QUANTIFICATION OF ANTIOXIDANTS AND ITS ENZYMATIC ACTIVITY ON *RIVEA HYPOCRATERIFORMIS* (Desr.) Choisy.

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ABSTRACT

Antioxidants are complexes found in the food that can retard or deter oxidation by preventing the initiation and propagation of oxidizing chain reactions. Medicinal plants have been used for centuries by man to manage diseases and have a host of antioxidant complexes. However, there is a little information concerning antioxidant activity in *Rivea hypocrateriformis* (Desr.) Choisy. Hence, the present study was undertaken to determine the antioxidants and its enzymatic activity on *R. hypocrateriformis*. Antioxidant quantification using different successive solvents (petroleum ether, chloroform, ethyl acetate, methanol and water) clearly justifies the presence of total phenols, total tannins and total flavonoids content in *R. hypocrateriformis*. Antioxidant enzymatic activity in *R. hypocrateriformis* analysed the various enzymes such as catalase (6.10 ± 1.12), glutathione reductase (1.80 ± 0.20), peroxidase (3.15 ± 0.13) and superoxide dismutase (2.10 ± 0.48) activity respectively.

KEY WORDS:

Antioxidant, catalase, glutathione reductase, petroleum ether and superoxide dismutase.

INTRODUCTION

Antioxidant research is an important topic in the medicinal field as well as in food industry. Plants and their products are found throughout human history as herbal supplements as botanicals,

nutraceuticals and drugs (Ekor, 2014). Natural antioxidants constitute the essential part in cell's defense mechanisms and they can be endogenous and exogenous which are commonly derived from plant sources and the efficacy is determined by plant species, variety, extraction and processing methods and growing environment (Said *et al*., 2002). Total phenolics, anti-oxidant, anti-tumor and enzyme inhibitory activity of Indian medicinal and aromatic plants has growing tendency in comparing the phytochemical constituents isolated from plants and their pharmacological actions (George and Joseph, 2009).

MATERIALS AND METHODS

Source of the plant sample:

Rivea hypocrateriformis is collected from Kottaram. It is a small village located in Kanyakumari district of Tamil Nadu. This village is used to be a resting place for the Travancore Maharajas. It is very close to Kanyakumari, Vattakottai and Marunthuvazh Malai. It is a mesmerizing beautiful place.

Chemicals:

Various chemicals used were DPPH (1, 1-diphenyl-2-pieryl-hydrazyl) (Sigma Chemicals, USA) and aluminium chloride. Ascorbic acid obtained from Sisco Research Laboratories Pvt. Ltd., India. Folin-Ciocalteu's phenol reagent and sodium carbonate from Merck Chemical Supplies (Damstadt, Germany). All chemicals and solvents were of analytical grade.

Preparation of plant extracts for antioxidants activity

The whole plant dry powder samples of *R. hypocrateriformis* is extracted successively with different solvents such as chloroform, ethyl acetate, petroleum ether, methanol and water. The successive extracts were evaporated to dryness, stored at 4^{o}C and used to determine residues were used for analysing antioxidant compounds and their free radical scavenging activity.

Preparation of plant extracts for antioxidant enzymes

The fresh whole plant material of *R. hypocrateriformis* was homogenized in a pre-chilled mortar and pestle with phosphate buffer (assay buffer diluted 10 times) at 1-4^{o}C and centrifuged. The sediment is stirred with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then the extraction was repeated once or twice. The extraction should not take more than 24hrs. The combined supernatants were used for the various antioxidant enzymes assays.

Determination of antioxidant compounds

To estimate the level of antioxidant compounds in the successive solvent extracts of *R. hypocrateriformis* the following standard methods were used. The flavonoid content was measured according to the method of Zhishen *et al*. (1999). The total phenols and total tannins were determined by using the methods of Siddhuraju and Becker (2003) and Siddhuraju and Manian (2007), respectively.

Total phenolics / Tannins (Siddhuraju and Becker, 2003)

Ten microliter aliquots of the extracts (10 mg/ 2 ml) were taken in test tubes and made up to the volume of 1ml with distilled water. Then 0.5 ml of Folin-Cio-Calteu phenol reagent and 2.5 ml of sodium carbonate solution (20 %) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank in a UV-Spectrophotometer to determine the total phenolic content in the solvent extracts of plant samples, tested. The analysis was performed in triplicate and the results were expressed as tannic acid equivalents.

Using the same extract, the tannins were estimated after treatment with polyvinyl polypyrrolidone (PVPP) (Siddhuraju and Manian, 2007). Hundred mg of PVPP was weighed into a 100 x 12 mm test tube and to this 1ml distilled water and then 1ml of the sample extracts were added. The content was vortexed and kept in the test tube at 4^{o}C for 15 min. Then the sample was centrifuged (3000 rpm for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolic other than tannins (the tannin would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured and expressed as the content of non-tannin phenolics on a dry matter basis. From the above results, the total tannin content in the solvent extracts of the sample was calculated by using the formula: Tannin (%) = Total phenolics (%) – Non-tannin phenolics (%).

Total flavonoid content (Zhishen *et al*., 1999)

About 0.5 ml aliquot of appropriately (10 mg/2 ml) diluted sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5 % $NaNO_2$ solution. After 6 min, 0.15 ml of 10 % $A1Cl_3$ solution was added and allowed to stand for 6 min, and then 2 ml of 4 % NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 ml, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus water blank in a UV-Spectrophotometer to determine the total flavonoid content in the solvent extract of plant samples, tested. The results were expressed as rutin equivalent.

Determination of antioxidant enzymes

Estimation of catalase activity (Luck *et al*., 1974)

The enzyme catalase has a double function and it catalyzes the following:

a. It decomposes hydrogen peroxide to give water and oxygen.

b. It oxidizes H^+ donors, for example methanol, formic acid, phenol with the consumption of one mole of peroxide.

Principle: The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250 nm. On decomposition of hydrogen peroxide by catalase, the absorption values decreases with time. The enzyme activity could be calculated based on this decrease in absorbance. But, this method is applicable only to enzyme solution, which do not absorb strongly at 230-250 nm.

Reagents

- Phosphate buffer 0.067 M (pH 7.0) – Dissolve 3.522 gm of KH_2PO_4 and 7.268 gm of K_2HPO_4. $2H_2O$ in distilled water and made up the volume to one litre.
- Hydrogen peroxide – Phosphate buffer – Dissolved 0.16 ml of H_2O_2 (10% w/v) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution should be about 0.5 at 240 nm with 1 cm light path.

Procedure:

Enzyme extract: The whole plant sample of *R. hypocrateriformis* is homogenized in a pre-chilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1-4^{o}C and centrifuged. The sediment was stirred with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then the extraction was repeated once or twice. The combined supernatant was used for the assay.

Assay: Read against a control cuvette containing 3 ml of H_2O_2 and the enzyme solution as in the phosphate buffer (M/150). Pipetted into the experimental cuvette 3 ml of H_2O_2 phosphate buffer was pipetted into an experimental cuvette and mixed in 0.01-0.04 ml sample with the glass or plastic rod flattened at one end. The time required for a decrease in absorbance from 0.45-0.4 nm was noted. This value was used for calculations. If time (t) was more than 60 seconds, the measurement was repeated with more concentrated solution of the sample. The concentration of H_2O_2 was calculated using the extinction coefficient 0.03 nm mole/ml.

Estimation of peroxidase activity (Reddy, 1995):

Principle: Peroxidase converts H_2O_2 to water and oxygen in the presence of hydrogen donor (pyrogallol or dianisidine) the oxidation of pyrogallol or dianisidine to coloured product called purpurogalli is measured calorimetrically.

Reagents:

- Pyrogallol – 0.05M phosphate buffer (pH 6.5)
- 1 % H_2O_2

Enzyme extract: One gram of the leaf sample of *R. hypocrateriformis* was measured with 5 ml (w/v) 0.1 M phosphate buffer (pH 6.5) in a homogenizer. The homogenate was centrifuged at 300 gm for 15 minutes and the supernatant was used as the enzyme source. All procedures were carried out at 0-5^{o}C.

Procedure: 3 ml of 0.05M pyrogallol solution and 0.5 to 0.1 ml of enzyme extract were taken in a test tube. The spectrophotometer was adjusted to read ‘0’ at 400nm, added 0.5 ml of 1 % H_2O_2 in the test cuvette and the change in the absorbance every 30 seconds up to 3 minutes was recorded.

Estimation of superoxide dismutase activity (Misra and Fridovich, 1972):

Principle: Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating systems and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can be assayed spectrophotometrically.

Reagents:

- 50 mM Potassium phosphate buffer (pH 7.8)
- 45 mM Methionine
- 5.3 mM Riboflavin
- 84 mM Nitro Blue Tetrozolium (NBT)
- 20 mM potassium cyanide

Enzyme extract: One gram of the whole sample of *R.hypocrateriformis* weighed separately with 5 ml (w/v) 0.1M phosphate buffer (pH 6.5) in a homogenizer. The homogenate was centrifuged at 300 gm for 15 minutes and the supernatant was used as the enzyme source. All procedures were carried out at 0-5^{o}C.

Procedure: The incubation medium contained a final volume of 3 ml, 50 mM potassium phosphate buffer (pH 7.8), 45 mM Methionine, 5.3 mM Riboflavin, 84 mM Nitro Blue Tetro;zolium (NBT) and 20 mM potassium cyanide. The tubes were placed in aluminium foil-lined box maintained at 25^{o}C and equipped with 15W fluorescent lamps. Reduced NBT was measured Spectrophotometrically at 600 nm after exposure to light for 10 min. The maximum reduction was evaluated in the absence of the amount of enzyme giving 50 % inhibition of the reduction of NBT.

Assay of glutathione reductase (David and Richard, 1983):

Principle: Glutathione reductase catalyses the conversion of oxidized glutathione or reduced glutathione employing NADPH as a substrate and the amount of NADPH utilized are a direct measure of enzyme activity.

Reagents:

- 0.12 M phosphate buffer, pH 7.2

- 15 mM EDTA
- 10 mM Sodium azide
- 6.3 mM oxidized glutathione
- 9.6 mM NADPH

Procedure:

Aqueous extract (20 %) of the whole plant sample of *R. hypocrateriformis* was prepared in 0.12 M phosphate buffer pH 7.2 and used as the source of the enzyme. The assay system contained 1 ml of 0.12 M potassium phosphate buffer, 0.1 ml of 15 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 6.3 mM oxidized glutathione and 0.1ml of enzyme source and water in the final volume of 2 ml. It was kept for three minutes. Then 0.1 ml of NADPH was added. The absorbance at 340 nm was recorded at an interval of 15 seconds for 2 to 3 minutes. For each series of measurements, controls were maintained with water instead of oxidized glutathione. The enzyme activity was expressed as micro moles of NADPH oxidized/minutes/gm sample.

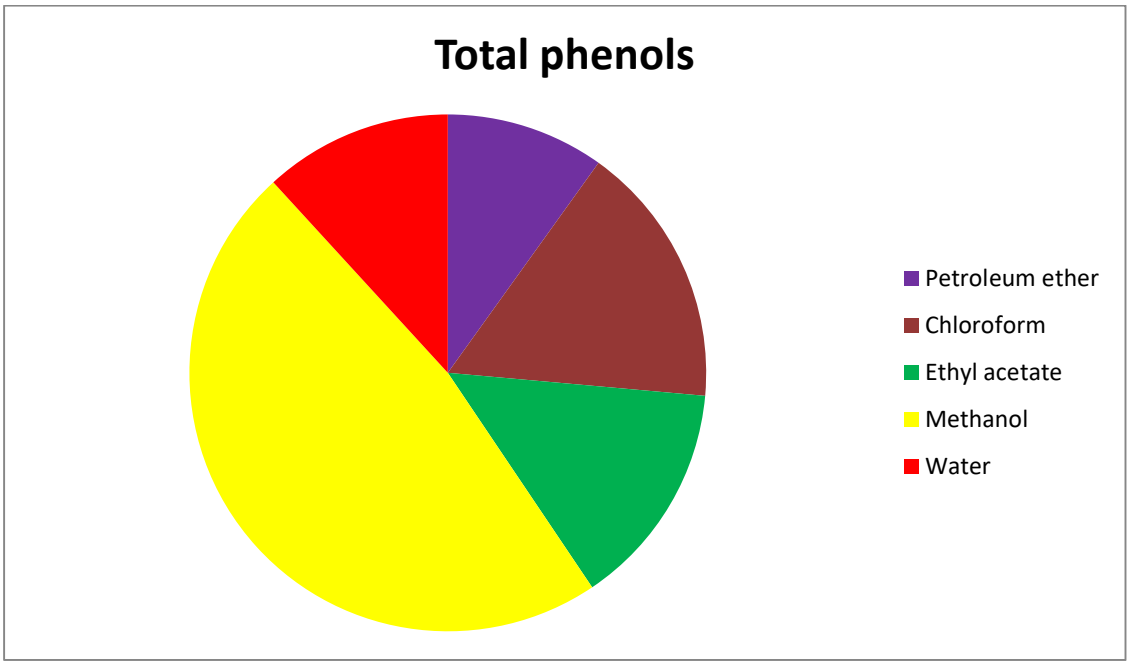
RESULT AND DISCUSSION

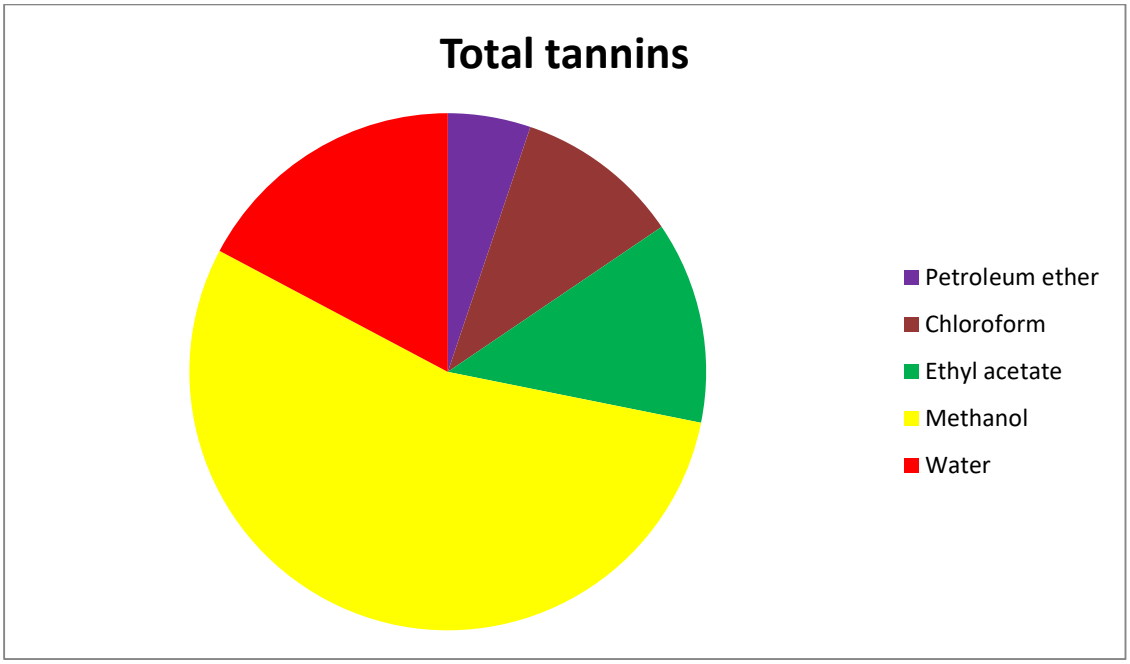
Table 1: Quantification of antioxidants in different successive solvent extracts of *Rivea hypocraterformis* whole plant samples

Whole plant successive solvent used	Amount estimated
1.Total phenols	
Petroleum ether	21.40±5.30
Chloroform	35.60±5.90
Ethyl acetate	30.50±4.00
Methanol	101.10±5.80
Water	25.30±6.10
2.Total tannins:	
Petroleum ether	9.10±2.20
Chloroform	18.00±6.60
Ethyl acetate	22.10±1.44

Methanol	95.60±2.30
Water	30.00±5.10
3.Total flavonoids	
Petroleum ether	1.40±0.01
Chloroform	0.35±0.04
Ethyl acetate	0.40±0.06
Methanol	4.60±2.10
Water	0.30±0.10

Figure 1: Quantification of antioxidants in different successive solvent extracts of *Rivea hypocraterformis* whole plant samples.




Total tannins
Petroleum ether
Chloroform
Ethyl acetate
Methanol
Water

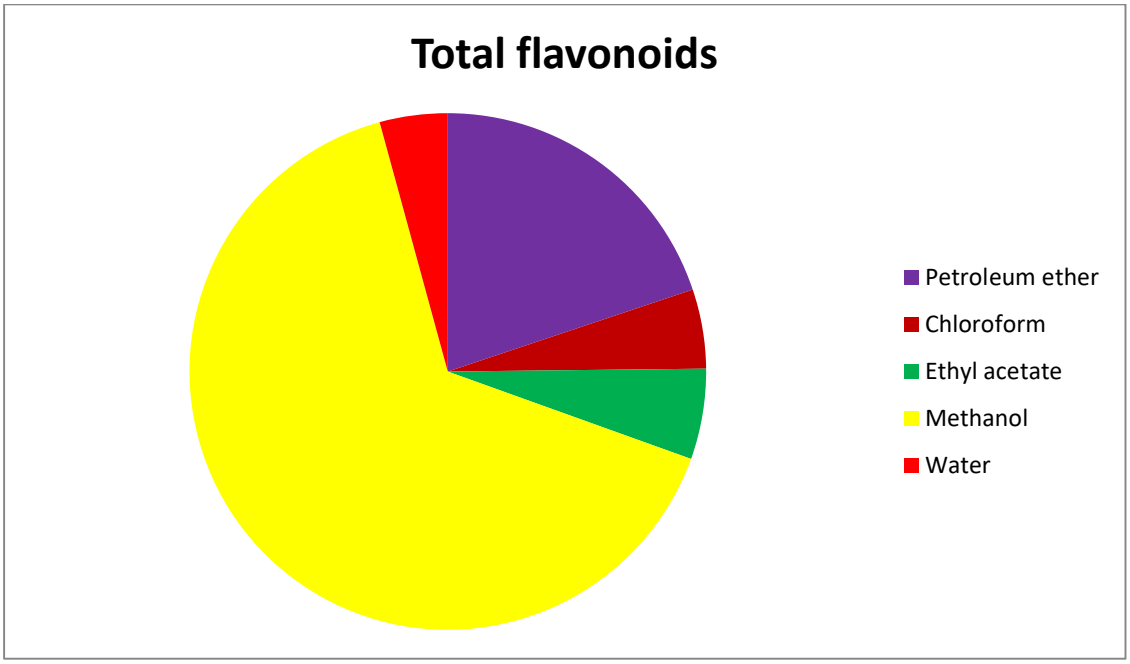
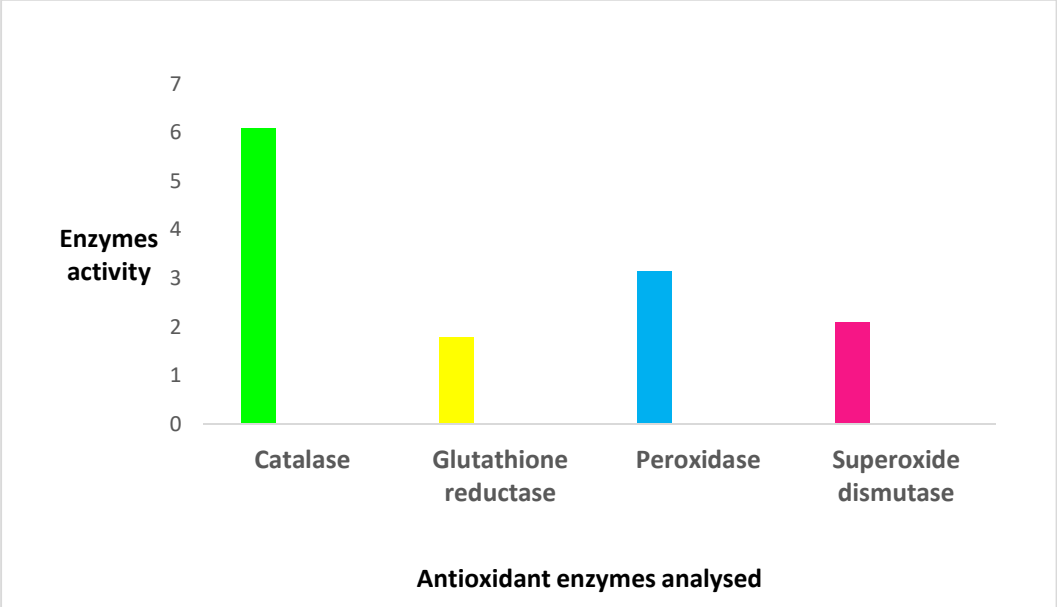

Total flavonoids
Petroleum ether
Chloroform
Ethyl acetate
Methanol
Water

Table 2: Evaluation of antioxidant enzymatic activity in *Rivea hypocrateriformis* whole plant sample

S.No	Antioxidant enzymes analysed	Enzymes activity
1	Catalase	6.10±1.12
2	Glutathione reductase	1.80±0.20
3	Peroxidase	3.15±0.13
4	Superoxide dismutase	2.10±0.48

Figure 2: Evaluation of antioxidant enzymatic activity in *Rivea hypocrateriformis* whole plant sample.



In traditional societies nutrition and health care are strongly interconnected and many plants have been consumed both as food and for medicinal purposes. The consumption of non-cultivated botanicals plays a central role in the diet, but very few ethnopharmacological and phytopharmacological studies have dealt exhaustively with the potential health benefits of such

diets. Quantification of antioxidants in different successive solvent extracts (petroleum ether, chloroform, ethyl acetate, methanol and water) of *R. hypocrateriformis* whole plant sample shows the presence of total phenol, total tannins and total flavonoids content and are presented in the table- 1 & figure- 1.

The results indicated that the total phenol content in the whole plant successive extracts of *R. hypocrateriformis* varied from 21.40 ± 5.30 to 35.60 ± 5.90 to 30.50 ± 4.00 to 101.10 ± 5.80 to 25.30 ± 6.10 mg/TAE/g extracts respectively. Methanol extract of *R. hypocrateriformis* showed the maximum total phenol content while, the petroleum ether extract showed the minimum total phenol content. The total phenol content in *R. hypocrateriformis* of the various successive solvent extracts is shown in the following descending order: methanol > chloroform > ethyl acetate > water > petroleum ether.

The total tannin content in the successive whole plant extract of *R. hypocrateriformis* was quantified and the data are shown in the table-1 & figure- 1. The total tannin content in the whole plant successive extracts of *R. hypocrateriformis* varied from 9.10 ± 2.20 to 18.00 ± 6.60 to 22.10 ± 1.44 to 95.60 ± 2.30 and 30.00 ± 5.10 mg/TAE/g extract respectively. Maximum tannin content was noted in the methanol extract of *R. hypocrateriformis* and the petroleum ether shows the minimum tannin content. Based on the concentration, the total tannin content of the solvent extracts are arranged in the following ascending order: petroleum ether < chloroform < ethyl acetate < water < methanol.

The total flavonoid content in the whole plant successive extracts of *R. hypocrateriformis* was estimated and the data are recorded in the table-1 & figure-1. The total flavonoid content in the whole plant successive extracts of *R. hypocrateriformis* varied from 1.40 ± 0.01 to 0.35 ± 0.04 to 0.40 ± 0.06 to 4.60 ± 2.10 and 0.30 ± 0.10 mg/TAE/g extract respectively. Among the five solvent extracts analysed, methanol extract shows the highest tannin content and the water extract shows the least flavonoid content. Based on the concentration, the total flavonoid content of the solvent extracts is arranged in the following descending order: methanol > petroleum ether > ethyl acetate > chloroform > water. Among the quantification of three antioxidant estimation using various solvent extracts, methanol extract shows the maximum value which is clearly tabulated in the table- 1.

The antioxidant enzymatic activity of the selected whole plant extract illustrates four enzymes activity such as catalase (6.10 ± 1.12 mg), glutathione reductase (1.80 ± 0.20 mg),

peroxidase (3.15 ± 0.13) and superoxide dismutase (2.10 ± 0.48). Among the four enzymes analysed for antioxidant activity, catalase enzyme shows maximum activity followed by peroxidase, superoxide dismutase and glutathione reductase respectively noted in the table- 2 & figure- 2.

The antioxidant activity is mainly due to phenolic components such as phenolic acids and phenolic diterpenes (Shahidi *et al*., 1992). It is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxidises (Osawa, 1994). Earlier authors (Tanaka *et al*., 1988) have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Duh *et al.*, 1999), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). The methanolic and aqueous extracts of *Lavandula stoechas* exhibited *invitro* antioxidant activity, but the latter was the most potent (Karabagias *et al*., 2019).

Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans *et al*., 1997; Jorgensen *et al*., 1999). Superoxide dismutase is a large protein consisting of two main domains (Dehury *et al.*, 2013). Catalase does not require any reductant for its catalytic activity (Oshino *et al*., 1975). Peroxidase enzyme work in the extracellular space for scavenging H_2O_2 (Hiraga *et al.,* 2001). Glutathione peroxidase is derived from the three amino acids such as glutamate, cysteine and glycine (Passaia *et al*., 2015).

The root and leaf extracts of *Anchusa italica* (Retz) exert significant antioxidant activity because of the high content of flavonoids and polyphenols (Mostafa *et al*., 2022). The antioxidant activity of the ethyl acetate extract of *Globularia alypum* L. shows higher antioxidant activity, namely IC50 = 12.3 ± 3.83 µg/ml for DPPH and 53.1 ± 3.83 µg/ml for FRAP assay (Fadou *et al*., 2021).

CONCUSION

Antioxidant, free radical scavenging and enzymatic activity of *R. hypocrateriformis* demonstrated highest reducing power. The plant shows shows significant antioxidant activity which is attributed to its high phenolic content. Therefore, the selected plant is regarded as

excellent source for bioactive compounds than can be further developed into drugs to combat oxidative stress.

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