Chapter 4:

Determination of in vitro antioxidant properties
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4.0 Determination of \textit{in vitro} antioxidant properties

4.1. 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 \textit{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 their 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 are derived from oxygen and nitrogen, and are generated in our body by various endogenous systems, exposure to different physicochemical conditions or pathophysiological states (Devasagayam \textit{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 the herbal system of medicine for centuries and have its roots in civilization of the world. Curing ailments by the natural source is highly effective because the ingredients interact simultaneously and can complement or damage disease causing agents or neutralize their possible negative effects. Plants provide boundless opportunities for novel drug because of the vast availability of phytochemical diversity. Earlier studies have mentioned the antioxidant properties of medicinal plants rich in phenolic compounds (Brown and Rice-Evans, 1998). Flavonoids, phenolic acids, tannins, tocopherols present in plants also act as antioxidants (Krings and Berger, 2001). Hence, the present study was undertaken to analyze the antioxidant activity of methanolic extract of *Rotula aquatica*.

*Rotula aquatica* is one of the 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 and bark of herb was known to possess diuretic, laxative and is used 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. aquatica* possess enormous phytomedical constituents, hence it’s possible that, the plant possesses even more potent medicinal application other than those reported earlier.
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Considering this, the goal of the present study has been to analyze methanolic extracts of *Rotula aquatica* for its antioxidant activity.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals and reagents required

All the chemicals and solvents were of analytical grade. Folin-Ciocalteu’s reagent, gallic acid, NaNO₂, AlCl₃, NaOH, quercetin, sodium nitroprusside, Ascorbic acid, ferric chloride, potassium ferricyanide, sodium dihydrogen phosphate, ferric chloride hexahydrate were purchased from Sisco Research Laboratories, India. Trichloroacetic acid, sulfanilamide, naphthylethlenediamine dihydrochloride (NED) were procured from HiMedia Pvt Ltd, India. DPPH (Diphenyl picryl hydrazyl), NADH, Nitroblue tetrazolium, phenazine methosulfate, tripyridyl-s-triazine were obtained from Sigma-Aldrich, Mumubai, India.

4.2.2. Solvent extraction of plant material

The dried powder of plant material (stem and root) was extracted with methanol by cold maceration as described in chapter 3.

4.2.3. Total phenolic contents in methanolic extract of *R. aquatica*

Total phenolics in plant extract was determined using spectrophotometric method (Singleton and Rossi, 1965). Briefly, 0.2ml of the diluted sample extract was transferred to tubes containing 1 ml of 1/10th 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 the concentration of polyphenols in samples which was expressed as gallic acid equivalents (GAE) in mg%.
4.2.4. Total Flavonoid content in methanolic extract of *R. aquatica*

Total flavonoid content was measured by the aluminum chloride colorimetric assay as described by Singleton and Rossi (1965). Extract (1 ml) was added to 1 ml of distilled water containing 75 μl of 5% NaNO₂ and incubated for 5 min. Later, 75 μl of 10 % AlCl₃ 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).

4.2.5. *In-vitro* antioxidant activity of crude extract of *R. aquatica*

4.2.5.1. DPPH (Diphenyl picryl hydrazyl) Scavenging activity

DPPH stable free radical scavenging activity was determined by the method described by Molyneux (2004). Various concentration (2-128 μg/ml) of methanol extract was made upto 2 ml using 0.1 mM 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

\[
\text{% Inhibition} = \left( \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100
\]

4.2.5.2. Nitric oxide radical scavenging assay

Nitric oxide generated from aqueous sodium nitroprusside solution interacts with oxygen to produce nitrite ions at physiological pH, which may be quantified by the Griess Illsovoy reaction (Panda *et al.*, 2009). The reaction mixture contains 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 the reference standard.

4.2.5.3. Superoxide radical scavenging activity

Superoxide radical is generated in Phenazine methosulfate-nicotinamide 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 Nitroblue tetrazolium, 15µM phenazine methosulfate and various concentration of extract (10-50 µg/ml) was incubated at 25°C for 5 min and the absorbance was measured at 562 nm. Quercitin was used as reference standard. The inhibition percentage was calculated as follows.

\[
\text{% Scavenging} = \left( \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100
\]

4.2.5.4. Ferric-reducing antioxidant power (FRAP) method

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

4.2.5.5. 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 10 min. The supernatant was mixed with distilled water (1ml) and the absorbance was read at 700 nm. The increase in the absorbance shows increase in reducing power.

4.2.6. 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 (DMRT) using Statistica software. All analyses and comparisons were evaluated at the 95% level of confidence (p<0.05).

4.3. RESULTS AND 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., 2009). 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. In this view, the present work was carried out to analyze the antioxidant potential of methanolic extract of Rotula
*R. aquatica* an important medicinal plant used in the preparation of formula to treat kidney stone.

### 4.3.1. Total phenolic content of methanol extract of *R. aquatica*

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*., 1989). In the present study, phenolic content was estimated in the methanolic extract of *R. aquatica* to correlate the antioxidant activity. Figure 4.1a represents the total amount phenolic content in the varied concentration of methanolic extract of *R. aquatica*. The phenolic content was calculated as gallic acid equivalent (*y* = 0.010x; *R*² = 0.9055). According to the results obtained, the methanolic extract of *R. aquatica* showed the total phenolic content of 263.75μg gallic acid equivalent/mg. Similarly, Priya *et al.* (2013) have reported the total phenolic content of methanolic extract of *R. aquatic*a stem to be 6.8g gallic acid equivalent/100g. Pallavi *et al.* (2016) evaluated chloroform and methanol extract of *R. aquatica* 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.
4.3.2. Total Flavonoid content in methanolic extract of *R. aquatica*

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 4.1b along with standard quercetin graph. A linear increase in the flavonoid content was observed with increase in concentration.
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. aquatica* (Pallavi et al., 2016).

Hidalgo *et al.* (2010) have reported that flavonoid synergistically interacts with free radicals to neutralize them. In the present study, an appreciable amount of flavonoid content was observed indicating the ability of *R. aquatica* 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).

### 4.3.4. DPPH scavenging activity

The DPPH radical scavenging activity of the methanolic extract of *R. aquatica* at different concentrations in comparison with reference compound ascorbic acid is presented in Figure 4.2a and 4.2b 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.

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.

Figure 4.2: DPPH scavenging activity of (a) Standard Ascorbic acid (b) methanolic extract of R. aquatic at various concentration. Values are Mean ± standard deviation

4.3.5. 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 aquatic showed an considerable 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 an IC50 value of 16 μg/ml corresponding to standard ascorbic acid with IC50 of 2 μg/ml (Figure 4.3a and 4.3b). 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.

![Graphs showing nitric oxide scavenging activity](image)

**Figure 4.3:** Nitric oxide scavenging activity of (a) Standard Ascorbic acid (b) methanolic extract of *R. aquatica* at various concentrations. Superoxide scavenging activity of (c) Standard quercetin (d) methanolic extract of *R. aquatica* at various concentrations. Values are Mean ± standard deviation.
4.3.6. Superoxide scavenging activity

The superoxide anion radical scavenging activity of the extracts from *Rotula aquatica* is represented in Figure 4.3d with corresponding standard quercetin (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 quercetin showed an IC50 value of 39 μg/ml.

Priya *et al.* (2013) observed an 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 an 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. The appreciable consumption of superoxide anions by methanolic extract of *R. aquatica*, in the present study indicate the protective effect over damage caused by these free radicals.

4.3.7. Ferrous reducing antioxidant assay (FRAP)

Figure 4.4a 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. 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*. 
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4.3.8. Total reducing potential

Ascorbic acid was used as a standard to compare the reducing potential of methanolic extract of *R. aquatica*. According to the result obtained, the reducing activity significantly (p< 0.05) increased as the concentration of extract increased (Figure 4.4b). Reductones present in the extract are the main reason for reducing capacity, 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).

4.3.9. 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 ($R = 0.987$, $p < 0.05$), superoxide scavenging activity ($R = 0.975$, $p < 0.05$), nitric oxide 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.

It has been proposed that the antioxidant activity increases 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* has 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).
4.4. OUTCOME OF THE CHAPTER

- The overall 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 also 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 an 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.