Antioxidant Activities of Methanolic and Aqueous Extracts from Leaves of *Martynia annua* Linn.

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ABSTRACT

The antioxidant activity of the methanolic and aqueous extracts of *Martynia annua* Linn. leaves were evaluated by several *in vitro* systems of assay, namely, reducing power assay, DPPH radical-scavenging activity, nitric oxide scavenging activity, H_2O_2 radical scavenging activity, superoxide radical scavenging assay, hydroxyl radical-scavenging activity, and total antioxidant capacity. Total phenolic content was measured by Folin–Ciocalteau reagent. The antioxidant property depends upon concentration and increased with increasing amount of the extract. The free radical scavenging and antioxidant activities may be attributed to the presence of phenolic and flavonoid compounds present in the extract. The results showed that the methanolic extract exhibited higher antioxidant activity than the aqueous extract. Chlorogenic acid is reported in this plant and a TLC densitometric method was developed for the quantification of Chlorogenic acid.

Keywords: *Martynia annua* Linn., antioxidant, chlorogenic acid, HPTLC.
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INTRODUCTION

Active (or reactive) oxygen species and free radical-mediated reactions have been implicated in degenerative or pathological processes such as aging, cancer, coronary heart disease and Alzheimer's disease (1). Meanwhile many epidemiological results point to an association between a diet rich in fresh fruit and vegetable and a decrease in the risk of cardiovascular diseases and certain forms of cancer in humans (2). Several reports concern the antioxidant activities of natural compounds in fruits and vegetables. These include phenolic compounds (3), anthocyanin (4), water extracts of roasted *Cassia tora* (5).

In cells, metabolic pathways normally couple to degrade free radicals. If the generation rates of free radicals are faster than degradation rates under environmental stresses, cells suffer oxidative stresses. Two distinct pathways, nonenzymatic or enzymatic, were found in plant cells as routes of free radical scavengers. The former included ascorbate or chlorogenic acids (6) or vitamin E; the latter included different forms of SOD to metabolize superoxide free radical to hydrogen peroxide. The hydrogen peroxide produced was further metabolized either by catalase or different forms of peroxidase such as glutathione peroxidase (7).

The genus *Martynia* comprises of 7 species native to Mexico. *Martynia annua* Linn., a native of Mexico belongs to the Martyniaceae family and common names include Devil's Claw (English), Bichhu (Hindi), Kakanasika (Sanskrit) and Vichchida (Gujarati). The leaves and fruits are biologically active part of this plant (8, 9). The leaves of the *Martynia annua* are edible and used as antiepileptic and antiseptic, applied locally to tuberculous glands of the neck; the juice of the leaves as a gargle for sore throat and the leaf paste for wounds of domestic animals (10, 11). Some important chemical constituents of *M. annua* include phenolic acids like chlorogenic acid, sinapic acid, p- hydroxybenzoic acid; Several flavonoids including apigenin, luteolin, apigenin-7-O-beta- D- glucuronide (12, 13).

In the present paper, we report our work on establishing antioxidant activity of *M. annua* leaves, in quenching free radicals that are generated in several *in vitro* models. We also report the presence and TLC densitometric quantification method of chlorogenic acid, in order to evaluate a relationship between the antioxidant activity

and the phytochemical constituents of the *M. annua* leaves.

MATERIALS AND METHODS

Plant material and extraction

The aerial parts of the plant were collected between the months of August-September 2008, from Anand district of Gujarat State, India. The collected plant was authenticated by a Taxonomist, Bioscience Department, Sardar Patel University, Vallabh Vidyanagar. The Voucher specimen of *Martynia annua* was deposited under DCP/Ma-2/23 in A.R.College of Pharmacy, Vallabh Vidyanagar. After collection, the leaves were washed thrice with water to remove dust and debris. The washed leaves were dried under shade and powdered (60#). The powdered leaves of *Martynia annua* (100 g) were soaked in petroleum ether (600 mL) and allowed to stand for 48 h, with occasional shaking. The macerate was decanted and filtered, through cloth, and then, through Whatman filter paper. The residue, called 'marc,' after the extraction with petroleum ether was dried and extracted exhaustively with methanol to yield a greenish brown semisolid (11.68 % W/W). The same procedure was followed for preparation of aqueous extract by taking chloroform water instead of methanol to yield a reddish brown solid (22.32 %W/W). The methanol extract of *Martynia annua* (MEMA) and aqueous extract of *Martynia annua* (AEMA) were used for evaluation of antioxidant activity.

Determination of total polyphenols

The Total phenolic content in the MEMA and AEMA extracts were determined with the Folin-Ciocalteau reagent according to the method of Chandler and Dodds (14) using gallic acid as a standard phenolic compound. Briefly, 1 mL of extract solution (1000 µg/mL) in a volumetric flask diluted with distilled water (46 mL). FC reagent (1 mL) was added and the contents of the flask were mixed thoroughly. After 3 minutes, 3 mL of Na_2CO_3 (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. The amount of total phenolic compounds in the plant extract was determined in micrograms of gallic acid equivalent, using the equation obtained from the standard gallic acid graph:

$$y = 0.0047\times - 0.0879,\ R^2 = 0.9798$$

Reducing Power Assay

The reducing power of MEMA and AEMA extracts were determined by the method of Oyaizu (15). The capacity of extract to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. For this purpose, different concentrations of plant extract (100–1000 µg/mL) in 1 ml of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 minutes Aliquots (2.5 mL) of Trichloro acetic acid (TCA, 10%) were added to the mixture. The 2.5 mL of solution was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The absorbance was measured at 700 nm by spectrophotometer. Increased absorbance of the reaction mixture indicates increased reducing ability.

DPPH free radical scavenging activity

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH (16). 0.1 mM solution of DPPH in methanol was prepared and 1.0 mL of this solution was added to 3.0 mL of extract solution in water at different concentrations (10–200 µg/mL). It was incubated at room temperature for 45 minutes and the absorbance was measured at 517 nm against corresponding blank solution. Ascorbic acid was taken as reference. Percentage inhibition of DPPH free radical was calculated based on the control reading, which contained DPPH and distilled water without any extract using the following equation:

$$\text{DPPH Scavenged (\%)} = (A_{cont} - A_{test}) / A_{cont} \times 100$$

Where A_{cont} was the absorbance of the control reaction and A_{test} was the absorbance in the presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in µg/ml) of extracts that inhibits the formation of DPPH radicals by 50%.

Nitric oxide scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction (17). 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract at various concentrations and the mixture incubated at 25°C for 150 minutes. From the incubated mixture 0.5mL was taken out and added into 1.0 mL sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 minutes. Finally,

1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 minutes. The absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radical scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100]$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract/Standard.

Hydrogen peroxide scavenging activity

H_2O_2 scavenging ability of extract was determined according to the method of Ruch et al. (18). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extract at the different concentrations (10–1000 µg/mL) in 3.4 mL phosphate buffer were added to a H_2O_2 solution (0.6 mL, 40 mM). The absorbance of the solution was measured at 230 nm against a blank solution containing the extract without H_2O_2. The percentage of H_2O_2 scavenging of extract and standard compounds was calculated as:

$$\% \text{ Scavenged} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where $A_{control}$ was the absorbance of the control, and A_{sample} was the absorbance in the presence of the sample of extract.

Superoxide radical scavenging activity

The scavenging activity of the MEMA and AEMA extracts towards superoxide anion radicals were measured by the method of Liu, Ooi, and Chang (19). 100 µL riboflavin solution [20 µg], 200 µL EDTA solution [12 mM], 200 µL methanol and 100 µL NBT (Nitro-blue tetrazolium) solution [0.1 mg] were mixed in test tube and reaction mixture was diluted up to 3 mL with phosphate buffer [50 mM]. The absorbance of solution was measured at 590 nm using phosphate buffer as blank after illumination for 5 minutes. This is taken as control. Different concentrations of extracts were taken, to each of this, 100 µL riboflavin, 200 µL EDTA, 200 µL methanol and 100 µL NBT was mixed in test tubes and further diluted up to 3 mL with phosphate buffer. Absorbance was measured after illumination for 5 minutes at 590 nm on UV visible spectrophotometer.

Hydroxyl radical (OH–) scavenging activity

The scavenging ability for hydroxyl radical was measured by Zhao et al. method (20). Stock solution of EDTA (1mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM) H_2O_2 (10 mM) and deoxyribose (10 mM) was prepared in distilled de- ionized water. The attempt was performed by adding up 0.1 mL EDTA, 0.01 mL of $FeCl_3$, 0.1 mL H_2O_2, 0.36 mL deoxyribose, 1 mL of sample extract (100–500 µg/mL) dissolved in distilled water, 0.33 mL of phosphate buffer (50mM, pH 7.4) and 0.1mL of ascorbic acid added. The mixture was incubated at 37°C for 1 h. A 1.0 mL of incubated mixture was mixed with 1.0 mL of 10% trichloro acetic acid and 1.0 mL of 0.5% thiobarbituric acid (in 0.025M NaOH containing 0.025% BHA) to urbanized the pink color measured at 532 nm. The hydroxyl radical scavenging activity is reported as percent inhibition of deoxyribose sugar dilapidation and is calculated as

$$I\% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

Where A_{blank} is the absorbance of control and A_{sample} is the absorbance of test.

Determination of Total antioxidant capacity

Total antioxidant capacity was measured according to the method reported by Prieto with slight modifications (21). In brief, 100 µg of extract and 100 µg of ascorbic acid (as standard) were taken in 0.1 mL of alcohol, combined separately in an eppendroff tube with 1.9 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 minutes. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1.9 mL of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under the same conditions as the rest of the samples. For samples of unknown composition, water-soluble antioxidant capacities are expressed as equivalents of ascorbic acid. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid. The experiment was conducted in triplicate and values are expressed as ascorbic acid equivalents in µg per mg of extract.

Quantification of marker compounds by HPTLC

TLC Conditions

Plate: Precoated HPTLC aluminum sheet (MERCK) Silica gel $60F_{254}$ (10cm×10cm)

Spotter: Camag Linomat V, a sample applicator as bands using the spray-on technique, Camag Switzerland.

Developing chamber: CAMAG glass twin trough chamber (20 × 10 cm)

Scanner: CAMAG TLC Scanner 3 and WINCATS software (version 4.06)

Experimental conditions: Temperature 37°C, relative humidity 40 %

Solvent system: Ethyl acetate: Formic acid: Methanol (9:2.4:1.5)

Preparation of sample solution

5.0 g of leaf powder of *M. annua* was extracted exhaustively with methanol (4 × 100 ml) and the extract was filtered, pooled and the combined extract was concentrated under vacuum and volume was made up to 25 ml in a volumetric flask.

Preparation of standard solutions

Standard solution of chlorogenic acid: Accurately weighed 10mg standard Chlorogenic acid was taken in 10 ml volumetric flask. Add 10ml of methanol and dissolved. (1mg/ml)

Calibration curve

Calibration curve for chlorogenic acid: Graded concentration of standard solution of Chlorogenic acid (solution A, 1mg/ml=1000 ng/µl) in which 1, 2, 3, 4, 5 and 6 µl volumes were applied on a precoated HPTLC silica gel 60 F_{254} (10×10cm) using Camag Linomat V automatic spotter. The concentrations of Chlorogenic acid were 1000, 2000, 3000, 4000, 5000 and 6000 ng/ spot.

Quantification of chlorogenic acid in the leaves of M. annua

10 µl of the sample solution was applied in triplicate on a pre-coated silica gel 60 F_{254} HPTLC plate. The plate was developed in the solvent system of Ethyl acetate: Formic acid: Methanol (9:2.4:1.5) and scanned at 329nm. The peak areas and absorption spectra were recorded. The amount of chlorogenic acid in the sample was calculated using the calibration curve.

Method validation

ICH guidelines (CPMP/ICH/381/95; CPMP/ICH/281/95) were followed for the validation of the analytical procedure. The method was validated for precision, repeatability and accuracy. Instrumental precision was checked by repeated scanning of the same spot of chlorogenic acid seven times and was expressed as coefficient of variance (% CV). The repeatability of the method was affirmed by analyzing 3000 ng/spot of chlorogenic acid after application on the TLC plate (n = 6) and was expressed as % CV. Accuracy of the method was tested by performing recovery studies at three levels (50%, 100% and 150% addition). The percent recovery as well as average percent recovery was calculated. For the evaluation of limit of detection and limit of quantification different dilutions of the standard solution of chlorogenic acid were applied along with methanol as blank and determined on the basis of signal to noise ratio.

RESULTS AND DISCUSSION

Total Polyphenols

Total Phenolic Content (TPC) was determined in comparison with standard gallic acid and the results expressed in terms of µg GAE/g dry sample. The TPC values for MEMA (42.53 ± 0.71) and AEMA (31.89 ± 0.86), all as µg Gallic Acid Equivalent (GAE)/g dry sample, respectively. As phenolics are responsible for antioxidant activity, generally, it is expected that extract/drug which contains high TPC would show highest total antioxidant capacity.

Reducing power assay

MEMA and AEMA had effective reducing power using the potassium ferricyanide reduction method when compared to the standard (Ascorbic acid) (Table 1). For the measurement of the reductive ability of MEMA and AEMA, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of MEMA and AEMA using the method of Oyaizu. At different concentrations MEMA (r^2 = 0.9852) and AEMA (r^2 = 0.9928) demonstrated powerful reducing ability. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing power increased as the MEMA concentration increased, indicating some compounds in *M. annua* is both electron donors and could react with free radicals to convert them into more stable products and to terminate radical chain reactions when compared with AEMA. The reducing capacity of a compound may serve as significant indicator of its potential antioxidant activity.

DPPH free radical scavenging activity

Table 2 illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of MEMA, AEMA and ascorbic acid (r^2= 0.8916). The scavenging effect of MEMA (r^2= 0.9524), AEMA (r^2= 0.9237) and ascorbic acid on the DPPH radical decreased in the order of ascorbic acid > MEMA > AEMA, which were 79, 57 and

Table 1: Reducing power ability of MEMA, AEMA and ascorbic acid

Concentration (µg/ml)	MEMA	AEMA	Ascorbic acid
100	0.394 ± 0.0023	0.120 ± 0.0031	0.340 ± 0.0025
200	0.654 ± 0.0035	0.189 ± 0.0016	0.620 ± 0.0103
400	1.151 ± 0.0041	0.301 ± 0.0027	0.780 ± 0.0032
600	1.546 ± 0.0118	0.446 ± 0.0024	0.991 ± 0.0019
800	1.884 ± 0.0021	0.599 ± 0.0013	1.201 ± 0.0118
1000	2.135 ± 0.0032	0.787 ± 0.0009	1.435 ± 0.0025

Values are mean ± S.E.M (n=3)

MEMA- Methanolic extract of *Martynia annua;* AEMA- Aqueous extract of *Martynia annua*

Table 2: Free radical scavenging activity of MEMA, AEMA and ascorbic acid in DPPH method

Concentration (µg/ml)	% free radical scavenging activity of MEMA	% free radical scavenging activity of AEMA	% free radical scavenging activity of Ascorbic acid
10	13.26 ± 1.45	6.51 ± 1.22	39.77 ± 0.86
20	20.91 ± 0.86	13.37 ± 1.73	49.37 ± 1.63
40	26.97 ± 2.17	19.77 ± 0.66	57.14 ± 1.22
60	33.83 ± 1.39	21.25 ± 1.43	65.49 ± 0.92
80	42.97 ± 0.84	30.63 ± 1.79	71.66 ± 1.18
100	57.83 ± 0.92	42.63 ± 2.64	79.89 ± 0.80
200	77.03 ± 1.74	54.51 ± 1.73	92.46 ± 1.29

Values are mean ± S.E.M (n=3)

MEMA- Methanolic extract of *Martynia annua;* AEMA- Aqueous extract of *Martynia annua*

Table 3: Nitric oxide scavenging activity of MEMA, AEMA and ascorbic acid

Concentration (µg/ml)	% radical scavenging activity of MEMA	% radical scavenging activity of AEMA	% radical scavenging activity of Ascorbic acid
100	29.87 ± 1.22	12.33 ± 0.91	38.18 ± 1.74
200	35.66 ± 0.75	18.62 ± 1.23	43.27 ± 2.18
400	39.43 ± 0.92	34.09 ± 2.78	49.37 ± 0.80
600	45.59 ± 2.37	39.69 ± 1.22	53.65 ± 1.67
800	55.84 ± 1.62	46.54 ± 0.87	62.70 ± 2.14
1000	66.10 ± 1.48	52.01 ± 0.21	72.77 ± 1.21

Values are mean ± S.E.M (n=3)

MEMA- Methanolic extract of *Martynia annua;* AEMA- Aqueous extract of *Martynia annua*

42 %, at the concentration of 100 µg mL^{1}, respectively. DPPH free radical scavenging activity of MEMA and AEMA also increased with increasing concentration. IC_{50} values (concentration of sample required to scavenge 50% free radical) were found to be 105.56 µg/mL, 164.56 µg/mL and 15.81 µg/mL for MEMA, AEMA and ascorbic acid (standard) respectively. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow colored diphenyl-picrylhydrazine. This method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radial form DPPH-H by the reaction. DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants (22).

Nitric oxide scavenging activity

The fractions of *M. annua* effectively reduced the generation of nitric oxide from sodium nitroprusside. A MEMA, AEMA and ascorbic acid (standard) exhibited 66.10%, 49.56% and 72.77% inhibition, respectively and the IC_{50} values were found to be 636.44 µg/ml, 912.34 µg/ml and 425.28 µg/ml for MEMA, AEMA and ascorbic acid (standard) respectively (Table 3). Nitric oxide is a free radical which plays an important role in the pathogenesis of pain, inflammation, etc. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent (23). The absorbance of the chromophore was measured at 546 nm in the presence of the fractions. All the fractions of *M. annua* decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. This may be due to the antioxidant principles in the fractions which compete with oxygen to react with NO^- thereby inhibiting the generation of nitrite.

Hydrogen peroxide scavenging activity

A 1000 µg/ml of MEMA, AEMA and ascorbic acid (standard) exhibited 78.45%, 70.02% and 84.74% inhibition, respectively and the IC_{50} values were found to be 357.69 µg/mL, 422.65 µg/mL and 199.82 µg/mL for MEMA, AEMA and ascorbic acid (std.) respectively as shown in Table 4. The composition of hydrogen peroxide into water may occur according to the antioxidant compounds. Since antioxidant compound/(s) present in the extract are good electron donors, they may accelerate the conversion of H_2O_2 to H_2O (24).

Superoxide radical scavenging activity

The Table 5 shows concentration dependent scavenging of superoxide radicals by methanolic and aqueous extracts of *Martynia annua*. A 1000 µg/ml of MEMA, AEMA and ascorbic acid (standard) exhibited 72.81%, 62.47% and 84.65% inhibition, respectively and the IC_{50} values were found to be 256.29 µg/mL, 589.58 µg/mL and 50.41 µg/mL for MEMA, AEMA and ascorbic acid (standard) respectively. Superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen both of which contribute to the oxidative stress (22). The capacity of both the extracts (MEMA and AEMA) to scavenge superoxide radical revealed that these extracts possess superoxide dismutase like activity. Although the activity was found to be lower than scavenging activity of ascorbic acid in all concentration ranges (200 – 1000 µg/mL).

Hydroxyl radical (OH–) scavenging activity

Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of deoxyribose by the free radicals generated by the Fenton reaction. All the fractions of *M. annua* and the ascorbic acid (standard) inhibited the production of hydroxyl radicals. A 500 µg/ml of MEMA, AEMA and ascorbic acid exhibited 67.25%, 52.40% and 75.55% inhibition respectively and the IC_{50} values were found to be 309.03 µg/ml, 471.22 µg/ml and 206.59 µg/ml for MEMA, AEMA and ascorbic acid respectively (Table 6). A concentration dependent inhibition against hydroxyl radical induced deoxyribose degradation was observed in the deoxyribose assay. The oxygen derived hydroxyl radicals along

Table 4: H_2O_2 radical scavenging activity of MEMA, AEMA and ascorbic acid

Concentration (µg/ml)	% radical scavenging activity of MEMA	% radical scavenging activity of AEMA	% radical scavenging activity of Ascorbic acid
10	15.41 ± 1.36	18.02 ± 1.07	28.83 ± 1.65
20	23.01 ± 3.17	23.77 ± 2.46	33.05 ± 0.95
40	28.07 ± 0.83	32.44 ± 1.28	38.57 ± 1.73
80	34.59 ± 1.29	38.57 ± 1.76	43.48 ± 1.97
100	46.17 ± 1.75	41.64 ± 0.68	54.53 ± 2.44
200	58.67 ± 1.44	51.61 ± 3.27	62.27 ± 3.57
400	62.19 ± 2.10	55.60 ± 2.64	67.71 ± 0.85
800	72.47 ± 1.68	66.95 ± 1.21	79.68 ± 1.08
1000	78.45 ± 0.68	70.02 ± 2.25	84.74 ± 1.33

Values are mean ± S.E.M (n=3)

MEMA- Methanolic extract of *Martynia annua;* AEMA- Aqueous extract of *Martynia annua*

Table 5: Superoxide radical scavenging activity of MEMA, AEMA and ascorbic acid

Concentration (µg/ml)	% radical scavenging activity of MEMA	% radical scavenging activity of AEMA	% radical scavenging activity of Ascorbic acid
200	47.55 ± 1.28	36.78 ± 0.85	55.12 ± 1.47
400	55.22 ± 0.94	45.42 ± 1.67	63.43 ± 1.56
600	61.09 ± 2.68	50.32 ± 1.44	69.93 ± 1.40
800	66.10 ± 3.33	56.61 ± 3.12	77.93 ± 0.88
1000	72.81 ± 2.10	62.47 ± 1.75	84.65 ± 1.23

Values are mean ± S.E.M (n=3)

MEMA- Methanolic extract of *Martynia annua;* AEMA- Aqueous extract of *Martynia annua*

Table 6: Hydroxyl radical scavenging activity of MEMA, AEMA and ascorbic acid

Concentration (µg/ml)	% radical scavenging activity of MEMA	% radical scavenging activity of AEMA	% radical scavenging activity of Ascorbic acid
100	34.93 ± 0.58	15.72 ± 2.10	41.05 ± 1.14
200	39.74 ± 1.75	21.83 ± 3.66	48.03 ± 1.89
300	47.16 ± 2.10	33.19 ± 2.85	59.83 ± 2.17
400	57.21 ± 1.43	44.54 ± 1.70	66.38 ± 1.55
500	67.25 ± 1.46	52.40 ± 0.82	75.55 ± 1.31

Values are mean ± S.E.M (n=3)

MEMA- Methanolic extract of *Martynia annua;* AEMA- Aqueous extract of *Martynia annua*

with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid (25). All the fractions of *M. annua* when added to the reaction mixture scavenged the hydroxyl radicals and prevented the degradation of deoxyribose.

Determination of Total antioxidant capacity

Total antioxidant capacity of each of methanolic and aqueous extract of *Martynia annua* Linn. is expressed as number of equivalents of ascorbic acid. One mg of methanolic and aqueous extracts contained 240.0 µg and 94 µg of ascorbic acid equivalents antioxidant capacity respectively. The assay was based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid (26).

Quantification of marker compounds by HPTLC

In the TLC densitometric method, it was observed that the best resolution of chlorogenic acid, avoiding interference from the other components of the extracts, was obtained in the solvent system of Ethyl acetate: Formic acid: Methanol (9:2.4:1.5). The identity of chlorogenic acid in the extracts was established by comparing the Rf (0.68) with the standard compound (Figure 1). HPTLC fluroscent image under the excitation wavelength 329 nm

Table 7: Method validation parameters for the quantification of Chlorogenic acid

Parameters	Chlorogenic acid
Specificity	Specific
Linear range (ng/spot)	1000 – 6000
Correlation coefficient (r^2)	0.9966
Linear regression	y = 6.1012x + 455.34
LOD (ng/spot)	203.21
LOQ (ng/spot)	615.78
% Recovery	96.75 – 99.36
Repeatability (RSD, n=6)	0.715%
Precision (% CV) Intraday (n=3) Interday (n=3)	0.66 – 1.55 0.82 – 1.40
Amount of Chlorogenic acid found in *Martynia annua* leaves (%w/w)	0.100 ± 0.0062

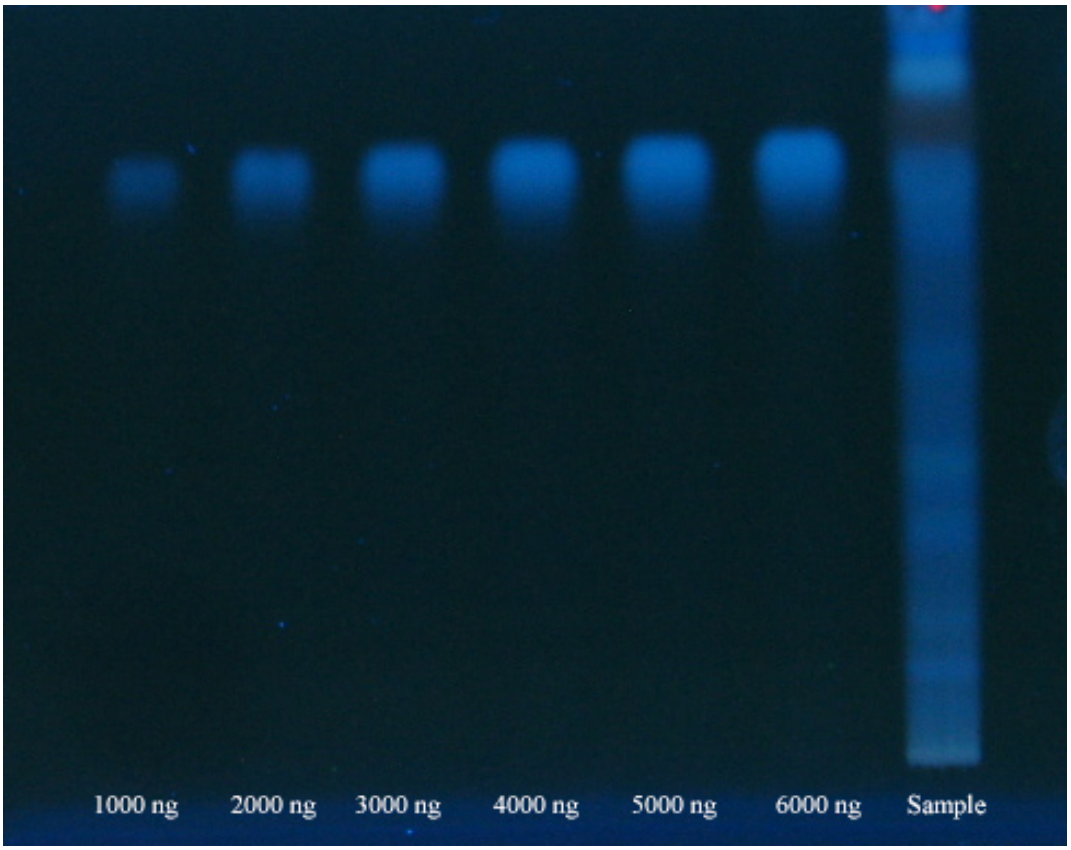

Figure 2. HPTLC fluroscent image under the excitation wavelength 329 nm of standard and sample extract containing Chlorogenic acid.

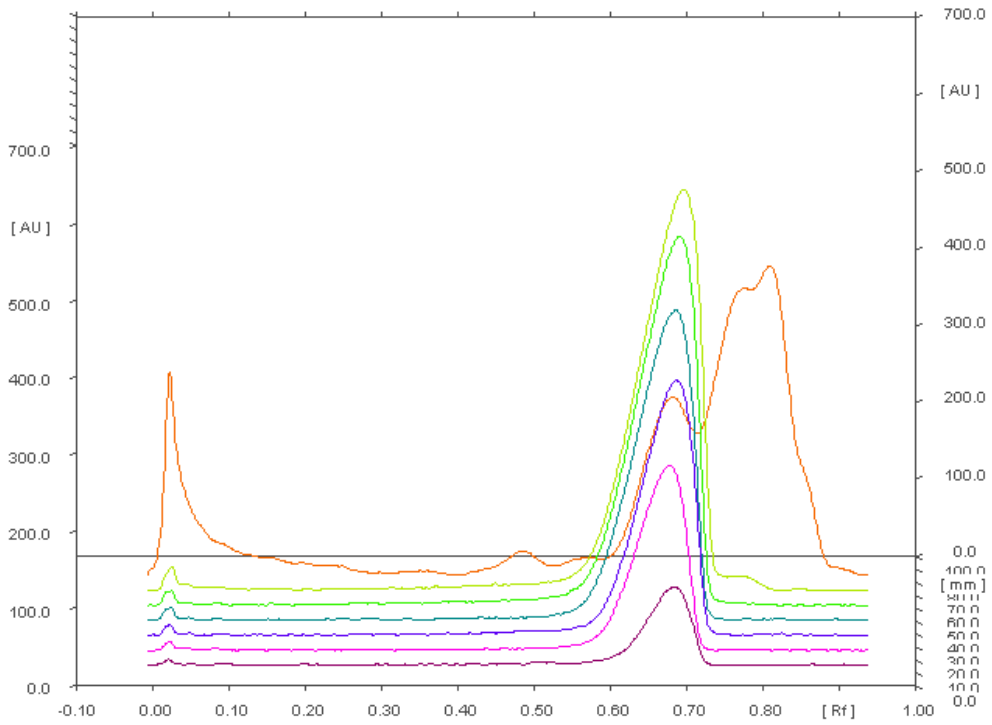

Figure 1. TLC densitometric chromatograms of extract of *Martynia annua* leaves with chlorogenic acid standards.

Table 8: Recovery study of Chlorogenic acid by HPTLC method

Marker compound	Amount present in the sample (ng)	Amount added (ng)	Amount found (ng)	Recovery (%)	Average recovery (%)
Chlorogenic acid	1000	500	1457 ± 74.18	97.13 ± 4.94	97.75
	1000	1000	1935.33 ± 15.57	96.75 ± 0.77	
	1000	1500	2484 ± 48.49	99.36 ± 1.93	

Values are mean ± SD (n=3)

of standard and sample extract containing chlorogenic acid was displayed in Figure 2. Linear relationship was obtained for chlorogenic acid in the range of 1000 – 6000 ng/spot with a correlation coefficient of 0.9966. The method was validated in terms of precision, repeatability and accuracy (Table 7). The limit of detection and limit of quantification were found to be 203.21 ng/spot and 615.78 ng/spot respectively. The content of chlorogenic acid estimated in the *M. annua* leaves extract was found to be 0.100 ± 0.0062 % W/W.

From the recovery studies that were carried out at three different levels i.e. 50 % addition, 100 % addition and 150 % addition of marker compound, the average percentage recovery obtained was 97.75 % (Table 8). Chlorogenic acid is reported for the first time in this plant. It was confirmed by TLC with a standard chlorogenic acid sample. The TLC densitometric method developed for the quantification of chlorogenic acid from *M. annua* leaves was found to be simple precise, accurate and robust and can be used for quality control of *M. annua* leaves.

CONCLUSION

In conclusion, the present study has demonstrated that *Martynia annua* methanolic and aqueous extracts are rich in phenolics and have a strong antioxidant activity and a radical-scavenging action in all of the tested methods. This suggests that *Martynia annua* leaf is a good source of natural antioxidants. The isolation, purification and mechanism action of chlorogenic acid and other components of *M. annua* are of interest for further investigation and will be carried out in future studies.

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