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RESEARCH ARTICLE

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Phytochemical Analysis and Antioxidant Activity of Ethyl Acetate Extract of *Martynia annua* Fruit

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ABSTRACT

Phytochemical analysis and antioxidant activity of ethyl acetate extract of Martynia annua fruits. *Martynia annua (M. annua)* commonly used in traditional medicines for cure of inflammation, tuberculosis, skin infection, epilepsy, burns, itching and sore throat. The fruit of M. annua was extracted with ethyl acetate by soxhlet method. The fruit extract (EAE) was subjected to phytochemical analysis,thin layer chromatography and determination of antioxidant activity. Phytochemical screening and thin layer chromatography of EAE confirmed the presence of alkaloids, steroids, carbohydrates, fat, volatile oil, tannin and phenolic compound. The EAE showed dose dependent antioxidant activity in DPPH, hydrogen peroxide assay and nitric oxide assay. The total phenolic and flavonoid content was found to be about 70- 90%. It is concluded that antioxidant activity of EAE of Martynia annua fruit produced dose related effect.

Keywords: *Martynia annua,* Phytochemical Screening, TLC Profiling, Antioxidant

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INTRODUCTION

From the dawn of the civilization traditional medicine has been used around the world. In rural area majority of the population prefer plant-based medicine in almost all the ailments[1]. In folk culture the natural resources form the basis of all the treatment. Different parts of plant like root, stem, leaf, fruit, seed oil, bark and latex have been used for preparing herbal formulations. They may also contain isolated phytochemicals which are secondary metabolites and are responsible for different pharmacological action on human body[2]. *M. annua* is road side weed growing in wastelands of tropical and subtropical area of America, Mexico, Burma and India. It has been used in Ayurvedic medicine since many years. It is called “kaakanaasikaa”, “Kaakaangi”, “Shirobal” in Sanskrit. Kaakanaasikaa means “dried seed”. Common name of *M. annua* is cat’s claw, devil claw and bichhuu. It belongs to Martyniaceae (or predaliaceae) family.

The leaf, root, seed oil and fruit of this plant have pharmacological activity. The leaves are eaten at time of epilepsy and tropically applied to tuberculous glands of the neck. Leaf extract is used as gargle for sore throat and leaf pastes are useful in wounds of domestic animal. Fruit is considered as alexiteric and anti-inflammatory (Fig.1). A mixture of fruit ash with coconut oil and is applied on burn and is also found effective in cse of bite of scorpion. An oil is extracted from seed, which is very effective in affection of skin and itching[3,4]. Ethanolic extract of root has shown antifertility activity[5]. For the mitigation of cold fever and pneumonia all the parts of plant is boiled in water and administered[6].



Figure 1: *Martynia annua* Fruit

Antioxidants are natural compounds that prevent cell damage caused by reactive oxygen species. During oxidation reaction cell produces free radicals which are highly reactive species and enters in cell membrane resulting in the degradation of cell or cell death. Antioxidants react with free radicals and inhibits unwanted oxidation reactions by oxidizing themselves[7]. Regular intake of antioxidant containing food such as- α-tocopherol, ascorbic acid, carotenoids, amino acid, protein, flavonoids and other phenolic compounds has shown to mitigate the threat of ailments that are chronic in nature (cancer, diabetes, cardiovascular disease etc.) several methods are used to measure the antioxidant activities. The *in vitro* antioxidant assays are ferric reducing capacity, DPPH radical scavenging, nitric oxide scavenging, hydrogen peroxide assay; reducing power assay[8].

In present study physicochemical analysis, phytochemical analysis, thin layer chromatography, DPPH assay, hydrogen peroxide and nitric oxide assay was performed with ethyl acetate extract of crushed fruit. Quantitative estimation of phenols and flavonoids was also performed.

MATERIAL AND METHOD

Collection and Preparation of Plant Material

Fruits of *M. annua* was procured in the month of September-October 2018 from medicinal garden of Columbia Institute of Pharmacy, Raipur, C.G. The fruit sample was thoroughly rinsed under tap water to cleanse dirt and dust. After the cleaning the fruit was shade-dried for 10 days till it was completely dried. The dried fruit was crushed to course powder and kept for further use in tightly closed box.

Extraction

500gm of the coarse powder was extracted with ethyl acetate in a soxhlet apparatus at 80˚C till complete extraction was achieved.The extract was concentrated in a water bath. The resulting substance was an oily dark coloured liquid. The final extract was stored in a tightly closed box after wheighing it and kept in a cool dark space for further studies.Calculate percentage yield of extract

$$\% \text{ Yield } = \frac{\text{Amount of pure product used (g)}}{\text{Amount of crude material used (g)}} \times 100$$

Preliminary Physicochemical Analysis

Physical Characteristics

Colour: Brown-Yellowish

Odour: odourless

Appearance*:* Oily nature

Solubility: EAE of *M. annua* was soluble in ethanol, methanol and ethyl acetate.

Phytochemical Characteristics

Phytochemical analysis was performed to identify chemical compounds present in test sample (Table. 1)[9, 10, 11].

Thin Layer Chromatography (TLC) of Extract

The extract was subjected to TLC studies for detection of compounds present in it. The TLC plates were prepared by using silica gel G. Silica gel was weighed and made to homogenous slurry with distilled water. A thin uniform layer of slurry was spread over the glass plates. The plates dried in room temperature. After that the plates were activated at 110˚C in hot air oven for about 30 minutes.

Preparation of sample

About ten milligram of material was taken and dissolved in ethanol for spotting on TLC plate.

Application of sample on TLC plate

Sample was spotted on TLC plate on 0.5 cm away from solvent phase. The spotting was done by capillary tube. After the plate is developed it is taken out and dried in air. A mark was put on the spot.

Table 1: Phytochemical Analysis of *Martynia annua*

Test/ reagent	Method	Observation	Ref.
Test for alkaloids			
Hager' test	Sample (0.5 ml) + Hager reagent (2 drops)	Yellow ppt.	12,13
Dragendorff's test	Sample (0.5 ml) + dragendorff reagent	Orange- brown ppt.	
Test for steroids			
Liebermann-Burchard's test	Sample (0.5 ml)+chloroform+acetic anhydride(0.5 ml) +few drops of conc. H_2SO_4 the wall of test tube.	Red-green colour show.	
Salkowski's test	Sample (0.5 ml) +Chloroform (0.5 ml) + conc. H_2SO_4 (0.5 ml)	Chloroform layer show red and greenish colour for acid.	
Test for carbohydrates			
Molisch's test	Eethyl Acetate extract +few drops of α-naphthol +conc. H_2SO_4 from the test tube	Violet ring is appearing.	14,15
Barfoed's test	Sample (0.5 ml) + barfoed reagent Heat in water bath and cool	Red ppt.	
Test for phenolic compounds/ Tannins			
Tannin	Sample (0.5 ml) + 5% ferric chloride solution	Deep blue black colour	
Phenol	Sample (0.5 ml) + dilute iodine solution	red colour formed	
Test for protein and amino acid			
Biuret's test	Sample (0.5 ml) +4% sodium hydroxide (0.5 ml) +few drops of 1% copper sulphate solution	Violet or pink colour appears	
Ninhydrin test	Sample (0.5 ml) + 1-2 drops of 5% Ninhydrin solution heated on water bath few minutes	Purple- bluish colour show.	
Test for glycoside			
Keller-killiani's test	Sample (0.5 ml) + glacial acetic acid (1ml) + few drops ferric chloride + H_2SO_4 from the wall.	Brown ring is appear	
Raymond's test	Sample (0.5 ml) + hot methanol	Violet colour show.	
Test for fat and oil			
Fat	Filter paper is permanently stained with oil.		16
Volatile oil	Volatile oil gives a characteristic odour		
Test for flavonoids			
Sinoda test	Sample (0.5 ml) + Magnesium powder + few drop of conc. H_2SO_4	Pinkish type red colour appeared	17
Lead acetate test	Sample (0.5 ml) + few drop 10% lead acetate	Shows yellowish precipitate	

Preparation of solvent system

Five different solvent systems were prepared. They are (i) water: ethyl acetate: chloroform: (1:5:3), (ii) cyclohexane:ethyl acetate (1:1), (iii) n-butanol: pyridine: water (8:4:3), (iv) methanol: water (6:3) and (v) chloroform: water (6:4)

Saturation of TLC chamber

TLC chamber was lined inside with filter paper on three sides. Solvent system was filled upto approximately 1cm of the base. The rim of the TLC chamber was then greased. It was then covered with glass plate. It was then left till the filter paper got evenly wet with the solvent. Following the same procedure five more chambers were made ready for experiment with different solvent systems respectively.

Development of TLC plate

TLC plates that were spotted earlier were put inside the saturated chambers. Plates were put in vertical position. TLC plates were put till the mobile phase ran upto approximately 80% of the starting point of thin layer. At this time plates were taken out and kept for drying in air.

Detection of TLC plate

The plates were seen after spraying with various reagents. The value of retention factor (Rf) was by the following formula: [18, 19,20]

Rf = Distance moved by the spot/ distance moved by solvent

In-vitro *Antioxidant Assay*

DPPH (1, 1-Diphenyl 2-Picryl Hydrazyl) Radical Scavenging Assay

Preparation of DPPH solution (1M)

1M solution of DPPH was made by using methanol and kept it in dark place.

Preparation of test and standard solution

For stock solution 5 mg of test or standard drug was dissolve in 100 ml methanol. From this stock solution, different concentration was prepared. Ascorbic acid was used as standard.

The reaction mixture was made in volumetric flask in which 1ml of standard sample of different concentration and 3ml of DPPH solution. The flasks were then vigorously shaken and incubated for half an hour at temperature. Similarly the procedure was repeated by taking test sample solution of different concentration. On completion of half an hour absorbance was measured. UV/Visible spectrophotometer at 517nm was used to determine inhibition percentage. The colour change was observed when it turned from deep violet- light yellow[21, 22, 23]. Percentage radical scavenging of DPPH was determined by calculation as shown below:

$$\%\ \text{DPPH radical scavenging} = \frac{A_1 - A_2}{A_1} \times 100$$

Where:

A_1 – Absorbance of standard

A_2 - Absorbance of sample

Hydrogen Peroxide (H_20_2) Scavenging Assay

H_20_2 solution was made with phosphate buffer (pH 7.4). Spectrscope was employed to find out concentration of H_20_2. Absorbance was found out with extinct coefficient for H_20_2. Different concentrations of extract in distilled water were added to H_20_2 solution (0.6ml, 40 mM). Absorbance of H_20_2 was measured at 230 nm after 10 minutes. It was compared with phosphate buffer alone. Solution of ascorbic acid was used as standard [24, 25]. The percent of H_20_2 scavenging was calculated by the equation given below-

$$\%\text{Hydrogen peroxide scavenging} = \frac{A_1 - A_2}{A_1} \times 100$$

Where:

A_1 – Absorbance of standard

A_2 - Absorbance of sample

Nitric oxide

Nitric oxide is formed in tissues in the presence of an enzyme nitric oxide synthases. It metabolizes arginine to citrulline. In this process Nitric oxide formed due to reaction of five electron oxidation. Reaction mixture was prepared by adding 0.5ml of standard sample at different concentrations respectively in volumetric flask and 2ml of (10mM) sodium nitroprusside dissolve in Phosphate buffere

saline (pH 7.4). Mixture was given a vigorous shake thereafter incubated at 25°C for 30 min. in room temperature. This procedure was repeated by taking test sample solution of different concentration. After half an hour 0.5 ml of sample that was incubated earlier was mixed with 0.5 mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H3PO4) was added. The absorbance was measured by UV/Visible spectrophotometer at 546nm. The calculation was done to find out percentage inhibition of nitric oxide by following formula[26].

$$\%\text{Inhibition nitric oxide assay} = \frac{A_C - A_T}{A_C} \times 100$$

Where:

A_C - Absorbance of control

A_T - Absorbance of test

Total Phenolic Content

Spectroscopic method was employed to find total phenolic content. EAM extract (1mg/ml) 1 ml was added to 1 ml 10% Folin-Ciocalteu's reagent that was dissolved in 13 ml of deionized water. To this 5 ml of 7% Na2CO3 solution was added to get the final reaction mixture. The resulting combination was given a strong shake. It was then kept in room temperature for 2 h in dark. In the same way blank solution without extract was also prepared. Spectrometer was employed to find out absorbance at 760 nm. This experiment was done thrice to find out mean value of absorbance. The curve of the test and blank were extrapolated on calibration line of obtained by gallic acid solution at different concentrations. Total phenol content was reported on the basis of gallic acid equivalent (mg GAE) per gram of the dried sample[27].

Total Flavonoid Content

Spectroscopy was employed to find total flavonoid content. About 100 ml of EAM extract (1mg/ml) was added to 100 mL of 20% aluminium trichloride prepared in methanol in which a drop of acetic acid was added. The blank solution was prepared by preparing a mixture of 100Ml of plant extract added to a drop of acetic acid and diluted in 5ml methanol. Spectrometer was employed to find out absorbance at 415nm. This experiment was done thrice to find out mean value of absorbance. Total flavonoid content was determined by extrapolating calibration line which was construed by gallic acid solution. The TFC was reported on the basis of gallic acid equivalent (mg GAE) per gram of the dried sample[28].

RESULTS AND DISCUSSION

Percentage Yield

The % yield, physical appearance and colour is mentioned in table 2. Ethyl acetate Extracts of *M. annua* was yellowish in colour and oily in nature. The % yield was found to be 10%.

Table 2: Percentage yield, physical appearance and colour of the *M. annua* fruit

Extract	Yield %	Physical appearance	Colour
M.annua	10	Oily	Brown-Yellowish

Phytochemical Analysis

It was performed to find out phytoconstituents present in the extract. The result is presented in table 3. The ethyl acetate extract of *M. annua* confirmed the presence of alkaloids, steroids, carbohydrates, fat, volatile oil, tannin, flavonoid and phenolic compound.

TLC Study

The TLC study of ethyl acetate extract of *M. annua* was performed by using different ratio of solvent system and visualizing agent. The detail of TLC study of ethyl acetate extract of M. annua is shown in table 4. The R_f value of Alkaloids, steroid, carbohydrates, tannin and Phenol were 0.97, 0.41, 0.30, 0.82 and 0.84, respectively.

The presence of Alkaloids, steroid, carbohydrates, tannin and Phenol in the plant material may produce numerous pharmacological activities namely antioxidant, anti-allergic, immunomodulating, anti-hepatotoxic, anti-viral, hypoglycemic, anti-microbial, anti-depressant, anti-diabetic, anti-cancer etc to the human body.

Antioxidant Assay

DPPH Scavenging Activity

The DPPH assay of fruit extract was determined by comparing with standard solution of ascorbic acid.

Absorbance figures were recorded at different concentrations (50-250µg/ml). The DPPH scavenging activity is shown in table 5 and the graph plotted between % inhibition Vs concentration µg/ml for fruit extract, from which, IC50 value was calculated using regression equation. *M. annua* showed 90.02 µg/ml scavenging activity shown in Fig.2 and Fig.3.

Table 3: Phytochemical screening of *M. Annua*

Compound	Test	EAE
Alkaloids	Hager reagent	+ve
	Dragendorff reagent	+ve
Steroids	Liebermann-burchard reaction	-ve
	Salkowski reaction	+ve
Carbohydrates	Molisch's test	+ve
	Barfoed test	-ve
Glycoside	Keller-Killiani test	-ve
	Raymond test	-ve
Fat	Filter paper staining test	+ve
Volatile oil	Organoleptic property	+ve
Protein	Biuret test	-ve
Tannin	Ferric chloride test	+ve
Amino acid	Ninhydrin test	-ve
Phenol	Iodine test	+ve
flavonoid	Sinoda test	+ve
	Lead acetate test	-ve

+ve presence of compound; -ve absence of compound

Hydrogen Peroxide Scavenging Activity

H2O2 assay of fruit extract was determined with the help of standard solution of ascorbic acid. Solutions of plant extract at concentrations of 50- 250µg/ml were tested for hydrogen peroxide scavenging activity. Concentration dependant inhibition was observed against hydrogen peroxide. Hydrogen peroxide scavenging activity of *M. annua* (EAE) and ascorbic acid at varying concentration are presented in table. 6 and the graph plotted between % inhibition Vs concentration µg/ml for fruit extract. The hydrogen peroxide scavenging activity of *M. annua* fruit was found to be 71.29 µg/ml shown in fig. 4 and fig. 5.

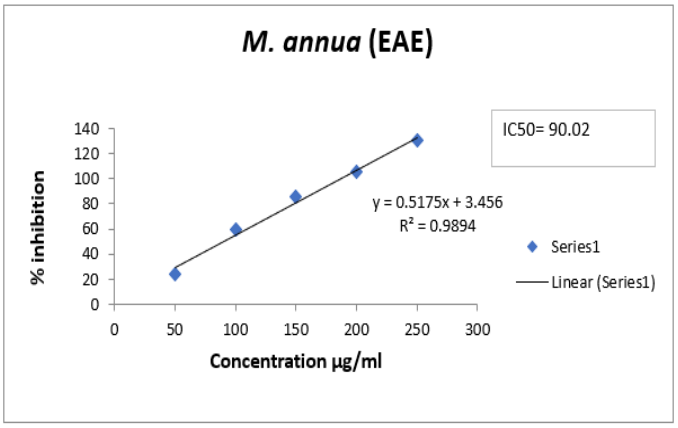


Figure 2: Scavenging effect of *M. annua* (EAE) on DPPH free radicals

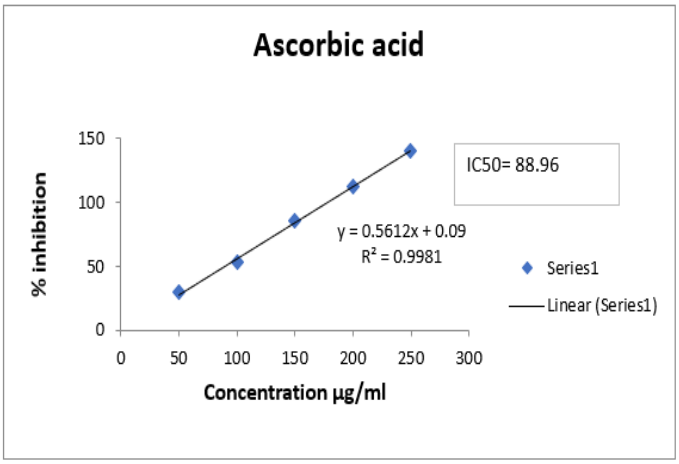


Figure 3: Scavenging effect of Ascorbic acid on DPPH free radicals

Nitric Oxide Scavenging Activity

Standard solution of ascorbic acid was used to determine nitric oxide scavenging of fruit extract. The absorbance was recorded at concentrations of 20-100µg/ml respectively. The percentage of nitric oxide scavenging activity presented in table 7 and the graph plotted between % inhibition Vs concentration µg/ml for fruit extract, from which, IC50 value was calculated using regression equation. M. annua showed 77.90 µg/ml scavenging activity shown in Fig.6 and Fig.7.

Total Polyphenols

Standard solutions of gallic acid at different concentrations were employed to find out total phenolic content (TPC). Result is presented in terms of mg GAE/g dry sample. TPC of EAE was found to be 90±0.26 mg GAE/g. Phenolics or polyphenols have been reported to show antioxidant activity. In general it is expected that extract/drug which contains high TPC would show significant antioxidant capacity.

Table 4: R_f value for various phytochemicals in EAE of fruit of *Martynia annua*

Phytochemical	Solvent System	Confirmatory Test	R_f Value
alkaloids	Water: EA: chloroform: (1:5:3)	Mayer's reagent	0.97
Steroid	Cyclohexane: ethyl acetate	Sulphuric acid	0.41
carbohydrate	n-butanol : pyridine : water (8:4:3)	Molisch's refagent	0.30
phenol	Methanol: water (6:3)	$FeCl_3$	0.84
Tannin	Chloroform : water (6:4)	$FeCl_3$	0.82

Table 5: Percentage of DPPH scavenging activity of *M. annua* (EAE) and ascorbic acid at varying concentration

Conc.(µg/ml)	%DPPH radical scavenging			
	EAE	IC_{50}	Ascorbic acid	IC_{50}
50	24.28±0.11		30.1±0.11	
100	59.5±0.29		53.08±0.18	
150	85.61±0.13		85.2±0.16	
200	105.2±0.48	90.02	112.1±0.24	88.96
250	130±0.16		140.9±0.11	

*All data presented in mean± SEM (n=6)

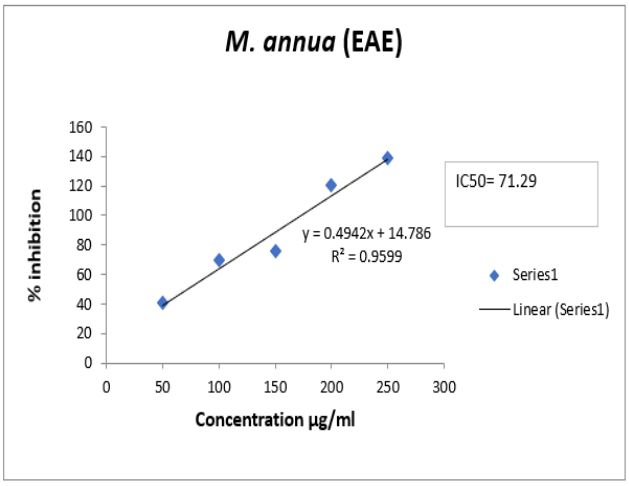


Figure 4: Free radical scavenging activity of *M. annua* (EAE) on Hydrogen peroxide assay

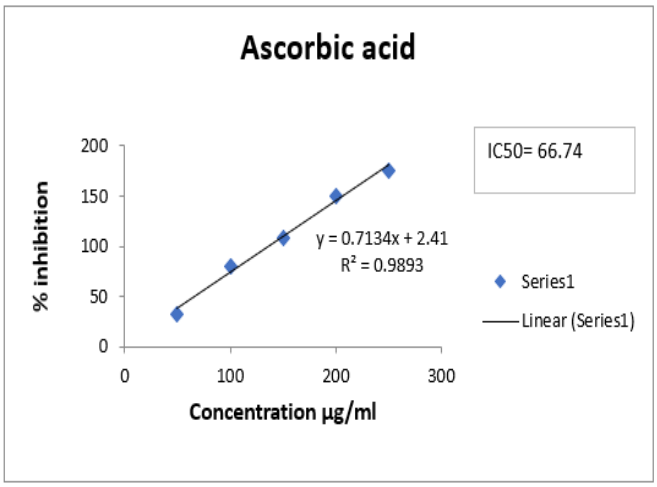


Figure 5: Free radical scavenging activity of Ascorbic acid on Hydrogen peroxide assay

Total Flavonoid Content

Standard solutions of gallic acid at different concentrations were employed to find out total flavonoid content (TFC). Result is shown in terms of mg GAE/g dry sample. The total flavonoid content of EAE was found to be 75±0.21 mg GAE/g.

Table 6: Percentage of hydrogen peroxide scavenging activity of *M. annua* (EAE) and ascorbic acid at varying concentration.

Conc.(µg/ml)	%Hydrogen peroxide scavenging			
	EAE	IC50	Ascorbic acid	IC50
50	40.5±0.20		32.1±0.10	
100	69.6±0.28		80.5±0.15	
150	75.48±0.37		108.6±0.16	
200	120.3±0.4	71.29	150.4±0.17	66.74
250	138.7±0.37		175.5±0.17	

*All data presented in mean± SEM (n=6)

Table 7: Percentage of Nitric oxide scavenging activity of *M. annua* (EAE) and ascorbic acid at varying concentration

Conc.(µg/ml)	% radical scavenging activity			
	EAE	IC_{50}	Ascorbic acid	IC_{50}
20	26.1±0.23		27.1±0.46	
40	27.8±2.5	77.90	44.8±2.6	
60	31±1.5		64.2±3.4	43.44
80	34.8±2.2		92.1±2.8	
100	41.2±3.6		110±4.2	

*All data presented in mean± SEM (n=6)

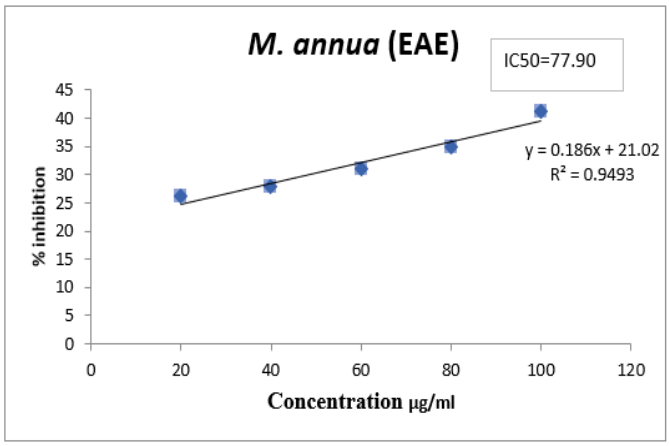


Figure 6: Free radical scavenging activity of *M. annua* (EAE) on nitric oxide assay

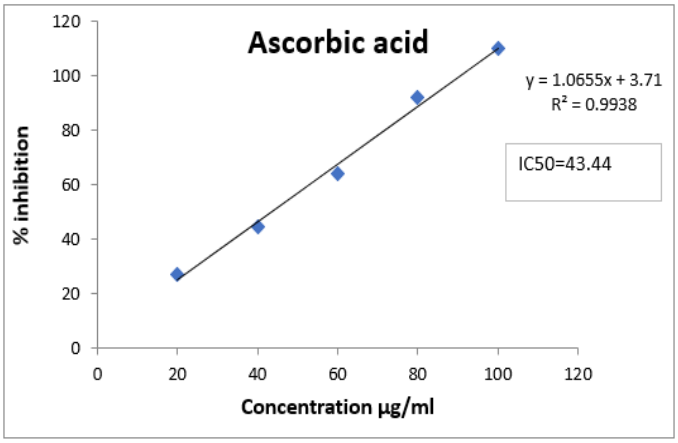


Figure 7: Free radical scavenging of Ascorbic acid on nitric oxide assay

DISCUSSIONS

Diverse plant parts of *M. annua* are used in treatment of many diseases. Ethyl acetate extract of the fruits of *M. annua* (EAE) was tested for phytochemical analysis by qualitative estimation as well as TLC, total phenolic content, total flavonoid content and antioxidant activities. EAE has shown presence of alkaloids, steroids, carbohydrates, fat, volatile oil, tannin, flavonoid and phenolic compounds in qualitative analysis as well as in TLC studies. It has also exhibited significant antioxidant activity. EAE in various concentrations (50, 100, 150, 200 and 250 µg/ml) has shown %DPPH radical scavenging of 24.28±0.11, 59.5±0.29 85.61±0.13 105.2±0.48 and 130±0.16 respectively, and standard drug (ascorbic acid) in same concentration has shown percentage of DPPH radical scavenging of 30.1±0.11, 53.08±0.18, 85.2±0.16, 112.1±0.24 and 140.9±0.11 respectively. The IC_{50} value of EAE and standard was found to be 90.02 and 88.96 respectively. In hydrogen peroxide scavenging assay EAE in different concentrations (50, 100, 150, 200 and 250 µg/ml) has shown hydrogen peroxide scavenging of 40.5±0.20, 69.6±0.28, 75.48±0.37, 120.3±0.4 and 138.7±0.37 percent respectively. In same concentrations the standard drug (ascorbic acid) has shown percentage scavenging of 32.1±0.10, 80.5±0.15, 108.6±0.16, 150.4±0.17 and 175.5±0.17 respectively. The IC_{50} value of EAE and standard was found to be 71.29 and 66.74 respectively. In nitric oxide scavenging assay EAE in different concentrations (20, 40, 60, 80 and 100 µg/ml) has shown nitric oxide scavenging of 26.1±0.23, 27.8±2.5, 31±1.5, 34.8±2.2 and 41.2±3.6 percent respectively. In same concentrations the standard drug (ascorbic acid) has shown percentage scavenging of 27.1±0.46, 44.8±2.6, 64.2±3.4, 92.1±2.8 and 110±4.2 respectively. The IC_{50} value of EAE and standard was found to be 77.90 and 43.44 respectively. The total phenolic content (TPC) of EAE was found to be 90±0.26 mg GAE/g. The total flavonoid content (TFC) of EAE was found to be 72±0.21 mg GAE/g Considerable amount of TPC and TFC are responsible for excellent DPPH hydrogen peroxide and nitric oxide activity.

CONCLUSION

Antioxidant activity has been shown to be one of the major parameter in recovery from several diseases. It can be concluded that EAE is potent inhibitor of free radicals. The results of this study propose that EAE of fruits of *M. annua* has significant amount of phenolics that may be the main reason for significant antioxidant activity. This further suggests that it has a great potential as a source of antioxidants to avoid the development of many diseases. However, further detailed investigations are needed for isolation and identification of pharmacologically active constituents present and to establish the traditional uses of this plant.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors equally contributed to this paper and approved for the publication.

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