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IN-VITRO EVALUATION OF THE TOTAL PHENOLIC, FLAVONOID CONTENTS AND FREE RADICAL SCAVENGING POTENTIAL OF *RUMEX HASTATUS*

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Keywords:

Free radical scavenging, Total phenolic content, Total flavonoid content, DPPH, *Rumex hastatus*

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ABSTRACT: Objective: The aim of this study was to screen various solvent extracts of leaves of *Rumex hastatus* to display *in-vitro* antioxidant activity, total phenolic, and flavonoid contents in order to find possible sources for future novel antioxidants in food and pharmaceutical formulations. **Materials and Methods:** A detailed study was performed on antioxidant activity of various *Rumex hastatus* leaves extracts like petroleum extract (PERH), chloroform extract (CERH), ethyl acetate extract (EAERH), methanolic extract (MERH) and aqueous extract (AERH) by *in-vitro* chemical analysis like DPPH, Reducing power, metal chelating, hydrogen peroxide and nitric oxide free radical scavenging assay. All these extracts were also subjected to a preliminary phytochemical screening test for various constituents. The total yield, total phenolic, and total flavonoid contents of all extracts were also determined. **Results:** Phytochemical screening shows the presence of triterpene, flavonoids, polysaccharides, steroids, tannins, cardiac glycoside, reducing sugar, and saponins in all extracts. The MERH exhibited the highest total phenolic content (121.7 ± 5.95 mg of GAE/g extract) and total flavonoid content (165.9 ± 3.99 mg of QE/g extract). IC_{50} values of ascorbic acid and MERH were found in DPPH (36.64 ± 0.53 µg/ml and 46.78 ± 0.44 µg/ml), metal chelating activity (45.81 ± 0.41 µg/ml and 53.94 ± 0.43 µg /ml), H_2O_2 activity (33.83 ± 1.92 µg/ml and 63.67 ± 1.34 µg/ml) and nitric oxide assay (62.95 ± 0.93 µg/ml and 80.87 ± 1.70 µg/ml) respectively. **Conclusion:** The results of the present study revealed that *Rumex hastatus* leaves act as an antioxidant agent due to its free radical scavenging activity.

INTRODUCTION: *Rumex hastatus* is a bushy shrub belonging to the Polygonaceae family, commonly known as 'khatimal' is a perennial shrub, widely distributed in India in Himachal Pradesh, Jammu and Kashmir, Uttaranchal and Kumaun [1].

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The name Rumex L. originated from "rums" (to suck) alluding to the habit of Romans sucking the leaves to allay thirst [2]. *R. hastatus* is historically taken for the treatment of sexually transmitted diseases, including AIDS, laxative, tonic agent, diuretic, against rheumatism, skin diseases, piles, bleeding of the lungs, cough, headache, fever [3-11].

The juice of the plant is employed for blood pressure, whereas the powder of roots is beneficial for abdominal pain. Leaves have a pleasing acidic taste and utilized in chutneys and pickles [12]. Anile part and contemporary tuber are utilized in

tonsillitis and sore throat [5, 13]. The main chemical constituents which are reported from the plant belong to various classes *viz*; Nepalin, nepodin, rumicin, anthraquinones, naphthalenes, flavonoids and phenolic compounds [4, 9-18]. Antioxidants from the natural original are extensively on use over the past years. Most diseases like cardiovascular diseases, inflammation, cancer, osteoporosis, degenerative diseases, *etc.* are coupled with Reactive Oxygen Species (ROS) production, Reactive Nitrogen Species production, and oxidative stress theory [19-21].

The free radicals mainly act by attacking the unsaturated fatty acid in the biological membranes, which extend to membrane lipid peroxidation, decrease in membrane fluidity and reduction of antioxidant defense enzymes, receptor activity, and damage to a membrane protein. These destructive processes finally trigger the cell inactivation or death [22, 23]. Antioxidants, therefore, can be used to reverse the harmful and pathological effects of the free radicals. The antioxidants generally scavenge the free radicals and detoxify the physiological system. Free radicals are normally generated during normal body metabolic function and also can be acquired from the environment. Free radicals can be oxygen radicals, such as superoxide radical (O_2-), hydroxyl radical (•OH), and non-free radical species, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($•O_2$) and are generated in many redox processes [14].

Superoxide dismutase, catalase, and glutathione peroxidase *etc*. are the enzymes of antioxidant defense system which trap and destroy these free radicals level of antioxidant defense enzymes, excess production of free radicals along with vitamin deficiency and increased lipid peroxidation are considered as the perpetrators for producing oxidative stress and associated with various pathological [23, 25, 26]. Local communities and folklore healers throughout the world use a wide range of plants and their parts for their medicinal properties. A large population from folk and tribal communities still uses a variety of plants for medicinal purposes due to either lack of advanced health care facilities in their remote regions or their traditional belief along with the success rate of these traditional medicines in different disease conditions. Moreover, a large number of bioactive drugs currently in use in the modern system of medicine came from medicinal plants only. Medicinal plants are now considered as potential source for new bioactive leads or drugs. The present study was designed to study the *in-vitro* antioxidant activities of extracts of leaves of *Rumex hastatus*. Preliminary phytochemical screening of the methanol extract was also done along with the determination of total phenolic and flavonoid content for a clear justification of the ayurvedic and traditional folklore use of this plant in liver disorders.

MATERIALS AND METHODS:

Plant Materials: The leaves of *Rumex hastatus* were collected from Mandi district of Himachal Pradesh in India and authenticated by routine pharmacognostic procedures by Dr. Suresh Kumar, Assistant Professor (Botany), HOD, Abhilashi Institute of Life Sciences, Nerchowk. A voucher specimen was retained and deposited at the crude drug repository of the herbarium of AILS, vide CDR accession no. AILS/3/RN/2011.

Drugs and Chemicals: Chemicals, such as Folin-Ciocalteau reagent, trichloroacetic acid (TCA), methanol, ammonium thiocyanate, dimethyl-sulphoxide (DMSO), gallic acid, butylated hydroxyanisole (BHA), were purchased from E. Merck (India) Limited. DPPH was procured from Sigma Chemicals Company, St Louis, MO, USA. Petroleum ether, chloroform, ethyl acetate methanol, gallic acid, N-(1-naphthyl) ethylene-diamine dihydrochloride, trichloroacetic acid, ferrozine, ascorbic acid and all other chemicals and solvents used were of analytical grade available commercially (SRL Mumbai, Himedia, E. Merck India).

Preparation of Extracts: The leaves of *Rumex hastatus* were dried in shade and coarsely powdered. Leaves 1 kg was successively extracted in the Soxhlet apparatus using petroleum ether (PERH), chloroform (CERH), ethyl acetate (EAERH), methanol (MERH) and water (AERH) as solvent for the complete extraction of the phytochemicals. The five extract were dried in Rota Evaporator at 45 °C and then lyophilized.

Preliminary Phytochemical Screening: Preliminary phytochemical screening for identification of the chemical constituents were carried out on the

methanol extract using chemical methods according to the methodology proposed elsewhere [27, 28].

Determination of Total Phenolic Content: Total soluble phenolic content in MERN was determined by using Folin–Ciocalteau method using gallic acid as a standard phenolic compound [29]. About 1.0 ml of extract (10 mg of extract) was diluted with 46 ml of distilled water in a volumetric flask. Folin-Ciocalteau reagent (1 ml) was added to the above mixture and mixed thoroughly. Three minutes later, 3.0 ml of 2% sodium carbonate was added, and the mixture was allowed to stand with intermittent shaking for 3 h.

The absorbance of the mixture was measured at 760 nm in a spectrophotometer (UV-1800 Shimadzu, Japan). The concentration of total phenols was expressed as mg/g of extract. The concentration of total phenolic compounds in the extract was expressed as gram of gallic acid equivalent (GAE) using an equation obtained from the equation of the regression line of standard gallic acid graph:

$$y = 0.008\ x - 0.032,\ r_2 = 0.997$$

Where y was the absorbance, and x was the concentration.

Total Flavonoid Content: A total of five extracts from the leaves of *Rumex hastatus* were used in the present study. The extracts and a quercetin standard were used for the determination of the flavonoid content by modified $AlCl_3$ method [30]. The crude extracts and quercetin standard (10-50 µg/ml) was added to.

Separately, 0.5 ml of each dilution was taken in a test tube and mixed with diluted with 1.5 ml methanol (95%), 0.1 ml of aluminum chloride (10% w/v), 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, absorbance of the mixture was noted at 415 nm on UV/Vis Spectro-photometer. The amount of aluminum chloride (10%) was substituted by the same amount of distilled water in the blank. The amount of total flavonoids was calculated as quercetin equivalents from the calibration curve of standard quercetin solution and expressed as milligram quercetin equivalents (mg QE)/g of extract using an equation obtained from the equation of regression line of standard quercetin graph:

$$y = 0.004\ x + 0.002,\ r_2 = 0.985$$

Where y was the absorbance, and x was the concentration.

***In-vitro* Antioxidant Activity:**

DPPH Radical Scavenging Activity of all the Successive Solvent Extracts: The free radical scavenging activity of the extract was measured by DPPH• using the method described previously [31]. A 0.1 mM solution of DPPH• in methanol was prepared, and 1 ml of this solution was added to 3 ml of extract solution in water at different concentrations (10-80 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer. The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH• scavenging effect (\%)} = 100 - [(A_0 - A_t / A_0) \times 100]$$

Where A_0 was the absorbance of the control reaction, and A_t was the absorbance in the presence of the standard sample or extract. All the tests were performed in triplicate, and the graph of IC_{50} was plotted with the mean ± SEM values. Ascorbic acid was used as a standard antioxidant compound.

Reducing Power Assay: The reducing power of the extract was determined according to the method described previously [32]. The different concentrations of extracts (20-100 µg/ml) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)6$] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer (UV -1800 Shimadzu, Japan). Higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a standard antioxidant compound.

Metal Chelating Activity: The chelating of ferrous ions by the methanolic plant extract was measured by the method described previously [33, 34]. Different concentrations of the MERH and EAERH (20-100 µg/ml) were added to a solution of $FeCl_2$ (0.05 ml, 2 mM). Then the reaction was initiated by the addition of 5 mM ferrozine (0.2 ml). The reaction mixture was then shaken vigorously and allowed to stand at room temperature for 10 min. The absorbance of the solution was then measured at 562 nm. The percentage inhibition of ferrozine-ferrous complex formation was calculated by using the following formula:

$$\% \text{ Inhibition} = (A_0 - A_t / A_0) \times 100$$

Where A_0 was the absorbance of the control (without extract) and A_t was the absorbance in the presence of the extract or standard. All the tests were performed in triplicate, and the graph of IC_{50} was plotted with the mean ± SEM values. Ethylenediaminetetraacetic acid (EDTA) was used as a standard chelating compound.

H_2O_2 Activity: The hydrogen peroxide-scavenging ability of examined extracts was determined according to the method of Ruch RJ35. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentrations of the MERH and EAERH (20-100 µg/ml) were added to 3.4 ml of phosphate buffer, together with 0.6 ml of H_2O_2 solution. The absorbance value of the reaction mixture was recorded at 230 nm. The blank solution contained only the phosphate buffer (4 ml). The percentage of H_2O_2 scavenging of examined extracts was calculated as

$$\% \text{ of scavenged } H_2O_2 = [(A_0—A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control (phosphate buffer with H_2O_2), and A_1 is the absorbance of the examined extracts. All the tests were performed in triplicate, and the graph of IC_{50} was plotted with the mean ± SEM values. Ethylenediaminetetraacetic acid (EDTA) was used as a standard chelating compound.

Nitric Oxide Radical (NO•) Scavenging Activity: Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by the Griess reaction [36]. The reaction mixture (1 ml) containing sodium nitroprusside (10 mM, 0.5 ml), phosphate buffer saline (0.25 ml) and various concentrations of Different concentrations of the MERH and EAERH (20-100 µg/ml) were incubated at 25 °C for 150 min.

After incubation, 0.25 ml of the reaction mixture mixed with 0.5 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 0.5 ml of naphthyl ethylenediamine dihydrochloride (0.1%) was added, mixed, and allowed to stand for 30 min at 25 °C. A pink colored chromophore is formed in diffused light. The absorbances of these solutions were measured at 540 nm against the corresponding blank solutions. Ascorbic acid (20-100 µg/ml) was used as a positive control. The NO• scavenging activity of MERH and EAERH are reported as % inhibition and was calculated as above.

RESULTS AND DISCUSSION:

Yield of Extracts: The extractive yields of the petroleum ether, chloroform, ethyl acetate, methanol and water extracts of the leaves of *Rumex hastatus* were 3.34%, 2.01%, 1.96%, 13.33% and 12.65% respectively **Table 2**. The highest amount of total extractable compounds was in the methanol extract, and the lowest was in petroleum ether.

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING

S. no.	Constituents	PERH	CERH	EAERH	MERH	AERH
1	Alkaloids	-	-	-	-	-
2	Saponins	-	-	√	√	-
3	Sterols	√	-	-	√	√
4	Carbohydrates	-	-	√	√	√
5	Tannins	-	√	√	√	√
6	Flavanoids	-	-	√	√	√
7	Fatty acid	√	-	-	-	-
8	Terpenoid	-	-	-	√	-
9	Glycosides	-	-	√	√	-

Preliminary Phytochemical Screening: All leaves extracts were subjected to preliminary phyto-chemical screening using different chemical methods and the results showed the presence of triterpene, flavonoids, polysaccharides, steroids, tannins, cardiac glycoside, reducing sugar and saponins. The test for alkaloid showed negative result in **Table 1**.

Determination of Total Phenolic Content: The total phenolic contents of the leaves extracts were evaluated using a modified Folin-Ciocalteu reagent method reported by Slinkard, K [28]. Due to their hydroxyl groups, phenols and phenolics are very important phytoconstituent with free radical scavenging ability. But the antioxidant effects do not necessarily always correlate with the presence of large quantities of phenolics and related compounds [28]. MERH, EAERH, AERH, CERH, and PERH were also subjected to assay for total phenolic content. The MERH exhibited the highest total phenolic content (121.7 ± 5.95 mg of GAE/g extract), followed by EAERH (82.21 ± 1.54 mg of GAE/g extract), AERH (51.96 ± 1.02 mg of GAE/g extract), CERH (20.33 ± 0.58 mg of GAE/g extract) and PERH (8.79 ± 1.81 mg of GAE/g extract). Gallic acid was used as a standard for the calculation of total phenolic content in **Table 2**.

Determination of Total Flavonoid Content: The determination of the total flavonoid content of the leaves extracts was estimated using a modified $AlCl_3$ method Lin, J [30]. The total flavonoid content of different extracts of the leaves of *Rumex hastatus* are also presented in **Table 2**. The MERH exhibited the highest total flavonoid content (165.9 ± 3.99 mg of QE/g extract), and the lowest was that of PERH **Table 2**. Quercetin (QE) was used as a standard for the calculation of flavonoid content.

TABLE 2: TOTAL PHENOLIC CONTENT AND TOTAL FLAVONOID CONTENT

Plant	Solvent used for extraction	Total phenol content (mg GAE/g of extract)	Total flavonoid content (mg QE/g of extract)	Yield (%)
Rumex hastatus	Petroleum Ether	8.79 ± 1.813	20.33 ± 1.302	3.34
Rumex hastatus	Chloroform	20.33 ± 0.579	28.67± 1.557	2.01
Rumex hastatus	Ethyl Acetate	82.21 ± 1.542	88.42 ± 1.949	1.96
Rumex hastatus	Methanol	121.7 ± 5.947	165.9 ± 3.99	13.33
Rumex hastatus	Water	51.96 ± 1.01	47.17 ± 0.961	12.65

Data are expressed as Mean ± SEM; n = 3

***In-vitro* Antioxidant Activity:**

DPPH Radical Scavenging Activity of all the Successive Solvent Extracts: Overproduction of free radicals beyond the body's ability to protect itself by antioxidant defense a system causes oxidative stress and forms the biological basis of chronic condition [37]. Results from this study revealed MERH as a powerful free radical scavenger, which can block the free radical-induced damage in the physiological system. Measuring the scavenging capability of stable DPPH radical for evaluation of antioxidant activity *in-vitro* is a widely used quick method compared to other methods. DPPH is a stable free radical, and it accepts an electron or hydrogen radical to become a stable diamagnetic molecule. A decrease in absorbance at 517 nm by scavenging of DPPH radicals by antioxidants was observed. Hence, DPPH is unremarkably used as a substrate to evaluate the antioxidant activity of different antioxidants. In this present experimental setup, Ascorbic acid was used as a standard radical scavenger. The results for PERH, CERH, EAERH, MERH, and AERH are shown in **Fig. 1**, respectively.

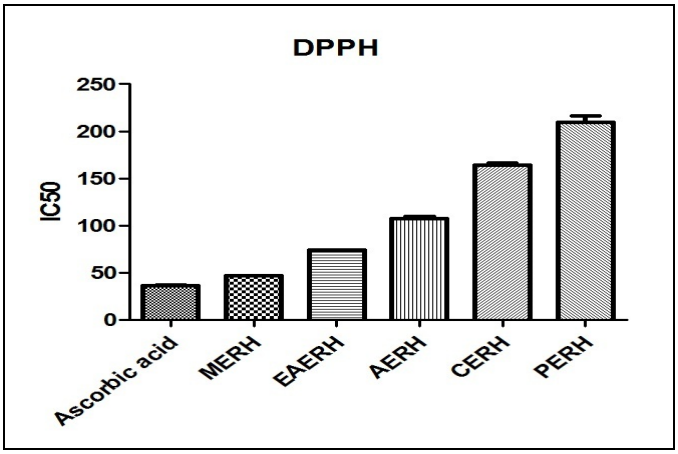

FIG. 1: DPPH SCAVENGING POTENTIAL OF DIFFERENT EXTRACTS. Data are expressed as Mean ± SEM; n = 3

The investigation showed that MERH has more ability to scavenge free radicals than other extracts. The IC_{50} values of the extracts and Ascorbic acid

were calculated using the linear regression equation. The calculated IC_{50} values of ascorbic acid and MERH were found to be 36.64 ± 0.53 µg/ml and 46.78 ± 0.44 µg /ml, respectively.

Reducing Power Assay: The reducing power of all extracts compared to Ascorbic acid is shown in **Fig. 2**. In the reducing power measurement, Fe^{3+}-Fe^{2+} transformation in the presence of extracts samples was investigated using the method of Oyaizu [32]. The reducing power of the extracts was found to be increased with increasing concentration of the extracts. The MERH shows higher reducing power than other extracts at all the concentrations under investigation and less than ascorbic acid. The reducing power of the extracts and standard ascorbic acid follow the order: Ascorbic acid > MERH > EAERH > AERH > CERH > PERH **Fig. 2**.

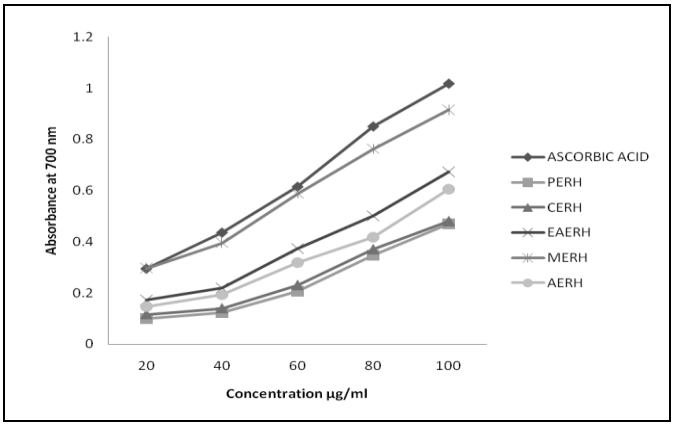

FIG. 2: REDUCING POWER ASSAY OF DIFFERENT EXTRACTS. Data are expressed as Mean ± SEM; n = 3

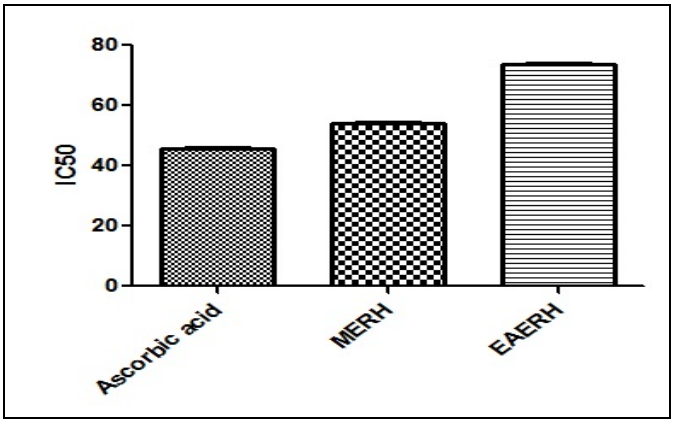

FIG. 3: METAL CHELATING ACTIVITY OF DIFFERENT EXTRACTS. Data are expressed as Mean ± SEM; n = 3

Metal Chelating Activity: The investigation showed that MERH has more ability to scavenge free radicals than EAERH **Fig. 3**. The IC_{50} values of the MERH, EAERH, and Ascorbic acid were calculated using the linear regression equation. The calculated IC_{50} values of ascorbic acid, MERH, and EAERH were found to be 45.81 ± 0.41 µg/ml, 53.94 ± 0.43µg/ml and 73.85 ± 0.27 µg/ml, respectively.

H_2O_2 Activity: Hydrogen peroxide gives rise to hydroxyl radicals. Removing hydroxyl radicals (·OH) is essential for the protection of the living system as they react with most biomolecules and other cellular components to cause tissue damage leading to cell death [38, 39]. The investigation showed that MERH has more ability to scavenge free radicals than EAERH **Fig. 4**. The IC_{50} values of the MERH, EAERH, and Ascorbic acid were calculated using the linear regression equation. The calculated IC_{50} values of ascorbic acid, MERH, and EAERH were found to be 33.83 ± 1.92 µg/ml, 63.67 ± 1.34 µg /ml and 114.5 ± 3.38 µg/ml, respectively.

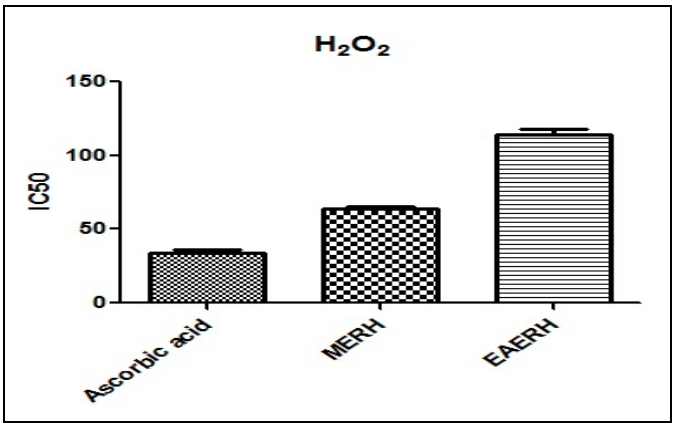

FIG. 4: METAL CHELATING ACTIVITY OF DIFFERENT EXTRACTS. Data are expressed as Mean ± SEM; n = 3

Nitric Oxide Radical (NO•) Scavenging Activity: Nitric oxide or reactive nitrogen species (RNS) are very reactive and cause severe pathological changes in the cellular structure and functional behavior of cellular components [40]. In this experimental design, nitric oxide scavenging by MERH and EAERH were evaluated by observing the reduction of linear time-dependent nitrite production in the sodium nitroprusside-PBS system. All the concentrations of the plant extract (20-100 µg/ml) were found to possess the nitric oxide scavenging activity. The nitric oxide scavenging activity of MERH was found to be lower than ascorbic acid and higher than EAERH.

The IC_{50} values of the MERH, EAERH, and Ascorbic acid were calculated using the linear regression equation. The calculated IC_{50} values of ascorbic acid, MERH, and EAERH were found to be 62.95 ± 0.93 µg/ml, 80.87 ± 1.7 0µg /ml and 117.9 ± 3.74 µg/ml, respectively **Fig. 5**.

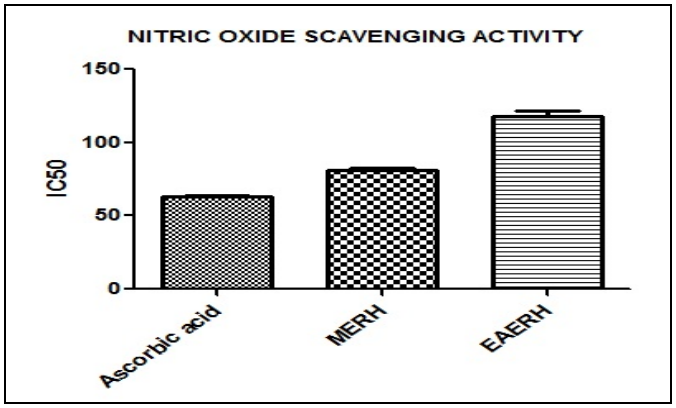

FIG. 5: METAL CHELATING ACTIVITY OF DIFFERENT EXTRACTS. Data are expressed as Mean ± SEM; n = 3

CONCLUSION: Based on the results from different *in-vitro* antioxidant models, it is evident that MERH has an effective and considerable antioxidant profile. The possible mechanism of action for these different antioxidant activities includes hydrogen donating ability, reducing ability, scavenging ability of DPPH, hydrogen peroxide, and nitric oxide radical. The presence of different phytoconstituents in MERH may be responsible for the antioxidant mechanisms. The preliminary phytochemical investigation and previous phytochemical work also suggested several phytoconstituents with potential antioxidant activity. It is therefore concluded that *Rumex hastatus* can be a good source of natural antioxidants. The results of this study are supportive of the usefulness of this plant in the Indian system of medicine and also its use as a functional food.

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CONFLICTS OF INTEREST: We declare that we have no conflicts of interest.

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