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IN-VITRO ASSESSMENT OF ANTIOXIDANT POTENTIAL OF ROTULA AQUATIC LOUR EXTRACT

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ABSTRACT

The present investigation was focused on the antioxidant activity of the methanolic extract of *Rotula aquatica*, a medicinal shrub trailing over gravel in stream beds. The extract exhibited significant ($p<0.05$) increase in the antioxidant activity with increase in concentration. The extract showed 91.89% DPPH scavenging activity and 74.35% inhibition of nitric oxide at a concentration of 128 µg/mL. Superoxide scavenging activity indicated that 38 µg/mL of extract could inhibit 50% of superoxides. FRAP assay and total reducing potential also signifies the antioxidant potential of the plant extract. The study demonstrated a statistical correlation ($r>0.95$) between phenol content and antioxidant activity. Overall, the work validates the ethnobotanical usage of the plant and its potential for application in pharmaceutical and food industries.

INTRODUCTION

In the present day life-style majority of human diseases are resultant effect of oxidative stress or oxidative phenomenon in the cell that effect cellular proteins, DNA and lipid (Halliwell *et al*., 1992). The study of antioxidant activity has gained much attention due to its importance in the field of medicine as well as in food processing industries, nutri-cosmetic and pharmaceutical companies to protect products from aging and decay. Antioxidants relieve from oxidative stress caused due to reactive oxygen species generated in biologic systems under certain abnormal condition. The reactive oxygen species, if not treated may leads to cellular death, many human diseases including cancer, development of neurodegenerative or cardiac disorders and faster aging. Thus antioxidants play a major role in interruption of radical chain processes and converting to low activity radicals that may be easily removed from the organism.

Free radicals and related species have attracted a great deal of attention. They are mainly derived from oxygen and nitrogen, and are generated in our body by various endogenous systems, exposure to different physicochemical conditions or pathophysiological states (Devasagayam *et al*., 2004). Normally human beings have self-defense mechanism with highly sophisticated and complex antioxidant protection system such as endogenous enzymes including superoxide dismutase, glutathione peroxidase and catalase, that functions interactively and synergistically to neutralize free radicals (Halliwell, 1994). However, under stress and abnormal physiological condition external source of antioxidant defense system is required to protect the organism from oxidative stress (Pietta, 2000). Synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene are being used to protect against free radicals, however high health risks and toxicity of the drugs have been reported recently. Therefore, there is an urgent need to replace them with alternative antioxidants that are safe, economical and powerful (Jamuna and Rai, 2011). Further, consumers anxiety to improve health by natural products with high potential, has motivated advance research on plant products to manage health condition.

People around the world have practiced herbal system of medicine for centuries and have its roots in civilization of the world. Curing ailments by natural source is highly effective because the ingredients interact simultaneously and can complement or damage disease causing agents or neutralize their possible negative effects. Plant extracts provide unlimited opportunities for new drug because of the vast availability of chemical diversity. Several studies have described the antioxidant properties of medicinal plants which are rich in phenolic compounds (Brown and Rice-Evans, 1998). Phytochemicals such as flavonoids, phenolic acids, tannins, tocopherols also act as antioxidants (Krings and Berger, 2001).

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Hence, the present study was undertaken to analyze the antioxidant activity of methanolic extract of *Rotula aquatic*.

Rotula aquatica is a species of rare rheophyte with aromatic flowering belonging to the family Boraginaceae and is represented by about 100 genera and 2000 species. It is widely distributed in lotic ecosystem of India, Sri Lanka, tropical south-eastern Asia and Latin America especially in sandy and rocky beds that trail over gravel in stream beds of rivers. *R. aquatica* Lour, is used as Pashanabheda, an Ayurvedic medicine to dissolve kidney stones. The root bark of herb was known to possess diuretic, laxative and in the treatment of kidney stones, piles and venereal diseases. The diuretic action of root is attributed to the presence of allantoin. A sterol named rhabdiol has been isolated from the roots (Pullaiah, 2006; Reddy and Sriniwasan, 2000). In Ayurveda, *R. aquatica* has been reported to be used for diabetes, (Christina *et al*., 2002), cardiotonic activity (Oudhia, 2007) and antiurolithiatic activity (Reddy and Sriniwasan, 2000).

From the literature survey it was found that the *R. aquatic* possess enormous phytomedical constituents, hence it's possible that, the plant possesses even more potent medicinal application other than those reported earlier. Considering this, the goal of the present study was to analyze methanolic extracts of *Rotula aquatica* for its antioxidant activity.

MATERIALS AND METHODS

Plant material collection and processing

The fresh stems and roots of *R. aquatica* Lour. were collected from areas Netravati river, Ujire during September 2010. The collected plant specimen was first identified based on the taxonomical characteristics and their authenticity was confirmed by comparing the voucher specimen at the herbarium collection centre of "Regional Medical Research Centre, ICMR, Belgaum". The herbarium was registered and preserved with accession no. RMRC-981. The stem and rots of the plant were washed to remove the adhering dust particles and dried under shade. The dried parts were then homogenized to fine powder and stored in airtight container.

Solvent extraction

The dried powder of plant material (stem and root) was extracted with methanol by cold maceration. The extraction was done for 72 h. The extract was then collected by filtration using whatsmann No 1 filter paper. The residue was subjected for repeated extraction for 3 times with a gap of 3 days each. The filtrates were pooled and concentrated to syrupy liquid under reduced pressure using super fit rotary vacuum evaporator, dried and stored in dissector until use.

Total phenolic contents in methanolic extract of R. aquatic

The concentration of phenolics in plant extracts was determined using spectrophotometric method (Singleton and Rossi, 1965). Briefly, 0.2ml of the diluted sample extract was transferred in tubes containing 1 ml of $1/10^{th}$ dilution of Folin-Ciocalteu's reagent in water. After incubating for 10 min, 0.8 ml of sodium carbonate solution (7.5% w/v) was added to the mixture. The tubes were then allowed to stand at room temperature for 30 min and the absorbance was read at 743 nm. The standard gallic acid curve ranging from 20-200 µg was taken for the calculation of concentration of polyphenols in samples which was expressed as gallic acid equivalents (GAE) in mg%.

Total Flavonoid content in methanolic extract of R. aquatic

Total flavonoid content was measured by the aluminium chloride colorimetric assay as described by Singleton and Rossi (1965). Extract (1 ml) was added to 1ml of distilled water containing 75 µl of 5% $NaNO_2$ and incubated for 5 min. Later, 75 µl of 10 % $AlCl_3$ was added and incubated for 5 min. Further, 0.5 ml 1M NaOH was added and incubated for 15 min. Absorbance was measured against the blank at 510 nm. The total flavonoid content was expressed as mg quercetin equivalents (QE).

In-vitro antioxidant activity of crude extract of R. aquatica
DPPH (Diphenyl picryl hydrayl) Scavenging activity

DPPH stable free radical scavenging activity was determined by the method described by Molneux (2004). Various concentration (2-128 µg/ml) of methanol extract was made upto 2ml using 0.1mM DPPH solution in methanol. The mixture was shaken and left for incubation in dark for 20 min. The absorbance was recorded at 517 nm in a UV-visible spectrophotometer. Ascorbic acid was used as the reference standard. The percentage of DPPH scavenging was calculated by comparing the absorbance values of the test samples with those of the controls (not treated with extract). The percentage of DPPH radical scavenging was measured by using the following formula

% Inhibition = (Abs control-Abs sample)/Abs control X 100

Nitric oxide radical scavenging assay

At physiological pH, nitric oxide generated from aqueous sodium nitroprusside solution interacts with oxygen to produce nitrite ions, which may be quantified by the Griess Illosovoy reaction (Panda *et al.*, 2009). The reaction mixture contain 10 mM Sodium Nitroprusside, phosphate buffered saline (pH 7.4) and various concentration methanol extract (2-128 µg/ml) in a final volume of 3 ml. After incubation for 150 min at 25˚C, 1ml sulfanilamide (0.33% in glacial acetic acid) was added and allowed to stand for 5min. Then 1ml of napthylethylenediamine di hydrochloride (NED) (0.1%w/v) was added and the mixture was incubated for 30 min at 25˚C. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540nm against blank sample. Ascorbic acid was used as standard.

Superoxide radical scavenging activity

Superoxide radical is generated in Phenzine methosulfate-nictoniamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by the reduction of nitro blue tetrozolium (NBT) to a purple formazan (Fontana *et al*., 2001). The reaction mixture (1ml) containing 20mM phosphate buffer (pH 7.4), 73µM NADH, 50µM Nitrobluetetrazolium, 15µM phenazine methosulfate and various concentration of extract (10-50 µg/ml) was incubated at 25 for 5 min and the absorbance was measured at 562 nm. Quercitine was used as reference standard. The inhibition percentage was calculated as follows.

% Scavenging= (Abs control-Abs sample)/Abs control X 100.

Ferric-reducing antioxidant power (FRAP) method

Ferrous reducing potential of methanol extract was determined by FRAP method described by Benzie and Strain (1996) with slight modification. Briefly, different concentration(200 to 1000 micro gram of methanol extract was taken. The FRAP solution was prepared by using 300 mM acetate buffer (pH 3.6), 10mM 2,4,6 tripyridyl-striazine solution in 40mM HCL and 20mM ferric chloride hexahydrate. The reaction was carried out by mixing 150µl of plant extract with 2850 µl of the FRAP solution. Reading was taken at 593nm.

Total reducing power method

Total Reducing power was measured according to the method described by Yen and Duh (1993). Different concentration (2-128 µg) of methanol extract was mixed with 0.5 ml of 0.2M Phosphate buffer (pH 6.6) and 500 µl of 1% potassium ferricyanide. The mixture was incubated at 50˚C for 20 min, then 500 µl of 10% trichloroacetic acid was added, and the mixture was centrifuged at 2500rpm for 10min. The supernatant was mixed with distilled water (1ml) and the absorbance was read at 700 nm. Increase in the absorbance shows increase in reducing power.

Statistical analysis

The experiments were carried out in triplicates and values are mentioned as mean ± standard deviation. The raw data obtained was subjected to One-Way ANOVA (Analysis of Variance) with Duncan multiple range test using Statistica software. All analyses and comparisons was evaluated at the 95% level of confidence ($p<0.05$).

RESULTS

Total phenolic content of methanol extract of R. aquatic

In the present study, phenolic content was estimated in the methanolic extract of *R. aquatic* to correlate the antioxidant activity. Figure 1a represents the total amount phenolic content in varied concentration of methanolic extract of *R. aquatic*. The phenolic content was calculated as gallic acid equivalent ($y = 0.010x$; $R^2 = 0.9055$).

Total Flavonoid content in methanolic extract of R. aquatic

The total flavonoid content in the methanolic extract of *R aquatica* extract was determined as quercetin equivalent with the equation $y = 0.0045x$; $R^2 = 0.9803$. The obtained data with various concentration of extract is presented in Figure 1b along with standard quercetin graph. A linear increase in the flavonoid content was observed with increase in concentration.

DPPH scavenging activity

The DPPH radical scavenging activity of the methanolic extract of *R aquatic* at different concentrations in comparison with reference compound ascorbic acid is presented in Figure 2a and 2b respectively. The radical scavenging activity of the extract was the highest (91.89%) at the maximum dose of 128 µg/mL, with an IC50 value of 40 ± 0.05 µg/mL. The effect was concentration dependent. Ascorbic acid showed an IC50 value of 6 ± 0.06 µg/mL.

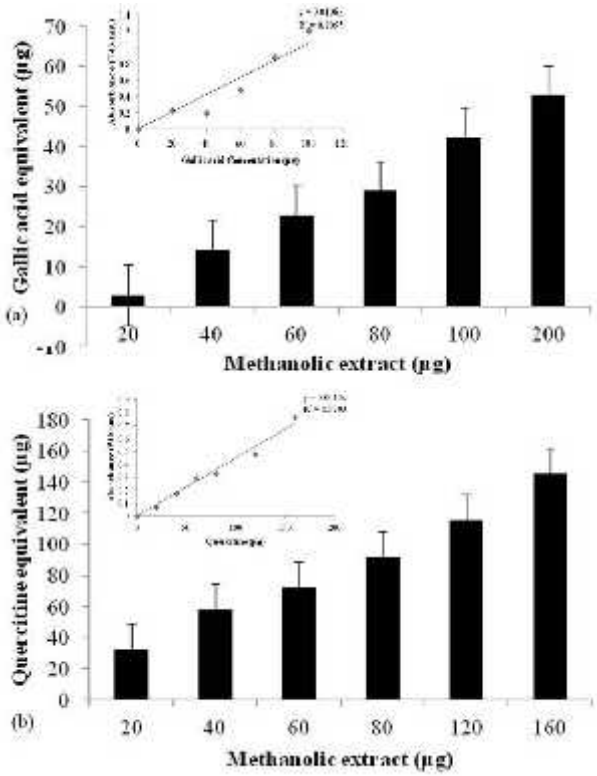

Figure 1 (a) Total phenolic content (b) Total flavonoids, of *R aquatica* extract at different concentration. Linear graph presented in the graph represents their respective standard graphs. Values are Mean ± standard deviation.

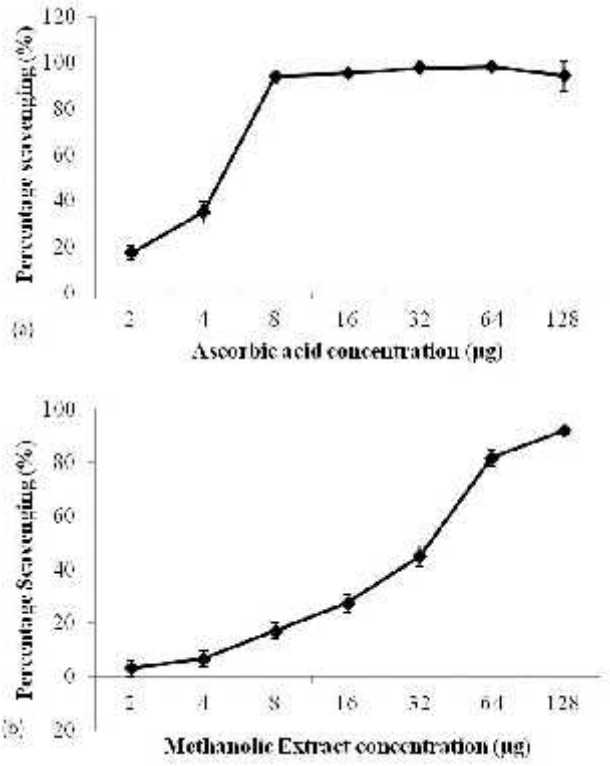

Figure 2 DPPH scavenging activity of (a) Standard Ascorbic acid (b) methanolic extract of *R aquatica* at various concentration. Values are Mean ± standard deviation

Nitric oxide scavenging activity

In the present study, nitric oxide radical quenching activity of methanolic extract was detected by comparing with the standard ascorbic acid. Accordingly, the extracts of *Rotula aquatica* showed an appreciable scavenging activity by competing with oxygen to react with nitric oxide resulting in the inhibition of anions. The extract exhibited maximum percent inhibition of 74.35% at a concentration of 128 µg/ml. The result shows IC50 value of 16 µg/ml corresponding to standard ascorbic acid with IC50 of 2 µg/ml (Figure 3a and 3b).

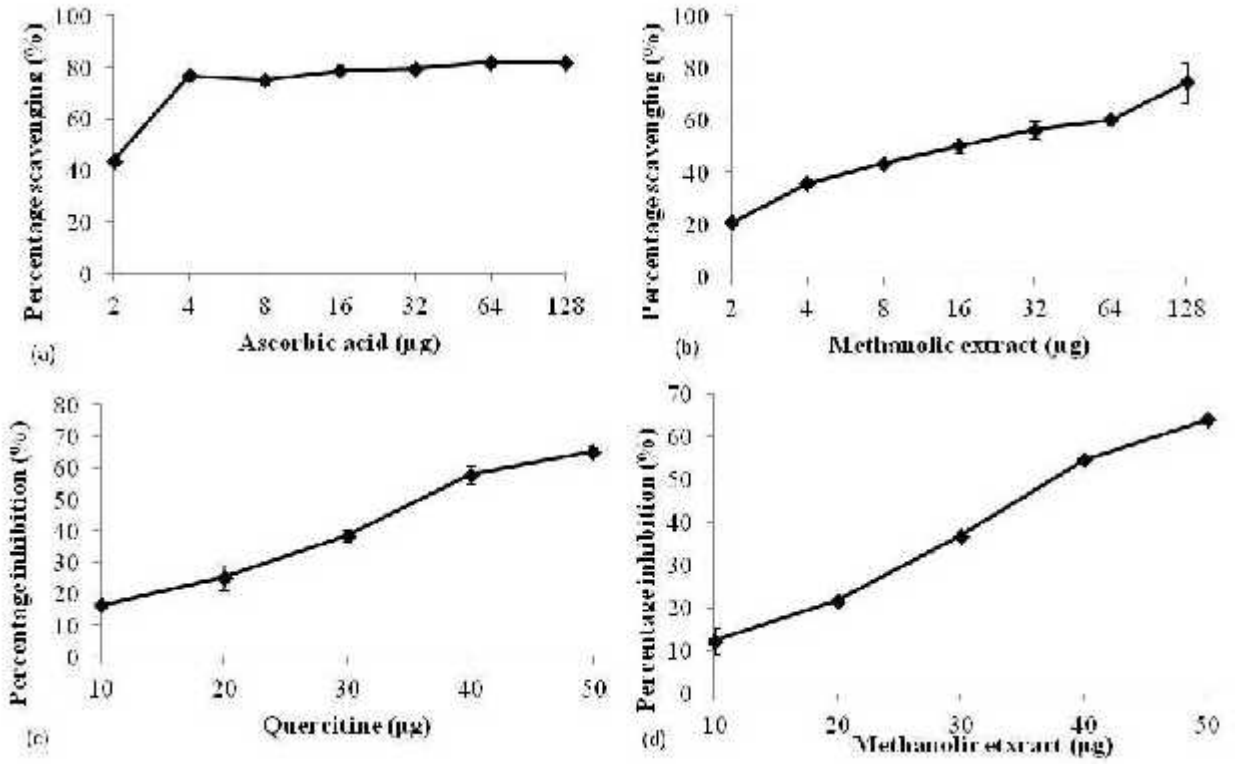

Figure 3 Nitric oxide scavenging activity of (a) Standard Ascorbic acid (b) methanolic extract of *R aquatica* at various concentration. Superoxide scavenging activity of (c) Standard quercitine (d) methanolic extract of *R aquatica* at various concentration. Values are Mean ± standard deviation.

Superoxide scavenging activity

The superoxide anion radical scavenging activity of the extracts from *Rotula aquatica* is represented in Figure 3d with corresponding standard quercitine (figure 3c). The superoxide scavenging activities of methanolic extract and reference compound increased markedly with increasing concentrations. Methanolic extract showed maximum percent inhibition (63.93%) at a concentration of 50 µg/ml with IC50 value of 38µg/ml. However the standard quercitine showed a IC50 value of 39 µg/ml.

Ferrous reducing antioxidant assay (FRAP)

Figure 4a shows the FRAP assay results of methanolic extract *R aquatica*. Methanolic extract shows dose-dependent ferrous reducing potential with 63.2% reduction at 1 mg/ml concentration.

Total reducing potential

The reduction potential of methanolic extract of *R aquatica* was compared with standard ascorbic acid. According to the result obtained, the reducing activity significantly ($p < 0.05$) increased as the concentration of extract increased (Figure 4b).

Correlation between the total phenolic content and antioxidant activity

The total phenolic content of methanolic extract of *R. aquatica* significantly correlated with its DPPH radical scavenging activity ($R = 0.987$, $p < 0.05$), super oxide scavenging activity ($R = 0.975$, $p<0.05$), nitricoxide quenching activity ($R = 0.955$, $p < 0.05$), ferrous reducing ability ($R = 0.958$, $p<0.05$) and total reducing potential ($R = 0.958$, $p < 0.05$). These results indicate that the phenolic contents present in methanolic extract of *R. aquatica* are responsible for its antioxidant activity.

DISCUSSION

Phytomedicine, is gaining prominence as the "drug of future" globally. World Health Organization (WHO) estimates that 75-80% of the people rely on plant based medicine for primary health care. India has been identified as a major resourceful area in the traditional and alternative medicines globally and the rich culture and natural biodiversity, offers a unique opportunity for novel drug discovery (Jachak and Saklani, 2007). Generally, antioxidants compounds have been identified as major health beneficial compounds reported from varieties of medicinal plants (Daniel, 2005).

Antioxidants are known to protect the body against free radical mediated toxicities and also reduce oxidative stress (Nuttall *et al*., 1999). Various plant species have been evaluated for antioxidant activities. The ability of the compounds to remove

oxidative stress is of interest in the development of health foods, nutritional supplements and herbal medicine.

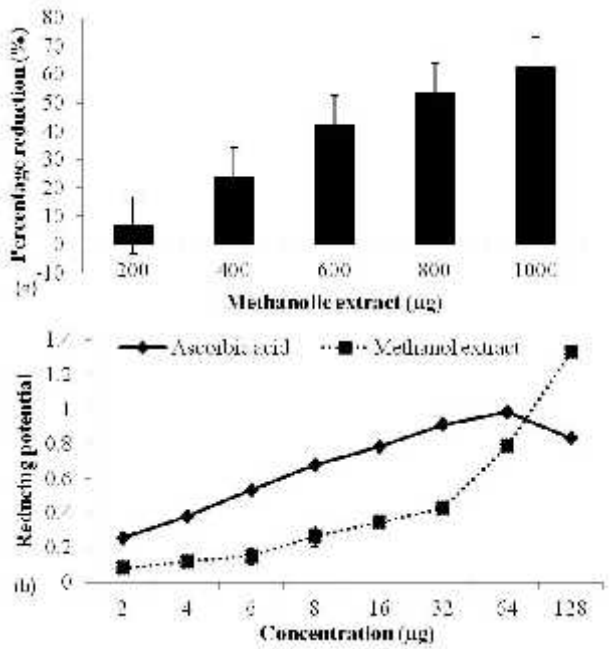

Figure 4 (a) FRAP assay showing percentage reduction of ferrous ions by methanolic extract of *R aquatica*. (b) Reducing potential of methanolic extract of *R aquatica* along with standard ascorbic acid. Values are Mean ± standard deviation

In this view, the present work was carried out to analyze the antioxidant potential of methanolic extract of *Rotula aquatic* an important medicinal plant used in the preparation of formula to treat kidney stone.

Polyphenols are widely distributed in plants and they are the major contributors for scavenging free radicals because of their phenolic hydroxyl groups (Hatano *et al*., 1998). In the present study, the methanolic extract of R aquatica showed the total phenolic content of 263.75µg gallic acid equivalent/mg. Similarly, Priya *et al*. (2013) has reported the total phenolic content of methanolic extract of *R aquatic* stem to be 6.8g gallic acid equivalent/100g. Pallavi *et al*. (2016) evaluated chloroform and methanol extract of *R aquatic* root and leaves for total phenol content and antioxidant activity. The methanolic extract of root showed highest concentration of phenolics (55.2±0.02 µg/mg) followed by chloroform extract (48.4±0.1µg/mg), while the leaf methanolic and chloroform extract recorded (44.9±0.03 µg/mg) and (34.7±0.3 µg/mg) respectively. The polyphenol antioxidant activity is due to the chemical structure and ability to donate/accept electrons, thereby delocalizing the unpaired electron within the aromatic structure (Ross and Kasum, 2002). Among the diverse roles of polyphenols, they protect cell constituents against destructive oxidative damage, thus limiting the risk of various degenerative diseases associated with oxidative stress by acting as potent free radical scavengers.

Flavonoid and their related compounds like flavonol, flavonones, isoflavones are well studied for various biological activities especially reduction of risk related to free radicals. They have attracted much attention because of their potential activities in the prevention of cancer, inflammation and coronary heart diseases (Garcia-Mediavilla *et al*., 2007). The methanolic extract of R aquatica showed 114.31 µg quercetin equivalent per 100 mg of sample. Similarly, total flavonoid content was found to be 2.60 g and 1.79 g quercetin equivalent/100 g methanolic and aqueous extract respectively of *R. aquatic* (Pallavi *et al*., 2016). Hidalgo *et al*. (2010) have reported that flavonoid synergistically interact with free radicals to neutralize them. In the present study, appreciable amount of flavonoid content was observed indicating the ability of *R. aquatic* extract in counteracting the deleterious action of reactive oxygen species. Flavonoids also have significant antioxidant activity under both *in vivo* and *in vitro* conditions (Pietta, 2000).

DPPH is a stable, nitrogen centered free radical, which upon accepting hydrogen from the antioxidants present in the polyphenolic extract, is converted into a stable diamagnetic molecule, diphenyl-picryl hydrazine (Knezevic *et al*., 2011). The methanolic extract of R aquatica demonstrated 91.89% reduction. The observed reduction of DPPH by the extract was either due to the transfer of a hydrogen atom or the transfer of an electron. Aswathanarayan and Vittal (2013) has reported an IC50 of 19.8 µg/ml in the methanolic extract of *R. aquatica*. Priya *et al*. (2013) studied the antioxidant activity of methanolic and aqueous extract of *Rotula aquatic* stem and reported DPPH IC50 value to be 50.98 µg/ml and 65.4 µg/ml respectively. Pallavi *et al*. (2016) evaluated DPPH scavenging activity of chloroform and methanol extract of *R. aquatic* root and leaves. The methanolic extracts of roots and leaves were found to possess IC50 values of 128.27µg/ml and 134.51µg/ml respectively, whereas chloroform extracts of roots and leaves showed 50% inhibition at a concentration of 140.64µg/ml and 198.01µg/ml respectively.

In the present study, methanolic extract showed nitric oxide scavening activity with IC50 value of 16 µg/ml. Correspondingly, Pallavi *et al*. (2016) observed IC50 values of 91.65µg/ml and 81.98µg/ml in chloroform and methanol extracts of *Rotula aquatica* roots respectively and IC50 of 99.90 µg/ml and 87.65µg/ml in the leaves extract. Nitric oxide is a diffusible free radical that react with superoxide anion and form potential cytotoxic molecule such as peroxynitrite. This has a major role in various biological functions including neuronal communication, vasodilatation, antimicrobial and antitumor activities (Shahidi *et al*., 2007). In this regards, the extract of *R. aquatica* showing nitric oxide quenching property signifies its potential in avoiding cytotoxic effect of nitric oxide.

The superoxide scavenging activity of the extract depicted an IC50 value of 38µg/ml. Priya *et al*. (2013) observed a IC50 for methanolic extract of *R aquatica* to be 65.41 µg/ml and IC50 value of 71.97 µg/ml for aqueous extract. Pallavi *et al*. (2016) reported the methanolic extracts of roots and leaves with IC50 values of 128.27µg/ml and 134.51µg/ml respectively, whereas chloroform extracts of roots and leaves with140.64µg/ml and 198.01µg/ml respectively. Superoxide anion plays a vital role in the production of other reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, or singlet oxygen which induce oxidative damage. Hence a noticeable consumption of superoxide anions by methanolic extract of R. aquatica in the present study indicate the protective effect over damage caused by superoxide free radicals. in the reaction mixture by the

antioxidants is indicated by the decrease of absorbance at 560 nm.

Further, the ferrous ion reducing potential of *R aquatica* extract was analyzed by FRAP method, wherein 63.2% reduction was observed with 1 mg/ml of the extract. FRAP assay is based on electron transfer reaction and generally reducing properties are associated with compounds that can donate hydrogen atoms to free radicals to convert them into stable non-reactive molecules and then terminate the free radical chain reactions (Gordon, 1990). In the present study, methanolic extract showed appreciable activity indicating ferrous ion reduction ability of *R. aquatica*.

The reduction capacity of the extract depends on the presence of reductones, which break the free radical chain by donating a hydrogen atom (Mohamed *et al*., 2009). In the present Study, an increase in reducing power with an increase in the concentration of the extract have been observed which is in agreement with the reports of Kumar *et al*., (2012) and Kalaivani and Mathew (2010).

The overall data reveal a positive correlation between total phenolic content and antioxidant activity. It has been proposed that the antioxidant activity increase proportionally with the polyphenol content, primarily because of their redox properties (Rasineni *et al*., 2008). Polyphenols are widely distributed in plants, and phenolic antioxidants act as free radical scavengers and metal chelators. Recently, bioflavonoids and polyphenols of plant origin have been used extensively for free radical scavenging and to inhibit membrane lipid peroxidation (Newairy and Abdou, 2009).

It is apparent from the study, that the methanolic extract of *R. aquatica* have the ability to quench free radicals. This may be due to the presence of phenolic compounds or flavonoid that acts as necessary radical scavenger and contribute to reduce oxidative stress. Similarly, the presence of active constituents viz. polyphenols, tannins, flavonoids, glycosides etc., identified in the plant, have been documented for their antioxidant activity and therefore may represent a huge importance to explore the novel compounds for treatment diseases associated with free radicals induced tissue damage (Jamuna *et al*., 2011; Vijayakumari *et al*., 2013).

CONCLUSION

Overall the results suggest a significant antioxidant activity of methanolic extract of *R. aquatica*. The obtained results suggest the positive correlation of phenolic content in *R. aquatica* for its antioxidative potential. The present study demonstrates that the methanolic extract of *R. aquatica* can neutralize free radicals, thus protecting from oxidative stress. Thus signifying its importance in food and nutraceutical industries. However, further studies are required to clarify the *in vivo* potential of this plant in the management of human diseases resulting from oxidative stress. Further, the safety of the plant extract as additive in the food system also has been investigated. The study on the isolation and characterization of antioxidant components in the plant may be therefore highly appreciated.

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