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IN-VITRO ANTIOXIDANT ACTIVITY AND DETERMINATION OF TOTAL PHENOLIC, FLAVONOID CONTENTS OF STEMS OF *ROTULA AQUATICA* LOUR.

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ABSTRACT: This research aims to detect the *in-vitro* antioxidant activity of the methanolic and aqueous stem extract of *Rotula aquatica lour* and to estimate its phenolic and flavonoid content. DPPH (2, 2-diphenyl-1-picryl hydrazyl), superoxide radical scavenging capacity and reducing power assay (RPA) were carried out to evaluate *in vitro* antioxidant activity. Phytochemical screening was carried out according to standard procedures followed by their TLC profiling was carried out. Total phenolic content was estimated by using Folin-Ciocalteu's reagent and total flavonoid content by Aluminium chloride colorimetric method. It was observed that DPPH and superoxide IC_{50} value of methanolic and aqueous stem extracts of *Rotula aquatica lour* were found to be (50.98±0.19)µg/ml, (73.67±0.12)µg/ml and (65.41±0.42)µg/ml, (71.97±0.31)µg/ml respectively and reducing power of the extract was increased with the increasing concentration. Total phenolic and flavonoid contents were found to be 6.80 g and 5.90 g gallic acid equivalent per 100 g and 2.60 g and 1.79 g quercetin equivalent per 100 g of methanolic & aqueous extract respectively by spectrophotometric method. These primary findings suggest that stem extract possess phenolic and flavonoid constituents that are responsible for antioxidant activity.

INTRODUCTION: In recent years, consumers desire to manage a festering health condition through improved diet. Plants have evolved different phytochemicals and enzymes as a reservoir of antioxidant defence to maintain recovery and metabolism. Anxiety about improving health, involving natural products with high potential, has potentiated advance research on antioxidants.

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Plant extract or plant derived antioxidant compounds improve human body's antioxidant defence and are preferable because of their safety over synthetic. Therefore, a large scale of research is based on discovering plants that protect against various kinds of ailments with antioxidant potential that may be used for human consumption [1].

Rotula aquatica lour is species of aromatic flowering shrub belonging to family *Boraginaceae*. It is mostly present in aquatic region. It is a rare rheophyte native to India, where it is a member of the lotic ecosystem of streams [2]. *Rotula aquatic lour* is also called as pashanbed; it is widely distributed in India, Sri Lanka, tropical south-eastern Asia and Latin America.

In India, it is distributed from Kumaun to Assam and from Western to Southern India. The plant contain Baunerol, steroid, alkaloid, and rhabdiol and allantoin. The medicinal values of plant lie in their component phytochemicals such as alkaloids, flavonoid, phenolic compounds and other nutrients like as amino acid, proteins, which produce a definite physiological action on the human body. A systematic search for useful bioactivities from medicinal plants is now considered to be a rational approach in nutraceutical and drug research. It is an Ayurvedic plant which is an important traditional medicine for kidney and bladder stones.

In Ayurveda, plant used for cancer, piles, diabetes, venereal diseases, kidney and bladder stones, cough, heart problems, blood disorders, fever, poisonings, ulcers and uterine diseases. It is used as laxative, diuretic, antioxidant, antihelmentic, astringent, bitter etc. The different parts of plant extract have been reported to acquire above activities. But so far, there are no reports made on the comparison of antioxidant activity and phytochemical properties of stems of *Rotula acquatica lour* in various organic and aqueous extracts [3].

MATERIALS AND METHOD:

Plant material: The stems of *Rotula aquatica lour* were collected from Sawantwadi and authenticated from Botanical Survey of India, Pune, Maharashtra. The stems were cleaned and dried at room temperature in shade, away from direct sunlight and coarsely powdered in grinder.

Preparation of extract: The powdered material was subjected to soxhlet extraction with various solvents ranging from non-polar to polar. The solvents used were petroleum ether, chloroform, methanol and water. Each time before extraction with next solvents the marc was air-dried. All the extracts were concentrated by distilling the solvent at low temperature. They were then weighed and percentages of different extractive values were calculated with respect to air-dried substance. The extracts were selected for phenolic, flavonoid content and antioxidant activity on the basis of phytochemical screening and literature survey.

Reagents Chemicals and Instruments: Quercetin, curcumin, riboflavin, DPPH and ascorbic acid were obtained from Hi Media Labs, Mumbai. Folin-Ciocalteu reagent, aluminium chloride, NBT was purchased from Research lab Mumbai. All organic solvents were of analytical grade and supplied from Research Lab, Mumbai. UV-Visible Spectrophotometer (JascoV-530) was used for antioxidant activity determination by DPPH method and for total phenolic, flavonoid content determination.

Preliminary Phytochemical Screening: The preliminary phytochemical screening of the different extracts of *Rotula aquatica lour* were carried out in order to ascertain the presence of its constituents by utilizing standard conventional protocols [4, 5].

TLC analysis: Thin layer chromatographic technique was used to separate the chemical compounds present in the drug. Various solvent systems were checked to separate the maximum number of chemical compounds in the drug. After performing TLC of the methanolic extract, Rf values were calculated for the spots which were seen under UV illuminator [6].

Determination of Total Phenolic Content: The concentration of phenolic in plant extracts was determined using spectrophotometric method. Methanolic solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteau's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$.

The samples were thereafter incubated in a thermostat at 45 ^oC for 45 min. The absorbance was determined using spectrophotometer at λmax = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolic was read (mg/ml) from the calibration line; then the content of phenolic in extracts was expressed in terms of gallic acid equivalent (mg/g of GA/g of extract). All the tests were performed in triplicate.

Determination of Total Flavonoid Content: Total flavonoid content was determined according to the procedure by Aluminium chloride colorimetric method. Extract solution (1 ml, 0.1 mg/ml) was placed in a 10-ml volumetric flask, and then 5 ml of distilled water was added followed by $NaNO_2$ solution (0.3 ml, 5%). After 5 min, $AlCl_3$ solution (0.6 ml, 10%) was added. After another 5 min, NaOH solution (2 ml, 1 M) was added and volume was made up with distilled water. The solution was mixed thoroughly and absorbance was measured at 510 nm. Total flavonoid contents were expressed as milligrams of quercetin equivalent per milligram of dry weight. All the tests were performed in triplicate [7, 8].

Antioxidant assays: Each sample was dissolved in 95% methanol to make a concentration of 1 mg/ml and then diluted to prepare the series concentrations for antioxidant assays. Reference chemicals were used for comparison in all assays.

DPPH Radical Scavenging Activity Assay: The free radical scavenging activity of the fractions was measured *in vitro* by 2, 2′- diphenyl-1-picryl hydrazyl (DPPH) assay according to the method described earlier. The stock solution was prepared by dissolving 24 mg DPPH with 100 ml methanol and stored at 20°C until required. The working solution was obtained by diluting DPPH solution with methanol to attain an absorbance of about 0.98±0.02 at 517 nm using the spectrophotometer. A 3 ml aliquot of this solution was mixed with 100 µl of the sample at various concentrations (10 - 500 µg/ml). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared as above without any sample. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

Scavenging effect (%) =

$$[(A_{cont.} - A_{test}) / A_{cont.}] \times 100$$

Where, A_{cont} is the absorbance of control reaction and A_{test} is the absorbance in the presence of extract. The antioxidant activity of the extract was expressed as $IC_{50.}$ The IC_{50} value was defined as the concentration (in µg/ml) of extract that inhibits the formation of DPPH radical by 50% [9].

Superoxide Anion Scavenging Assay: The assay for superoxide anion radical scavenging activity was supported by riboflavin-light-NBT system. Briefly, 1 ml of sample was taken at different concentrations (20 to 100 µg/ml) and mixed with 0.5 ml of phosphate buffer (50 mM, pH 7.6), 0.3 ml riboflavin (50 mM), 0.25 ml PMS (20 mM), and 0.1 ml NBT (0.5 mM). Reaction was started by illuminating the reaction mixture using a fluorescent lamp. After 20 min of incubation, the absorbance was measured at 560 nm. Curcumin was used as standard. The scavenging ability of the plant extracts were determined by the following equation[10].

Scavenging effect (%) =

$$[(A_{cont.} - A_{test}) / A_{cont.}] \times 100$$

Assay of Reducing Power: 1 ml of plant extract solution (final concentration 100-500 mg/l) was mixed with 2.5 ml phosphate buffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide $[K_3Fe(CN)_6]$ (10g/l), then mixture was incubated at 50°C for 20 minutes. Two and one-half, 2.5 ml of tri-chloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml Fecl3 (1g/l) and absorbance measured at 700nm in UV-Visible Spectrophotometer (Jasco V-530 UV-Visible Spectrophotometer). Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was expressed as mean. Increased absorbance of the reaction mixture indicates stronger reducing power [11, 12].

RESULT AND DISCUSSION:

Preliminary Phytochemical Screening: Petroleum ether, chloroform, methanol and aqueous extracts of *Rotula aquatica lour* were subjected to Preliminary phytochemical studies. This study reveals the presence of phenolic, tannins, flavonoid, glycosides, steroids, alkaloids and Saponins.

TLC Profile: TLC of the methanolic extract was developed in the following mobile phases for phenolic and flavonoid compounds.

1) Mobile phase- Benzene: Methanol (9:1) **Fig. 1 & 2** and observed under UV 366 nm showed nine spots at Rf value 0.39, 0.32, 0.27, 0.24, 0.21, 0.17, 0.13, 0.08 (blue colour).

FIG. 1

FIG. 2

TLC PROFIE OF METHANOLIC EXTRACT OF *ROTULA AQUATICA LOUR*

2) Mobile phase for ethyl acetate fraction of methanolic extract- Chloroform: Acetone: Formic acid (7.5 : 1.65 : 0.85) **Fig. 3** and observed under UV 366 nm showed twenty spots at Rf value 0.95, 0.91, 0.84, 0.75, 0.69, 0.61, 0.57, 0.54, 0.43, 0.38, 0.34, 0.28, 0.26, 0.21, 0.19, 0.15, 0.13, 0.09, 0.06 and 0.04 (blue color).

Total Phenolic content: Total phenolic content was estimated by Folin-Ciocalteu method. Total content of phenolic compound was calculated as Gallic acid equivalent. The obtained observations are mentioned in **table 1** and plotting graph absorbance vs concentration (in **fig. 4**). Total Phenolic content was found to be 6.80 g & 5.90 g gallic acid equivalent per 100 g methanolic & aqueous extract respectively by using the equation (y = 9.2819x + 0.3645).

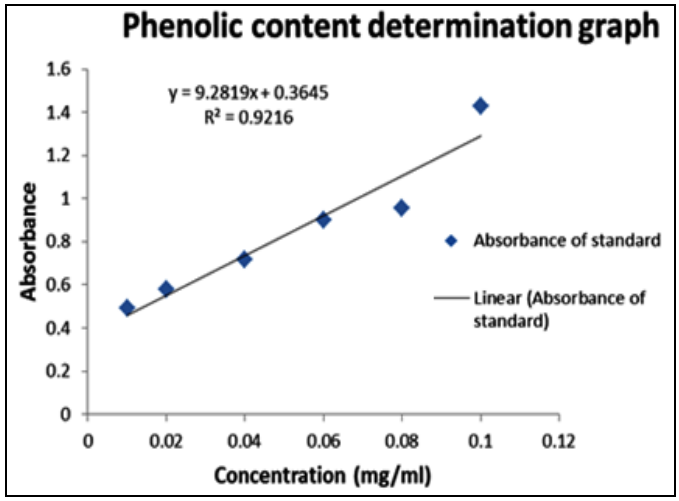

FIG. 4: GRAPH OF ABSORBANCE AGAINST CONCENTRATION FOR TOTAL PHENOLIC CONTENT

TABLE 1: PHENOLIC CONTENT OF METHANOLIC AND AQUEOUS EXTRACT OF STEMS OF *ROTULA AQUATICA LOUR*

S. No.	Sample	Concentration (mg/ml)	Absorbance
1.	Gallic Acid	0.01	0.4896
		0.02	0.5786
		0.04	0.7198
		0.06	0.8997
		0.08	0.9536
		0.1	1.4278
2.	***Rotula aquatica lour*** Methanolic extract	1	0.9956
3.	***Rotula aquatica lour aqueous extract***	1	0.9128

Total Flavonoid content: The total flavonoid content was estimated by Aluminium chloride colorimetric method. In this method flavonoid content was determined as quercetin equivalent. The obtained observations are mentioned in **table 2** and plotting graph absorbance vs concentration (**fig. 5**). Total flavonoid content was found to be 2.60 g & 1.79 g quercetin equivalent per 100 g methanolic & aqueous extract respectively by using the equation (y = 8.4325x + 0.2309).

TABLE 2: FLAVONOID CONTENT OF METHANOLIC AND AQUEOUS EXTRACT OF STEMS OF *ROTULA AQUATICA LOUR*

S. No.	Sample	Concentration (mg/ml)	Absorbance
1.	Quercetin	0.01	0.2103
		0.02	0.3997
		0.04	0.6982
		0.06	0.7688
		0.08	0.9225
		0.1	0.9997
2.	***Rotula aquatica lour*** Methanolic extract	1	0.4559
3.	***Rotula aquatica lour aqueous extract***	1	0.3745

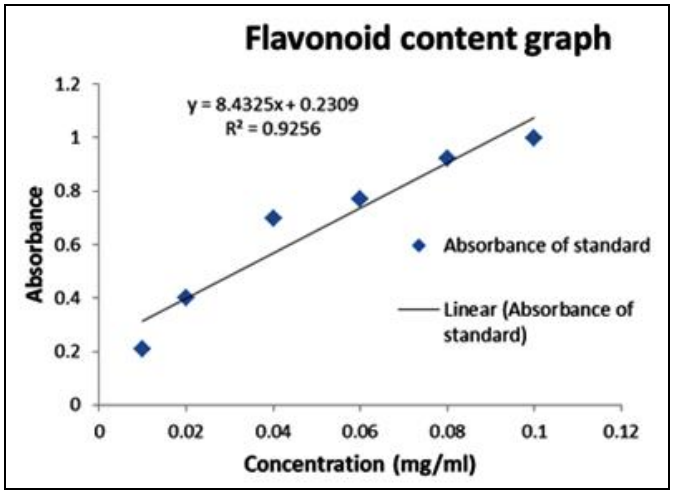

FIG. 5: GRAPH OF ABSORBANCE AGAINST CONCENTRATION FOR TOTAL FLAVONOID CONTENT

Antioxidant activity:

DPPH method: In order to determine the extent of scavenging effect, methanolic and aqueous extract of the stems of *Rotula aquatica lour* was tested for antioxidant activity using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical. Extract has showed antioxidant activity (**Table 3, Fig. 6**).

Methanolic extract showed significant antioxidant activity [(50.98±0.19) µg/ml= IC_{50}] than aqueous extract [(73.67±0.12)µg/ml=IC_{50}] as compared to standard ascorbic acid [(31.13±0.28) µg/ml=IC_{50}].

TABLE 3: DPPH % RADICAL SCAVENGING ACTIVITY OF METHANOLIC AND AQUEOUS EXTRACT OF STEMS OF *ROTULA AQUATICA LOUR*

Conc. (µg/ml)	% Radical Scavenging Activity (Mean±SD)		
	Standard (Ascorbic acid)	Methanolic extract	Aqueous extract
20	33.27±1.12	25.62±1.03	20.81±1.96
40	55.10±1.87	39.28±1.75	32.08±1.85
60	73.50±1.23	57.65±0.58	49.38±1.33
80	82.48±1.75	68.22±0.34	61.18±1.85
100	92.35±0.65	83.12±0.72	75.22±1.72
IC_{50}	31.13±0.28	50.98±0.19	73.67±0.12

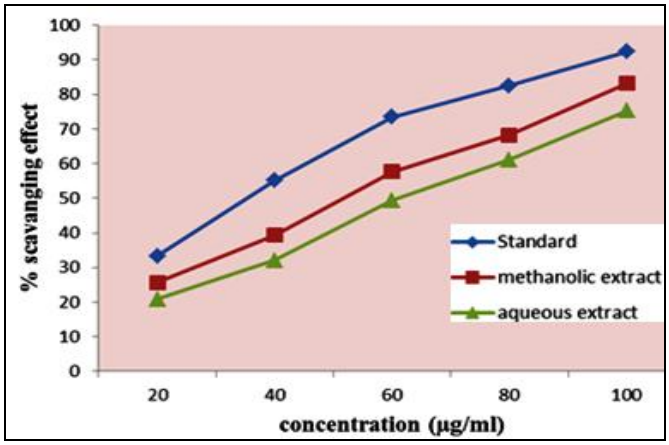

FIG. 6: GRAPH FOR DPPH % RADICAL SCAVENGING EFFECT

Superoxide radical scavenging activity: In order to determine the extent of scavenging effect, methanolic and aqueous extract of the stems of *Rotula aquatica lour* was tested for antioxidant activity by using riboflavin-light-NBT system. Extract has showed antioxidant activity (**Table 4, Fig. 7**).

Methanolic extract showed significant antioxidant activity [(65.41±0.42) µg/ml= IC_{50}] than aqueous extract [(71.97±0.31)µg/ml=IC_{50}] as compared to standard ascorbic acid [(24.03±0.07) µg/ml=IC_{50}].

Assay of Reducing Power: The reductive capabilities of methanolic and aqueous extract of the stems of *Rotula aquatica lour* were compared to ascorbic acid.

The reducing power of stems of *Rotula aquatica lour* extracts was very potent and the reducing power of the extracts was increased with quantity of sample (**Table 5 & Fig. 8**).

TABLE 4: SUPEROXIDE % RADICAL SCAVENGING ACTIVITY OF METHANOLIC AND AQUEOUS EXTRACT OF STEMS OF *ROTULA AQUATICA LOUR*

Conc. (µg/ml)	% Inhibition (Mean±SD)		
	Standard (Curcumin)	Methanolic extract	Aqueous extract
20	41.61±1.56	16.63±1.08	12.78±1.78
40	62.86±0.57	30.01±1.48	23.42±1.62
60	77.02±1.38	45.86±1.58	41.68±1.35
80	87.29±1.67	70.62±1..04	63.84±1.25
100	92.78±1.65	80.82±0.78	73.81±1.52
IC_{50}	24.03±0.07	65.41±0.42	71.97±0.31

TABLE 5: ASSAY OF REDUCING POWER

Sr. No.	Concentration (µg/ml)	Absorbance of standard	Absorbance of test	
			Methanolic extract	Aqueous extract
1	100	0.1234	0.0912	0.0711
2	200	0.1594	0.1378	0.1034
3	300	0.1962	0.1614	0.1343
4	400	0.2197	0.1798	0.1634
5	500	0.2450	0.1878	0.1799

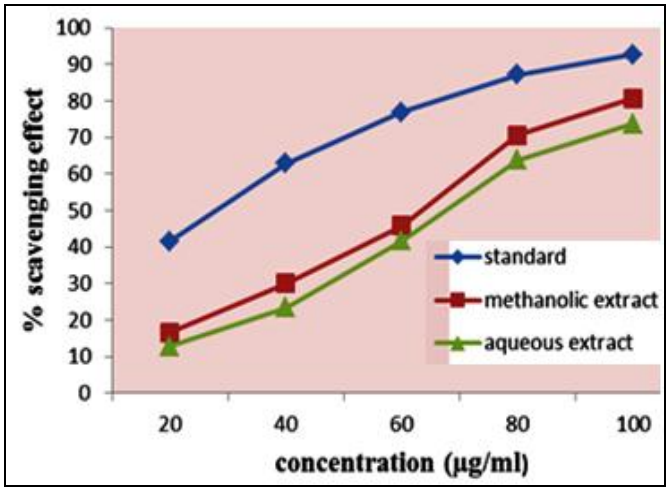

FIG. 7: GRAPH FOR SUPEROXIDE % RADICAL SCAVENGING EFFECT

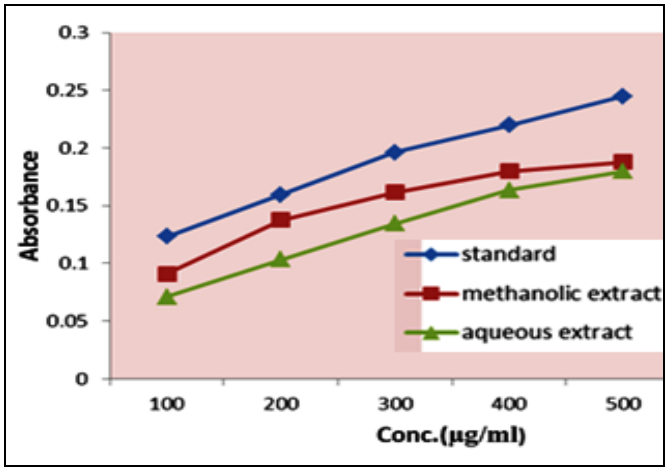

FIG. 8: GRAPH FOR ASSAY OF REDUCING POWER

CONCLUSION: The methanolic extract of stems of *Rotula aquatica lour* have indicated strong antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress induce diseases such as diabetes, liver diseases, cancer, ageing etc. which would be beneficial to the human health. This may be related to the high amount of phenolic and flavonoid compounds present in this plant methanolic extract.

We have also established the relationship of total phenolic, flavonoid contents and the free radical scavenging activity. Further studies are needed to clarify the *in vivo* potential of this plant in the management of human diseases resulting from oxidative stress and suggested that this plant could be used as an additive in the food industry to provide good protection against oxidative damage.

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