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PHYTOCHEMICAL SCREENING AND ANTIOXIDANT POTENTIAL OF ROOT OF *MENTHA ARVENSIS* L. FROM KASHMIR REGION

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Mentha arvensis, DPPH, Reducing power, Nitrous oxide, Metal chelating, H_2O_2

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ABSTRACT: To evaluate phytochemical constituents and antioxidant potential of aqueous and hydroalcoholic extracts of root of *Mentha arvensis* L from Kashmir region. The antioxidant activity of aqueous and hydroalcoholic extracts of *Mentha arvensis* L was evaluated by using 1, 1-diphenyl, 2-picrylhydrazyl (DPPH) scavenging, reducing power, metal chelating, hydrogen peroxide scavenging and nitrous oxide scavenging assays. The total phenolic content (mg/g) was found to be 9.12 and 211.11 mg/g in aqueous and hydroalcoholic extracts respectively and total flavonoid content (mg/g) was found to be 32.14 and 230.18 mg/g in aqueous and hydroalcoholic extracts respectively. The percentage inhibition values of DPPH scavenging for aqueous and hydroalcoholic extracts was found to be 35.83 and 57.63 respectively. The percentage inhibition values of Nitrous oxide scavenging for aqueous and hydroalcoholic extracts was found to be 39.11 and 63.25 respectively. The percentage inhibition values of H_2O_2 scavenging for aqueous and hydroalcoholic extracts was found to be 21.72 and 39.66 respectively. The percentage inhibition values of metal chelating activity for aqueous and hydroalcoholic extracts was found to be 46.1 and 65 respectively. The reducing power of aqueous and hydroalcoholic extracts was found to be 0.75 and 1.48 respectively at concentration of 0.25mg/ml. The results indicate that the hydroalchol extract of root of *Mentha arvensis* L has good antioxidant potential than aqueous extract and it can be regarded as promising candidates for natural plant sources of antioxidants with high.

INTRODUCTION: Reactive oxygen species (ROS) are continuously produced by human body such as superoxide anion radical, hydroxyl radical and hydrogen peroxide by many enzymatic systems through oxygen consumption. The production of large amounts of these ROS may be dangerous because of their high reactive nature towards numerous molecules such as proteins and lipids thereby causing number of disorders in humans including atherosclerosis, arthritis, cellular aging, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [1, 2].

Enzymes such as superoxide dismutase, catalase, and glutathione peroxidise and also non-enzymatic counterparts such as glutathione, ascorbic acid, and α-tocopherol act as antioxidant-defence mechanisms

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of the body. Non-nutritive phytochemicals such as carotenoids, alkaloids, vitamins, minerals, flavonoids and other phenolics which also possess antioxidant activity and may protect body against free radical damage[3]. During injury the increased production of reactive oxygen species results in consumption and depletion of the endogenous scavenging compounds. Flavonoids may have an additive effect to the endogenous scavenging compounds by interfering with different free radical producing systems and hence, can prevent injury caused by free radicals in various ways. One way is the direct scavenging of free radicals.

Flavonoids act as electron donors towards radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical. Some flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the peroxynitrite which is highly reactive oxygen-derived radical. Flavonoids can inhibit LDL oxidation in *vitro* by scavenging radicals [4].

The evidence that ROS cause various disorders has brought the attention of scientists to an appreciation of antioxidants for prevention and treatment of diseases, and maintenance of human health [5]. Inherent antioxidative mechanism and many of the biological functions such as the antimutagenic, anti-carcinogenic, and anti-aging responses originate from this property are present in human body [6, 7].

Antioxidant compounds deactivate free radicals, often before they attack targets in biological cells [8]. Recently there is increase in interest in naturally occurring antioxidants for use in food, cosmetic and pharmaceutical products, because they possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance [9, 10].

Mentha arvensis Linn belonging to family Lamiaceae is native to the temperate regions of Europe and western and central Asia, east to the Himalaya and eastern Siberia, and America. It is an herbaceous perennial plant growing to 10–60 cm (rarely to 100 cm) tall. The leaves are in opposite pairs, simple, 2–6.5 cm long and 1–2 cm broad, hairy, and with a coarsely serrated margin. The flowers are pale purple (occasionally white or pink), in clusters on the stem, each flower 3–4 mm long. The plant is widely distributed throughout India and leaves of the plant are extensively used in traditional system of medicine for various ailments like carminative, digestive, expectorant, cardiotonic, diuretic, dentifrice, jaundice, hepatalgia, inflammation of liver, peptic ulcer, diarrhoea, bronchitis and skin diseases [11-14].

The plant has been shown to possess anti – inflammatory and sedative – hypnotic activity [15], hepatoprotective and antioxidant activity [16], antibacterial [17] and antifertility action [18]. The plant consist essential oils of monoterpenes like menthol, menthone, carvone and pulegone major constituents. This plant also possesses anti-Candida [19] and also radio protective activity against gamma radiation [20].

In Kashmir, the powder of aerial parts mixed with dilute curd is given to cure cough, sore throat, indigestion and constipation [21,] also the leaves are used in the treatment of Diarrhoea and Asthma [22]. The objective of this study is to evaluate the hydroalcoholic and aqueous extracts of the root of *Mentha arvensis* for their antioxidant potential.

MATERIALS AND METHODS:

Collection of Plant material and preparation of various extracts of *M. arvensis*: The plant *Mentha arvensis* L**.** was collected in July-August 2012 from the fields and orchids of Narabal, Budgam, J&K. The plant was authenticated by the centre of Plant taxonomy, Department of Botany, University of Kashmir, Hazratbal. The plant (Root) material (500 g) was dried under shade and crushed to coarse powder and the powdered drug material was taken in a percolator for (cold extraction) successive extraction using hydroalcoholic (water: methanol, 1:1) and aqueous solvents. The different fractions were dried under reduced pressure to get the crude dried fractions. The yield of dried fractions of hydroalchol and aqueous extracts of root of *Mentha arvensis* was 13.5 and 16.7 gm respectively.

Source of Chemicals: All the chemicals were purchased from a local dealer and were HiMedia Laboratories Pvt. Ltd. Mumbai, India made.

Phytochemical evaluation: Various chemical tests were carried out on above two extracts using standard procedures to identify the constituents

such as alkaloids, glycosides, phenolics, terpenoids and steroids, flavonoids, saponins, carbohydrates, proteins, fats and tannin. The results for presence of various constituents in aqueous and hydroalcoholic extracts of root of *Mentha arvensis* L. are given in **Table 1**.

Tannins: To 0.5 ml of extract solution 1 ml of water and 1-2 drops of ferric chloride solution was added. Blue colour was observed for Gallic tannins and green black for catecholic tannins [23].

Alkaloids: Alkaloid solution produces white yellowish precipitate when few drops of Mayer's reagents are added [24]. Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent [25]. The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation.

Saponins: 20 ml Water is added to 150mg extract and shaken vigorously; layer of foam formation indicates the presence of Saponins [26].

Glycosides: To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid are added, and observed for a reddish brown coloration at the junction of two layers and the bluish green colour in the upper layer [26].

Terpenoids and Steroids: Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet color was observed for terpenoids and green bluish color for steroids [26].

Flavonoids: 2 g plant material was extracted in 10 ml alcohol or water. To 2 ml filtrate few drops of concentrated HCl followed by 0.5 g of zinc or magnesium turnings was added. After 3 minutes magenta red or pink colour indicated the presence of flavonoids [27].

Phenolics: To 2 ml of alcoholic or aqueous extract, 1 ml of 1% ferric chloride solution was added. Blue or green colour indicates phenols [28].

Carbohydrates: To 2ml of test solution add 2-3 drops of Molish reagent; add 2ml of conc. H_2SO_4 along the sides of test tube to form two layers. Violet ring at the junction of two liquids indicate the presence of carbohydrates [29].

Proteins: To 2ml of test solution add 2ml of 4% NaOH, to this add few drops of biuret reagent .Violet or pink colour indicates the presence of proteins [30].

Fats & oils: 1 ml of the extract was added to a filter paper. These extract was allow it for evaporation on filter paper and the appearance of transparency on filter paper indicates the presence of fats &oils [31].

TABLE 1: SHOWING PRESENCE OF VARIOUS PHYTOCONSTITUENTS.

Tests	Aqueous	Hydroalcoholic (1:1,water: methanol)
Alkaloids	-	+
Tannins	+	-
Phenolics	-	++
Flavonoids	+	++
Cardiac Glycosides	+	-
Terpenoids	+	+
Steroid	+	+
Saponins	+++	+
Carbohydrates	++	+
Proteins	+	+
Fats	+	+

(-) absent; (+) present in a negligible quantity; (++) present in moderate quantity; (+++): present in a considerable quantity

Antioxidant Activity:

1. **Determination of DPPH frees radical scavenging:** The free radical scavenging capacity of different extracts of *Mentha arvensis* was determined using DPPH method[32]. Freshly prepared DPPH (2,2-diphenyl-1-picrylhydrazyl), solution was taken in test tubes and extracts were added followed by serial dilutions (50µg/ml to 250µg/ml) to every test tube so that the final volume was 3 ml and after 30 min, the absorbance was read at 517 nm using a spectrophotometer. Ascorbic acid was used as standard. Control sample was prepared containing the same volume without any extract and standard and the absorbance was read at 517 nm using a spectrophotometer. Methanol was served as blank.

2. **Determination of reducing power:** The reductive capability of the extract was quantified by Oyaizu method [33]. One ml of (Extract) different concentrations of hydroalcoholic and aqueous extracts was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K3 Fe (CN) 6]. Similar concentrations of standard ascorbic acid were used as standard. The mixture was incubated at 50°C for 20 min. Then, the reaction was terminated by adding 2.5 ml of 10% trichloroacetic acid. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of 0.1%FeCl3. Blank reagent is prepared as above without adding extract. The absorbance was measured at 700 nm in a spectrophotometer against a blank sample. Increased absorbance of the reaction mixture indicated greater reducing power.

3. **Determination of the Total Phenolic and Flavonoid content:** The concentration of phenolics in plant extracts of *Mentha arvensis* was determined using standard method [34]. Crude extracts of *Mentha arvensis* were dissolved in the concentration of 1mg/ml. The reaction mixture was prepared by mixing 0.5 ml of methanol solution of extracts, 2.5ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5ml of 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5ml methanol 2.5ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5ml of 7.5% $NaHCO_3$. The samples were then incubated for 45mins at a temperature of 45degrees. Absorbance was measured at 765nm. The samples were prepared in triplicates for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for standard solution of Gallic acid and for control all reagents except extract was used [35].

   The content of flavonoids in the plant extract was determined using standard procedure. The sample contained 1ml of aqueous solution of the extract in the concentration of 1mg/ml and 1ml of 2% $AlCl_3$ solution dissolved in methanol. Same procedure was repeated for other extract of *Mentha arvensis.* The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at 415nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The content of flavanoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract) [36].

4. **Nitric oxide radical inhibition assay:** Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction [37].In this assay, Griess Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and *Mentha arvensis* extracts (25 to 125 mg/ml) or standard solution (rutin, 0.5 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink coloured chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Rutin was used as a standard.

5. **Metal chelating activity:** The ferrous level was monitored by measuring the formation of the ferrous ion-ferrozine complex [38].The reaction mixture containing 1.0 ml of different concentrations of *Mentha arvensis* extracts (1.0 ml) were added to 0.1 ml of 2 mM ferrous chloride and 0.2 ml of 5 mM ferrozine to initiate the reaction and the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The positive controls were those using ascorbic acid and all tests and analysis were run in triplicate. The percentage chelating effect of Ferrozine-Fe2+ complex formation was calculated. The chelating activity was calculated as

$$\% \text{ Chelating Activity} = [(A_1 - A_2) / A0] \times 100$$

Where A0 represents the absorbance of the control (without extract) and A_1 represents the absorbance of reaction mixture, A_2 represents the absorbance without $FeCl_2$.

6. **Scavenging of Hydrogen Peroxide:** The ability of the extracts to scavenge hydrogen peroxide was determined according to our recently published papers [39, 40]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (0.1-1 mg/ ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds were calculated as follows:

$$\% \text{ Scavenged } [H_2O_2] = [(Ao - A1)/Ao] \times 100$$

Where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard [39, 41].

RESULTS: Plants and plant derived products are good source of antioxidant compounds. Increased concentrations of free radicals in the body lead to various pathological conditions such as atherosclerosis, arthritis, Alzheimer's disease, cancers, etc. Intake of antioxidants can prevent the detrimental effects resulted from the imbalance in the antioxidant-pro-oxidant ratio. Antioxidants can be of both synthetic and natural origins. Natural antioxidants that are obtained from plants contain mainly phenolic compounds. Utilization of natural antioxidants from plants does not provoke adverse effects, while synthetic antioxidants are found to induce genotoxic effects.

DPPH frees radical scavenging activity: The ability of different extracts of root of *Mentha arvensis* to scavenge DPPH free radical was calculated as percentage inhibition which was found to be 35.83% and 57.63% for aqueous and hydroalchol extracts respectively at concentration 250 µg/ml, whereas percentage inhibition of ascorbic acid at the same concentration was 99.16% **Figure 1**.

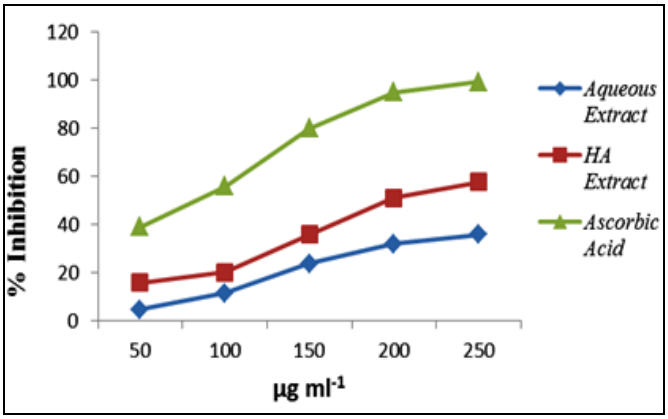

FIGURE 1: DPPH SCAVENGING ACTIVITY OF ROOT OF *MENTHA ARVENSIS* EXTRACTS

Reducing power capacity: The hydroalcohol extract showed good reducing power than aqueous extract when compared with standard ascorbic acid. The reducing power shown by aqueous extract was 0.75 and 1.48 by hydroalchohal extract at concentration of 0.25 mg/ml as compared to 2.69 shown by standard ascorbic acid at same concentration **Figure 2**.

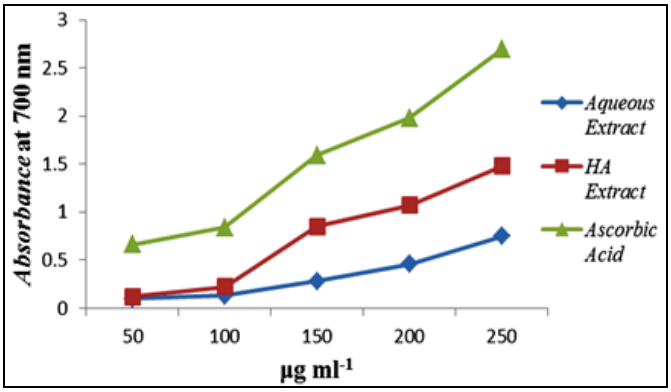

FIGURE 2: REDUCING POWER CAPACITY OF ROOT OF *MENTHA ARVENSIS* EXTRACTS

Total Phenolic and Flavonoid content: The content of phenolic compounds (mg/g) in Gallic acid equivalent was found to be 9.12 and 211.11 mg/g in aqueous and hydroalcoholic extracts of root of *Mentha arvensis* respectively **Figure 3**.

The total Flavonoid content (mg/g) in Rutin equivalent was found to be 32.14 and 230.18 in aqueous and hydroalcoholic extracts of root of *Mentha arvensis* respectively **Figure 4**.

TABLE 2: TOTAL AMOUNT OF PHENOLIC AND FLAVONOID CONTENT OF THE VARIOUS EXTRACT OF ROOT OF *MENTHA ARVENSIS*, [MEAN ± S.E.M. a]

Extract	Total phenolics mg/g plant extract (in GAE)	Total flavonoid mg/g plant extract (in RE)
Aqueous	9.12 ± 0.12	32.14 ± 2.31
Hydroalcoholic	211.11 ± 3.05	230.18 ± 2.43

(a): average of three determinations

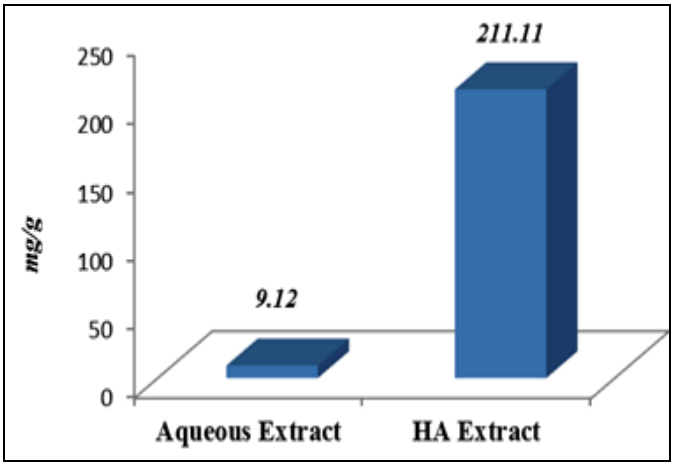

FIGURE 3: TOTAL PHENOLIC CONTENT OF ROOT OF *MENTHA ARVENSIS* EXTRACTS

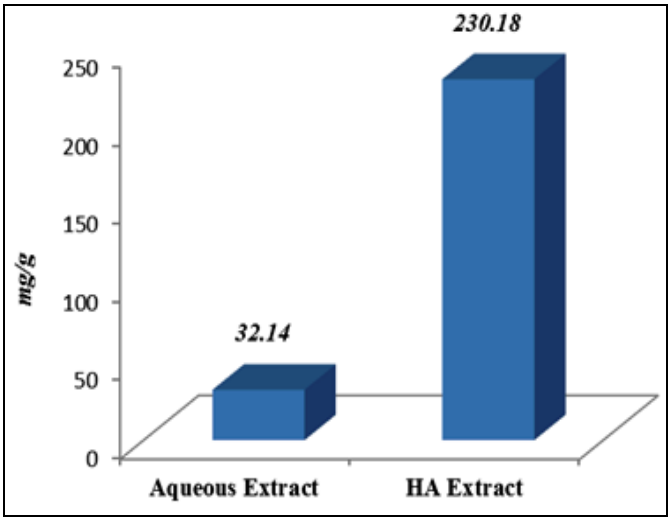

FIGURE 4: TOTAL FLAVANOID CONTENT OF ROOT OF *MENTHA ARVENSIS* EXTRACTS

Nitric oxide radical inhibition ability: The ability of different extracts of root of *Mentha arvensis* to scavenge Nitric oxide radical was determined by percentage inhibition which was found to be 39.11 and 63.25 for aqueous and hydroalcoholic extracts respectively at concentration 250 µg/ml, whereas percentage inhibition of standard rutin at the same concentration were 98.74 **Figure 5**.

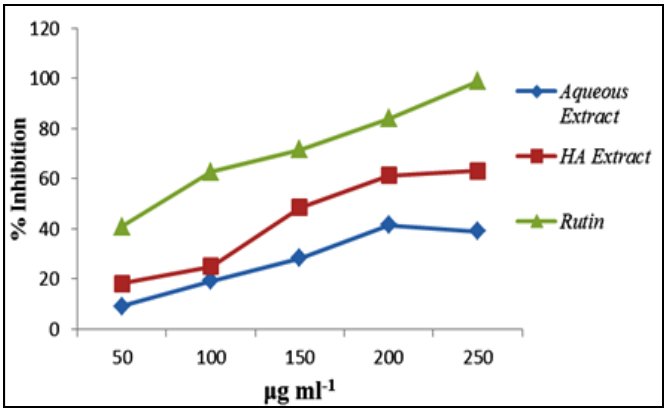

FIGURE 5: NITRIC OXIDE RADICAL SCAVENGING ABILITY OF ROOT OF *MENTHA ARVENSIS* EXTRACTS

Metal chelating ability: The metal chelating activities of various extracts of root of *Mentha arvensis* were concentration dependent. The absorbance of Fe^{2+}-ferrozine complex was linearly decreased with concentration dependently. The percentage of metal chelating capacity at the concentration of 500 µg/ml was 46.1 and 65 for aqueous and hydroalchol extracts of root of *Mentha arvensis* respectively. The percentage inhibition for standard ascorbic acid was 98.8 at same concentration **Figure 6**.

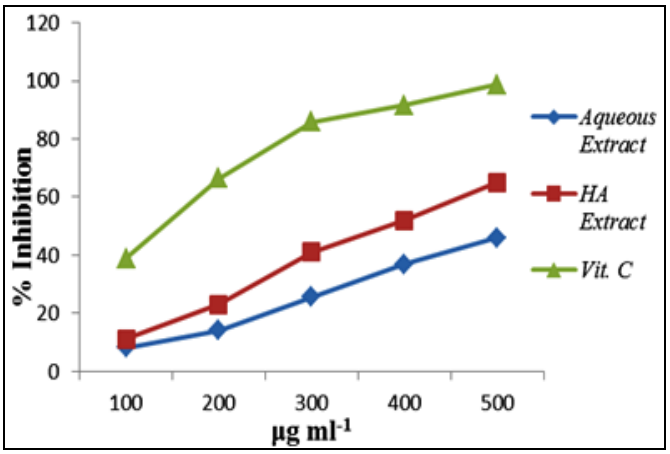

FIGURE 6: METAL CHELATING ABILITY OF VARIOUS EXTRACTS OF ROOT OF *MENTHA ARVENSIS*

Scavenging of Hydrogen Peroxide: The scavenging of hydrogen peroxide by various extracts of various extracts of root of *Mentha arvensis* was expressed as percentage scavenging. The aqueous and hydroalcoholic extracts showed 21.75 and 39.66 percentage inhibition respectively at concentration 1mg/ml as compared to standard ascorbic acid 96.43 at same concentration **Figure 7**.

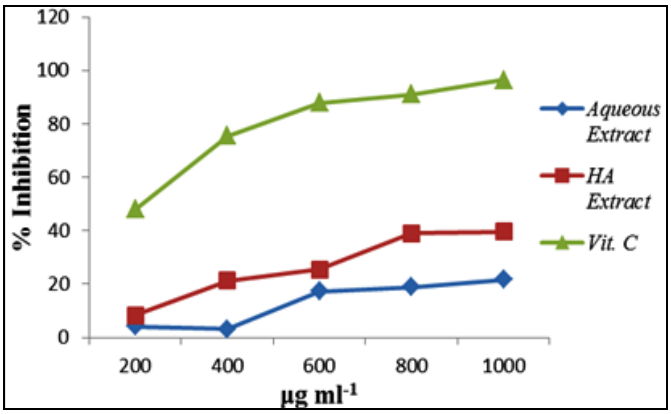

FIGURE 7: HYDROGEN PEROXIDE SCAVENGING ABILITY OF VARIOUS EXTRACTS OF ROOT OF *MENTHA ARVENSIS*

DISCUSSION: Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight free radicals and protect from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms. The results obtained from the preliminary phytochemical screening (**Table 1)**, it was observed that the aqueous and hydroalcoholic extracts of root of *Mentha arvensis* contains phenolics **Figure 3** and flavonoids **Figure 4** in moderate quantity along with other phytoconstituents (**Table 2)**. This depicts that the crude drugs may have antioxidant effect due to its polyphenolic property which needed to be further investigated. The correlation between phenolic content and antioxidant capacity is a well-documented study [42].

The antioxidant activity of *Mentha arvensis* extracts could be attributed to its flavonoidal content. Various oxidizing species like super oxide anion ($O_2^{-\bullet}$), hydroxyl radical or peroxy radicals are scavenged by flavonoids. They also act as quenchers of singlet oxygen[43]. The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavenging potential [44]. DPPH is a purple colour dye having absorption maxima of 517 nm and upon reaction with a hydrogen donor the purple colour fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance [45].

Among the various two extracts of root of *Mentha arvensis* hydroalcholic extract showed good electron donating capacity towards DDPH radical with percentage inhibition 57.63 than aqueous extract 35.83, at concentration of 250 µg/ml as compared to standard ascorbic acid (percentage inhibition 99.16) at same concentration **Figure 1**. The ability of two fractions to convert Fe^{3+} into Fe^{2+} determines their reducing power ability. Reductones are the antioxidants compounds which have reducing ability, which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom [46].

The antioxidant principles present in the extracts of root of *Mentha arvenis* caused the reduction of Fe^{3+}/ferric form to the ferrous form, and thus proved the reducing power ability. The reducing power shown by aqueous extract was 0.75 and 1.48 by hydroalcoholic extract at concentration of 0.25 mg/ml as compared to 2.69 shown by standard ascorbic acid at same concentration **Figure 2**.

Nitric oxide radical inhibition assay proved that hydroalcoholic extract of root of *Mentha arvensis* is a potent scavenger with percentage inhibition of 63.25 than aqueous extract 39.11 at concentration 250 µg/ml, whereas percentage inhibition of standard rutin at the same concentration were 98.74 **Figure 5**. The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. The fractions which scavenge nitric oxide compete with oxygen leading to reduced production of nitric oxide [47].

The metal chelating ability of the hydroalcoholic and aqueous extracts of *Mentha arvensis* was measured by the formation of ferrous ion ferrozine complex. Ferrozine reacts with ferrous ions forming a red coloured complex which selectively absorbs at 562 nm [48]. The ability of a chelating agent to form σ bond with a metal, may act as effective as secondary antioxidants, because they reduce the redox potential thereby stabilising the oxidised form of the metal ion [49]. The results of our study demonstrate that metal chelating activities of two extracts of root of *Mentha arvensis* were concentration dependent. The absorbance of Fe^{2+}-ferrozine complex was linearly decreased with concentration dependently. The percentage of metal chelating capacity at the concentration of 500 µg/ml was 46.1 and 65 for aqueous and hydroalcoholic extracts of root of *Mentha arvensis* respectively.

The percentage inhibition for standard ascorbic acid was 98.8 at same concentration **Figure 6**. Hydroxyl radicals are very toxic to the cell and hydrogen peroxide is a precursor for hydroxyl radicals but it is not particularly reactive with most biologically important molecules [50].

Thus, scavenging of H_2O_2 is a measure of the antioxidant activity of the extracts of root of *Mentha arvensis*. The extracts scavenged hydrogen peroxide which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralising it into water. The aqueous and hydroalcoholic extracts showed 21.75 and 39.66 percentage inhibition respectively at concentration 1mg/ml as compared to standard ascorbic acid 96.43 at same concentration **Figure 7**.

CONCLUSION: On the basis of the results obtained in the present study, it is concluded that a out of two extracts hydroalcoholic (1:1, methanol: water) extract of root of *Mentha arvensis*, which contains moderate amounts of phenolics and flavonoid compounds, exhibits high antioxidant and free radical scavenging activities. It also chelates iron and has reducing power. These in vitro assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various diseases associated with oxidative stresses. However, the components responsible for the antioxidative activity are currently unclear. Therefore, further investigations need to be carried out to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the in vivo antioxidant activity of this extract needs to be assessed prior to clinical use.

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