150 minutes. From the incubated mixture 0.5 ml was taken out and added into 1ml sulfanilic acid reagent (0.33% in glacial acetic acid) and incubated at room temperature for 5 minutes. Finally, 1ml of *N*-(1-Naphthyl)ethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 minutes. The absorbance at 540nm was measured with a spectrophotometer. The Nitric oxide radicals scavenging activity was calculated as explained previously.

Hydroxyl radical scavenging activity

The scavenging activity for hydroxyl radical was measured by Fenton reaction (Yu *et al.*, 2004). The hydroxyl radicals were generated in reaction mixture containing various concentrations of extracts with 60 μ l of 1.0 mM FeCl₃, 90 μ l of 1mM 1,10-phenanthroline, 2.4ml of 0.2M-phosphate buffer (pH 7.8) and 150 μ l of 0.17 M H₂O₂. Adding the H₂O₂ started the reaction. After incubation at room temperature for 5 minutes, the absorbance of mixture at 560 nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated as explained previously.

Fe²⁺ chelating activity

The chelating activity for the extracts for ferrous ions Fe²⁺ was measured according to the method of Dinis *et al.*, 1994. To the different concentrations of the extracts, 1.6ml of deionised water and 0.05ml of 2 mM FeCl₂ were added.

After 30 seconds, 0.1ml of 5mM ferrozine was added. Ferrozine reacted with divalent iron to form stable magenta complex species that were very soluble in water. After 10minutes at room temperature, the absorbance of the Fe²⁺Ferrozine complex was measured at 562nm. The chelating activity of the extract for Fe²⁺ was calculated as: Chelating rate (%) = $((A_0 - A_1) / A_0) \times 100$ where, A₀ is the absorbance of the control (without test samples) and A₁ is the absorbance of test samples.

RESULTS AND DISCUSSION

Extraction Yield

Methanolic and chloroform extracts of both leaves and roots of *Rotula aquatica* were weighed and the yields obtained were noted. *R. aquatica* roots methanol extract showed 38.97g and leaves methanol extract revealed 30.59g. In chloroform extracts of *R. aquatica*, roots showed 15.52g whereas leaves showed 18.56g (Table 1).

Table 1: Extraction yield of roots and leaves of *Rotula aquatica*.

Sl No.	Plant Extracts	Chloroform Extract	Methanol extract
1.	<i>Rotula aquatica</i> Roots	15.52g	38.97g
2.	<i>Rotula aquatica</i> Leaves	18.56g	30.59g

Qualitative phytochemical analysis

The preliminary qualitative phytochemical analysis revealed that both the methanolic and chloroform extract of *Rotula aquatica* roots and leaves showed the presence of carbohydrates, proteins, amino acids and glycosides. Steroids and triterpenoids are present in chloroform extracts whereas absent in methanolic extracts of both. Saponins showed presence only in methanol extracts of root and leaves. Quinones were present in both leaf extracts and in root chloroform extract. Alkaloids, tannins and polyphenols are present in chloroform and methanol extracts of both leaves and roots. Flavonoids showed positive for chloroform extract of root and methanol extracts of both leaves and root. The results of the analysis are shown in Table 2.

Table 2: Results of Qualitative analysis for Phytoconstituents in *Rotula aquatica*.

Tests	leaf extracts		root extracts	
	Chloroform Extract	Methanol Extract	Chloroform Extract	Methanol Extract
Carbohydrates	+	+	+	+
Proteins	+	+	+	+
Amino Acids	+	+	+	+
Steroids	+	-	+	-
Triterpenoids	+	-	+	-
Glycosides	+	+	+	+
Saponins	-	+	-	+
Alkaloids	+	+	+	+
Quinones	+	+	+	-
Flavonoids	-	+	+	+
Tannins & Polyphenols	+	+	+	+

Evaluation of *in vitro* antioxidant activities

Total Phenolics

The total amount of phenolic contents was present in varied concentrations in the different extracts of *Rotula aquatica*. The methanolic extract of root showed highest concentration of phenolics (55.2 \pm 0.02 μ g/mg) followed by chloroform extract (48.4 \pm 0.1 μ g/mg), while the leaf methanolic and chloroform extract recorded (44.9 \pm 0.03 μ g/mg) and (34.7 \pm 0.3 μ g/mg) respectively (Fig 2a and b).

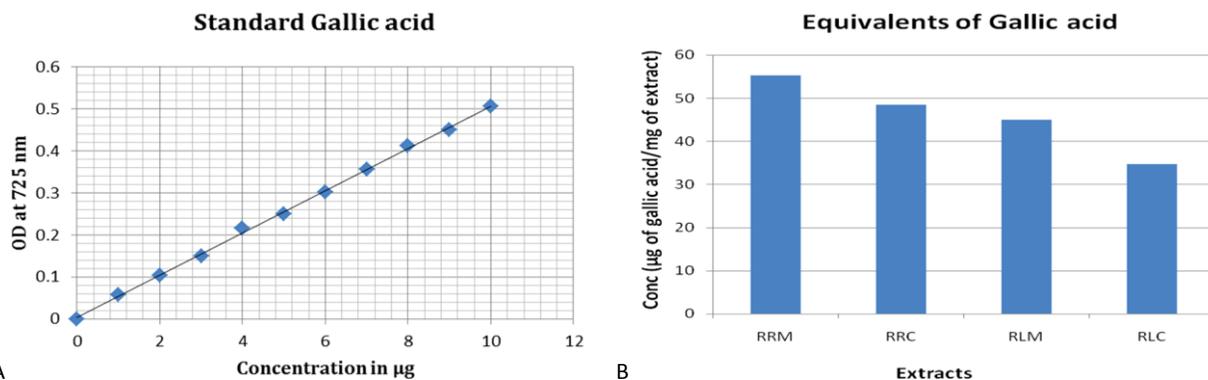


Fig. 2: Total phenolics of *Rotula aquatica* root extracts in Equivalents of gallic acid (a) Standard curve of gallic acid (b) Equivalents of gallic acid (RRM-*Rotula aquatica* root methanol extract, RRC- *Rotula aquatica* root chloroform extract, RLM-*Rotula aquatica* leaf methanol extract and RLC- *Rotula aquatica* leaf chloroform extract).

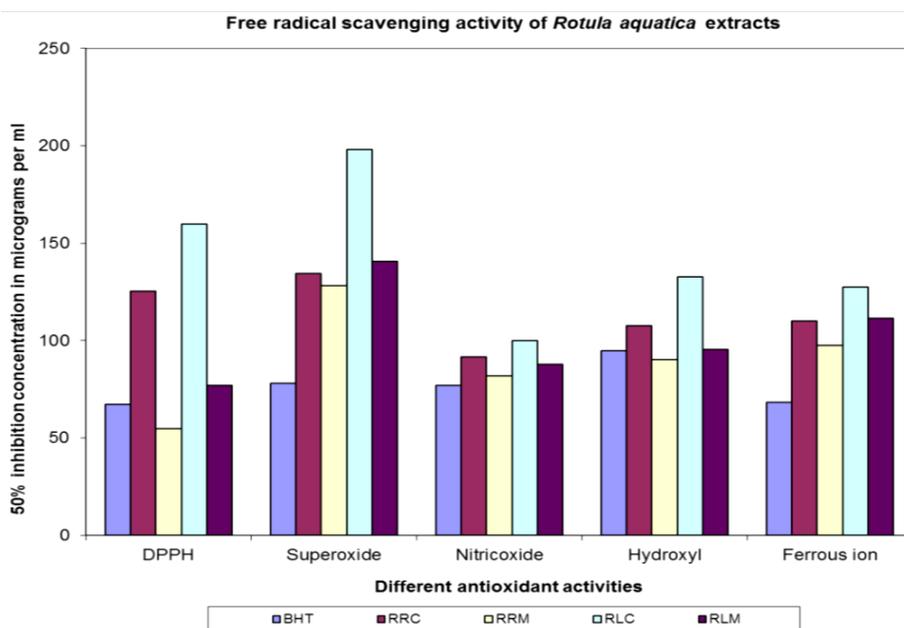


Fig. 3: Different radical scavenging activities of *Rotula aquatica* extracts at different concentrations (BHT-Standard, RRC- *Rotula aquatica* root Chloroform extracts and RRM-*Rotula aquatica* root methanol extract, RLC- *Rotula aquatica* leaf chloroform extract and RLM-*Rotula aquatica* leaf methanol extract)

DPPH radical scavenging activity

DPPH radical scavenging activity of the different extracts at varying concentrations were measured along with standard BHT. The methanolic extract of roots and leaves exhibited significant DPPH radical scavenging activity, with 50% inhibition (IC_{50}) at a concentration of 54.68 $\mu\text{g}/\text{ml}$ and 77.01 $\mu\text{g}/\text{ml}$ respectively. While the chloroform extracts of roots and leaves showed IC_{50} value of 125.31 $\mu\text{g}/\text{ml}$ and 159.74 $\mu\text{g}/\text{ml}$ respectively. The IC_{50} value of methanolic extract of root was found to be less than that of BHT (67.38 $\mu\text{g}/\text{ml}$) (Fig 3). It is evident from the results that roots methanol extract had the strongest radical scavenging activity compared to the other extracts.

Superoxide anion scavenging activity

The superoxide anion radical scavenging activity of the extracts from *Rotula aquatica* assayed by the PMS/ NADH system

is shown in Fig.3. The methanolic extracts of roots and leaves were found to possess good scavenging activity with IC_{50} values of 128.27 $\mu\text{g}/\text{ml}$ and 134.51 $\mu\text{g}/\text{ml}$ respectively, whereas chloroform extracts of roots and leaves showed 50% inhibition at a concentration of 140.64 $\mu\text{g}/\text{ml}$ and 198.01 $\mu\text{g}/\text{ml}$ respectively. BHT showed 50% inhibition at 78.07 $\mu\text{g}/\text{ml}$.

Nitric oxide radical scavenging activity

The extracts of *Rotula aquatica* showed a better activity in competing with oxygen to react with nitric oxide and thus the inhibition of anions.

The chloroform and methanol extracts of *Rotula aquatica* roots exhibited an IC_{50} values of 91.65 $\mu\text{g}/\text{ml}$ and 81.98 $\mu\text{g}/\text{ml}$, however the corresponding leaves extract showed 50% inhibition at a concentration of 99.90 $\mu\text{g}/\text{ml}$ and 87.65 $\mu\text{g}/\text{ml}$ respectively whereas BHT has lower IC_{50} value of 77.05 $\mu\text{g}/\text{ml}$ Fig 3.

Hydroxyl radical scavenging activity

All the extracts shown the scavenging ability at varying concentration ranges against the hydroxyl radicals generated in a Fenton reaction system. Among the extracts, hydroxyl radical scavenging was found to be high in roots methanol extract followed by roots chloroform, leaves methanol and chloroform extracts with IC₅₀ values of 90.20µg/ml, 107.64µg/ml, 95.49µg/ml and 132.62µg/ml respectively. The scavenging activity of different extracts measured against hydroxyl radical is as shown in **Fig 3**. The IC₅₀ value of methanolic extract of root was found to be less than that of BHT (90.20µg/ml).

Ferrous ion chelating activity

The methanol and chloroform extracts of roots possessed potent iron chelating activity with IC₅₀ values of 97.33µg/ml and 110.13µg/ml compared to methanol and chloroform extracts of leaves with 50% inhibition concentrations of 111.28µg/ml and 127.38µg/ml respectively, while the reference antioxidant BHT, showed relatively higher ion chelating activity with IC₅₀ at 68.32µg/ml (**Fig 3**).

In recent years, potent free radical scavengers have attracted a tremendous interest as possible therapeutics against free radical mediated diseases. Free radicals are constantly generated in cells, either as accidental by-products of metabolism or deliberately (Jamuna *et al.*, 2010). When an imbalance between free radical generation and body defense mechanisms occurs, oxidative damage will spread over all the cell targets (DNA, lipids, and proteins). It has been reported that a series of human illness such as cancer, atherosclerosis, cardio and cerebrovascular diseases, diabetes, immune system impairment, neurodegenerative diseases can be linked to the damaging action of extremely reactive free radicals (Mruthunjaya *et al.*, 2008). There is increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural antioxidants contained in spices, herbs and medicinal plants (Osawa, 1994 and Noda *et al.*, 1997).

In the present study, *Rotula aquatica* roots and leaves extracts were tested for different phytoconstituents along with its total phenolics and antioxidant potentials. The result of qualitative phytochemical analysis revealed that, *Rotula aquatica* chloroform and methanolic extracts are bestowed with the presence of several bioactive compounds *viz.* glycosides, alkaloids, flavonoids, tannins and polyphenols which therefore encourages further antioxidant studies.

From the observations it was reported that the root methanol extract possessed high level of polyphenolic compounds. Further, free radical scavenging activity of methanolic and chloroform extracts of *Rotula aquatica* roots and leaves were assessed using various *in vitro* radical scavenging activities such as DPPH, superoxide anion, nitric oxide, hydroxyl and iron chelating activity at different concentrations. From the results it was observed that the methanolic root extract showed highest

antioxidant potential followed by methanolic leaf, chloroform root and chloroform leaf extracts in terms of *in vitro* antioxidant parameters experimented. It is significant to note that the root extract showed a higher potency than the standard in scavenging of DPPH free radical.

It is obvious from the study, that the investigated extracts have the ability to quench free radicals. This may be due to chemical structure that can provide the necessary component as a radical scavenger and they contribute directly to antioxidative action in this plant extract. (Hatano *et al.*, 1989; Duh *et al.*, 1999 and Bhanu Priya *et al.*, 2013). This feature perhaps may be due to the presence of active constituents *viz.* polyphenols, tannins, flavonoids, glycosides *etc.*, identified in the plant, which have been documented for their antioxidant activity and therefore may be of immense value for further studies to unravel novel compounds and subsequent treatment strategies for disorders associated with free radicals induced tissue damage (Geissman, 1963; Saravana Kumar *et al.*, 2008; Jamuna *et al.*, 2011 and Vijayakumari *et al.*, 2013).

CONCLUSION

The present study suggest that the *Rotula aquatica* extracts possess potent antioxidant activity, which might be helpful in preventing or slowing the progress of various oxidative stress- related diseases. Further investigation on the isolation and identification of antioxidant component(s) in the plant may be therefore highly rewarding.

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