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PHYTOCHEMICAL & PHARMACOLOGICAL EVALUATION OF MARTYNIA ANNUA L. FOR IN-VITRO ANTI-OXIDATIVE & IN-VIVO ANTI-DIABETIC SCREENING

Vivekanand Katare and Chandra Kishore Tyagi*

Faculty of Pharmacy, Sri Satya Sai University of Technology & Medical Sciences, Sehore - 466001, India.

ABSTRACT

The preliminary phytochemical analysis revealed the existence of various secondary metabolites with varied percentage. Among the four different extracts of *Martynia annua* L. aerial parts, ethanolic extract showed the presence of maximum number (10/13) of compounds. The powdered drugs are evaluated for its physico-chemical parameters like total ash, acid insoluble ash, water soluble ash and moisture content.

Keywords: *Martynia annua* L., Anti-oxidative Potentials, Phytochemical Screening, Rat Plasma, Anti-Diabetic.

INTRODUCTION

Martynia annua L. (Martyniaceae) is one of the medicinal herbs used by native people of Mexico since ancient time for numerous therapeutic purposes. The plant is inborn to Mexico but now well familiarized throughout India on waste lands [1]. In India *Martynia annua* L. is well recognized small herbaceous annual plant. It is commonly known as Devil's claw or Cat's claw denotes to the inner woody capsule which splits open at one end into two curved horns or claws [2-7]. In Ayurveda, the plant is known as Kakanasika in Sanskrit. In Hindi it is called as Bichhu and in Gujarati it is known as Vinchudo, which is being used in Indian traditional medicines for epilepsy, inflammation and applied locally for tuberculosis glands of camel's neck [8-11]. The Martyniaceae family has three genera and these genera have sticky, hairy leaves, orchid-like flowers and woody, beak shaped pods. The seeds of the yellow-flowered Ibicellalutea, which is native to South America, are not commercially available in United Status, although the species occurs as an occasional weed in California's Central Valley. Introduced members of the Martynia and Proboscidea genera are often found growing as weeds in the South-western United Status [12]. So *Martynia annua* L. belongs to Mexico and Central America. It is naturalized throughout tropical regions of the world.

MATERIALS AND METHODS

Plant Material Collection and Authentication

The fresh bark of *Martynia annua* were collected from the field area of Bhopal district M.P. India. For identification and taxonomic authentication, plant material was submitted in Department of Botany, Saifia College, Bhopal, India. Its authenticity was confirmed and authenticated by Dr. Zia-Ul-Hasan. Collected plant materials were shade-dried and coarsely powdered.

Preparation of Extract

Shade-dried and coarsely powdered 100gm powder from bark of *Martynia annua* were soaked in 500 ml of methanol [methanol/drug mass ratio 5:1] separately. It was kept at room temperature for 48 hours with intermittent mixing. Methanol extract of plants (MEMA) obtained after 48 hours of soaking was filtered using Whatman paper. The extracts, which was thus obtained, was evaporated to make it into the powder form to re-dissolve in methanol.

In-vitro Antioxidant Assay

Few different assays were performed to determine the anti-oxidative power of *M. annua* extracts (1–250µg/mL) as described below. In each of these assays, ascorbic acid was used as a reference substrate. The ability of the extract to scavenge or inhibit free radicals was expressed as percentage inhibition and was calculated using the following formula:

Corresponding Author: **Chandra Kishore Tyagi** Email: kishore198012@gmail.com

$$Percentage\ inhibition = \frac{(A_0 - A_t)}{A_0} \times 100$$

Where, A_0 is absorbance of the control group (without plant extract) and A_t is absorbance of *M. annua* extracts. All determinations were carried out in triplicate with the readings taken in duplicate.

These assays were performed to screen the best extracts in terms of its anti-oxidant potential. The percent inhibition of oxidation of different standard compounds was studied and the final selection was made.

DPPH Radical Scavenging Activity

DPPH radical scavenging activity of aqueous extract was determined according to the method by Katalinic *et al.* In brief, 0.5mL of 0.1mM DPPH solution was prepared in methanol just before use. 1.0 mL of aqueous extract was added at different concentrations (1-250µg/mL) to DPPH solution. Double distilled H_2O was used in the control group instead of samples, with the same procedures applied. The ability of the substrate to reduce the stable radical DPPH from deep purple to yellow coloured diphenylpicryl hydrazine indicates its anti-oxidative potential. The mixture was shaken vigorously and left to stand for 30min in the dark, and absorbance was measured at 517 nm using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Lower absorbance at 517 nm represents higher DPPH scavenging activity.

Superoxide Radical Scavenging Activity

Measurement of superoxide radical scavenging activity of *M. annua* extract followed the method by Xiang and Ning. In brief, superoxide anions were generated in non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of NBT in the presence of different concentrations (1-250 µg/mL) of the extract. The reaction was initiated by adding 0.75mL of PMS (120 µM) to the mixture. The absorbance was measured at560 nm by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) following 5-minute incubation at room temperature. Ascorbic acid was used as standard comparator. Percentage inhibition of superoxide radical formation (scavenging of superoxide radicals) was determined as per the formula given in the section above.

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of *M. annua* extract was measured according to a modified method by Eswar Kumar *et al.* The reaction mixture contained 60 µL of 1.0mM$FeCl_2$, 90 µL of 1mM 1,10-phenanthroline, 2.4mL of 0.2M phosphate buffer (pH 7.8), 150 µL of 0.17M hydrogen peroxide(H_2O_2), and 1.5mL of different concentrations of the extract(20–300 µg/mL). H_2O_2 was added at the start of the reaction. After incubation at room temperature for 5 min. absorbance of the mixture was measured by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 560 nm.

***In-Vivo* anti-diabetic Potential**

Experimental Animals

Adult male Wistar rats weighing180–210 g was obtained from Animal House. Five to six animals were housed together under a standard environmental condition of temperature 25 ± 2°C, relative humidity between 45 and 55%, and 12 hr light/dark cycle. Rats had free access to standard food pellet and water *ad libitum*. The experimental protocol was in accordance with guidelines given by CPCSEA.

Study Design

Selection of diabetic model for oxidative stress

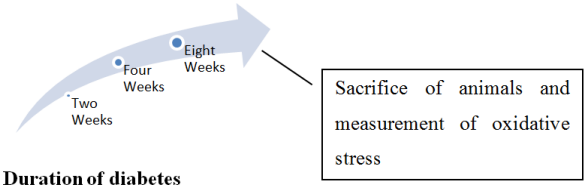

Fig. 1. Study Design for setting the model

Induction of Diabetes

Diabetes was induced in overnight fasted rats by a single intraperitoneal (*i.p.*) injection of freshly prepared STZ (55 mg/kg body weight, in ice-cold 0.1 M citrate buffer, pH 4.5, in a volume of 0.1 ml per rat).

Seventy two hours after STZ administration, the plasma glucose level of each rat was determined for confirmation of diabetes. Rats with plasma glucose level above 300 mg/dl were considered as a diabetic and used subsequently for further study. The values of lipid per-oxidation, GSH content and SOD and catalase activity are depicted in the above tables. Statistical analysis with ANOVA indicated significant (P< 0.001) oxidative stress in plasma and liver of 8-week diabetic rats. Hence, 8-week duration of diabetes was considered as optimum for further studies.

Estimation of LPO

LPO was estimated by thio-barbituric acid (TBA) reaction with malondialdehyde (MDA), where the latter was a product formed from membrane lipid per-oxidation. In brief, 2.5mL homogenate, 0.5 mL of 0.9% NaCl, and 1.0mL 20% w/v TCA were added into the mixture. The mixture was then centrifuged for 20minutes at 4000 ×g at 4°C. 0.25mL TBA reagent was added to 1.0 mL supernatant and the mixture was incubated at 95°C for 1 hour and cooled under running tap water prior to addition of 1mL n-butanol. After a thorough mixing, the mixture was centrifuged for 15 minutes at 4000 ×g at 4°C. The organic layer was transferred into a clear tube and absorbance was measured at 532 nm with a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). The rate of lipid per-oxidation was expressed as μ moles of MDA formed/gram wet weight of the tissue.

Estimation of SOD Activity

SOD activity was assayed according to the method by Misra and Fridovich. The assay procedure involves inhibition of epinephrine auto-oxidation to

adrenochrome in an alkaline medium (pH 10.2), which was markedly inhibited in the presence of SOD. 1.5 mLcarbonate buffer (0.05M, pH 10.2) and 0.5mL ethylene-diamine-tetra-acetic acid (EDTA) (0.49M) were added to 0.5mLsupernatant. The reaction was initiated by the addition of0.4mL epinephrine (3 mM). Changes in absorbance were recorded at 480 nm for one min at 15 sec interval, 3min each, by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). SOD activity levels were expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50%, which was equal to 1 U per milligram of protein.

Estimation of CAT Activity

CAT enzyme activity was determined on the basis of hydrogen peroxide decomposition. The reaction solution contained 2.5mL of 50mM phosphate buffer (pH 5.0) and 0.4mL of 5.9mM H_2O_2.The reaction was initiated by adding 0.1mL enzyme extract. Changes in the absorbance of the reaction solution were monitored every 30s and was read by using a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 240 nm. The enzyme activity levels were expressed as µmol of hydrogen peroxide (H_2O_2) metabolized/mg protein/min.

Estimation of GSH content

Reduced glutathione was estimated by Ellman method.

Experimental Design

Animals were randomly divided into five groups with six rats in each group:

Group 1: Diabetic: received vehicle only.

Group 2: Standard antioxidant (Vitamin C 500 mg/kg/day).

Groups 3: Treated with standard anti-diabetic agent (Metformin 500 mg/kg/day).

Group 4, 5 and 6: *M. annua* aqueous extract at 100, 200, and 500 mg/kg/day.

Group 7 and 8: *M. annua* ethanolic extract at 100, 200, and 500 mg/kg/day.

All the animals were given above-mentioned treatments after induction of diabetes for duration of 8 weeks. All doses were administered on body weight basis in a dose volume of 10 ml/Kg and the solutions were freshly prepared in 0.5 % CMC or distilled water.

RESULTS AND DISCUSSIONS

***In vitro* Antioxidant Assay**

DPPH radical scavenging activity

The DPPH assay revealed that all the extracts of *Martynia annua* possess potent antioxidant activity. The activity was found to be concentration dependent with linear increased in the concentration of 10-100 µg/ml. The ether extract produced 11.33 % inhibition at 1 µg/ml which increased in concentration dependent manner and reached maxima at 250 µg/ml. Similarly, aqueous extract produced 12 % inhibition of DPPH oxidation at 1µg/ml and reached maxima at 250 µg/ml. The ethanolic extract was found to produce marked inhibition at all the concentrations employed and was higher than other extracts used. The maximal inhibition was found to be at 100 µg/ml. Finally it was comparable to that of Ascorbic acid that was employed as reference standard. In conclusion the ethanolic extract was found to possess potent concentration dependent antioxidant activity, which was comparable to that of ascorbic acid.

Table 1. Percentage Inhibition of DPPH oxidation by *Martynia annua* Extract at Different Concentration.

Sr. No.	Conc. (µg/ml)	Ether Extract (Mean±SEM)		Aq. Extract (Mean±SEM)		EtOH Extract (Mean±SEM)		Ascorbic acid (Mean±SEM)	
1	1	11.33	3.72	12.83	2.64	22.67	1.75	32.33	1.51
2	5	17.17	4.26	16.83	2.64	32.33	1.51	40.83	2.64
3	10	29.67	5.32	28.00	4.86	40.67	3.01	47.50	5.01
4	25	45.17	6.05	38.50	5.09	48.67	4.41	63.00	3.41
5	50	61.00	4.77	53.83	8.42	63.50	3.08	80.33	3.01
6	100	70.17	3.43	70.67	5.13	80.00	3.16	93.50	3.67
7	250	83.00	4.73	91.83	4.92	93.50	3.67	98.67	1.97

Each value represents mean ± SEM of n=6 observations

Table 2. Percentage Inhibition of Superoxide Radical by *Martynia annua* Extract at Different Concentration.

Sr. No.	Conc. (µg/ml)	Ether Extract (Mean±SEM)		Aq. Extract (Mean±SEM)		EtOH Extract (Mean±SEM)		Ascorbic acid (Mean±SEM)	
1	1	10.17	3.43	11.83	1.33	22.33	1.37	24.33	2.50
2	5	22.00	1.79	18.00	2.10	33.83	1.72	34.33	2.34
3	10	34.50	2.07	28.00	2.10	40.67	3.01	49.17	3.97
4	25	44.50	2.07	37.67	2.16	45.83	1.72	62.00	2.19
5	50	60.33	3.39	55.83	3.31	64.50	3.39	73.17	2.86
6	100	70.50	3.62	72.83	2.32	80.00	2.28	86.67	2.58
7	250	84.50	2.81	93.67	3.44	91.67	2.42	97.00	3.41

Each value represents mean ± SEM of n=6 observations

Table 3. Percent Inhibition of Hydroxyl Radical Oxidation by *Martynia annua* Extract at Different Concentration.

Sr. No.	Conc. (µg/ml)	Ether Extract (Mean±SEM)		Aq. Extract (Mean±SEM)		EtOH Extract (Mean±SEM)		Ascorbic acid (Mean±SEM)	
1	1	17.50	1.87	18.67	2.50	16.67	2.58	17.50	1.87
2	5	32.17	1.94	31.17	1.47	30.33	1.63	30.33	1.03
3	10	47.50	3.39	43.50	2.43	39.17	1.47	47.50	1.87
4	25	60.83	4.62	61.83	2.64	46.50	1.05	61.50	1.38
5	50	73.67	2.73	70.67	1.97	58.50	2.88	73.17	2.86
6	100	83.83	2.79	83.83	2.64	73.33	3.44	86.00	2.00
7	250	94.33	2.50	95.00	1.90	97.50	1.87	97.00	3.41

Each value represents mean ± SEM of n=6 observations

Table 4. General Features of Experimental Rats

Sr. No.	Parameter	Groups			
		Vehicle control	Diabetes Two weeks	Diabetes four weeks	Diabetes eight weeks
1	Initial blood glucose (mg/dl)	90.8 ± 11.1	401.3 ± 84.1***	259.4 ± 53.8***	359.4 ± 53.8***
2	Final blood glucose (mg/dl)	85.3 ± 10.3	386.5 ± 43.2***	312.6 ± 56.6***	312.6 ± 56.6***
3	Terminal Body weight (g)	260.5 ± 8.9	191.3 ± 15.9***	223.3 ± 16.3***	243.3 ± 16.3***
4	Food intake (g/rat/day)	7.16 ± 1.33	9.44 ± 0.88***	9.8 ± 1.47***	10.8 ± 1.47***
5	Water intake (ml/rat/day)	29.50 ± 5.3	50.5 ± 6.2***	47.6 ± 7.1***	57.6 ± 7.1***

Table 5. Oxidative Stress Parameters in Rat Plasma

Sr. No.	Parameter	Groups			
		Vehicle control	Diabetes Two weeks	Diabetes four weeks	Diabetes eight weeks
1	LPO (TBARS nM/mg protein)	15.3 ± 031	17.8 ± 2.8	21.3±4.8	30.2 ± 2.5
2	GSH (µg/mg protein)	1.03 ± 0.09	0.85 ± 0.15	0.88 ± 0.22	0.65 ± 0.09
3	SOD (U/mg protein)	42.2 ± 5.2	49.2 ± 3.2	40.1 ± 1.2	30.1 ± 2.5
4	Catalase SOD (U/mg protein)	125.2 ± 10.2	130.1 ± 8.5	135.3 ± 5.1	90.1 ± 2.3

Table 6. Oxidative Stress Parameters in Rat Liver.

Sr. No.	Parameter	Groups			
		Vehicle control	Diabetes Two weeks	Diabetes four weeks	Diabetes eight weeks
1	LPO (TBARS nM/mg protein)	1.53 ± 0.31	1.8 ± 0.8	2.1±0.8	3.2 ± 0.5**
2	GSH (µg/mg protein)	16.03 ± 1.2	15.2 ± 1.5	8.8 ± 2.2*	6.5 ± 0.9**
3	SOD (U/mg protein)	12.2 ± 2.2	18.3 ± 3.2	20.1 ± 1.2*	5.1 ± 2.5*
4	Catalase SOD (U/mg protein)	75.2 ± 10.2	85.1 ± 8.5	105.3 ± 5.1	55.1 ± 2.3**

Table 7. Effect of Various Treatments on the Diabetes Induced Oxidative Stress in Rat Plasma

Sr. No.	Parameter	Groups (Diabetes 8 weeks)								
		Control	Metformin	Vitamin C	Aqueous Extract			Ethanolic Extract		
			500 mg	100 mg	100 mg	200 mg	500 mg	100 mg	200 mg	500 mg
1	LPO (TBARS nM/mg protein)	3.2 ± 0.5	3.7 ± 0.2	1.2 ± 0.7	3.1 ± 0.1	3.7 ± 0.5	1.8 ± 0.1*	3.9 ± 0.5	2.2 ± 0.2*	0.9 ± 0.5**
2	GSH (µg/mg protein)	6.5 ± 0.9	8.5 ± 1.9	10.5 ± 0.9*	6.9 ± 1.1	5.2 ± 0.8	11.1 ± 1.0*	6.5 ± 0.9	6.5 ± 0.9	6.5 ± 0.9
3	SOD (U/mg protein)	5.1 ± 2.5	7.1 ± 2.5	10.1 ± 2.5*	6.1 ± 1.5	7.1 ± 2.5	11.1 ± 0.5*	6.1 ± 2.3	7.5 ± 1.5	15.1 ± 1.5*
4	Catalase SOD	55.1 ±	75.1 ± 2.3*	85.1 ±	59.1 ±	61.0 ±	85.1 ±	25.1 ±	55.9 ±	85.1 ±

	(U/mg protein)	2.3		3.5*	1.3	5.1	5.3*	15.1	2.9	3.3*
5	Blood glucose (mg/dl) Initial	321.1 ± 10.2	301.1 ± 20.2	251.1 ± 16.2	311.1 ± 11.3	301.1 ± 10.2	283.1 ± 15.2	295.1 ± 18.7	315.1 ± 9.2	333.1 ± 13.5
6	Blood glucose (mg/dl) Final	352.1 ± 15.2	152.1 ± 25.2**	298.1 ± 10.2	352.1 ± 16.5	322.1 ± 11.1	201.1 ± 1.2*	312.1 ± 11.2	281.1 ± 11.2*	221.1 ± 15.2**

All the values are mean ± SD of 5-6 observations each. One-way ANOVA followed by Tukey's test (*P<0.05, **P<0.01, and ***P<0.001 vs. respective control group)

Superoxide Radical Scavenging Activity

The superoxide radical scavenging assay revealed that all the extracts of *Martynia annua* possess potent antioxidant activity. The activity was found to be concentration dependent with linear increased in the concentration of 10-100 µg/ml. All the extracts produced comparable results at all concentrations used with about 20 % inhibition at 1 µg/ml which increased in concentration dependent manner and reached maxima at 250 µg/ml. This was found to be comparable to that of Ascorbic acid that was employed as reference standard. In conclusion, in this assay all the extracts exhibit potent concentration dependent antioxidant activity, which was comparable to that of ascorbic acid.

Hydroxyl Radical Scavenging Activity

The superoxide radical scavenging assay revealed that all the extracts of *Martynia annua* possess potent antioxidant activity. The activity was found to be concentration dependent with linear increased in the concentration of 1-250 µg/ml. All the extracts produced comparable results at all concentrations used with about 20 % inhibition at 1 µg/ml which increased in concentration dependent manner and reached maxima at 250 µg/ml. This was found to be comparable to that of Ascorbic acid that was employed as reference standard. In conclusion, in this assay all the extracts exhibit potent concentration dependent antioxidant activity, which was comparable to that of ascorbic acid.

***In vivo* anti-diabetic Potential**

At the end of 8-weeks of various treatments the animals of different groups were sacrificed and the oxidative stress parameters were measured in there plasma. The results revealed that the standard viz., metformin and Vitamin C were able to control either the oxidative stress of glucose alone and were not as effective as *Martynia annua* extract in reducing the LPO levels, and increasing the GSH levels in diabetic rats.

Similarly, the SOD and catalase activities were also increased after the treatments with aqueous and ethanolic extracts. Treatment with Vitamin C did reduce the LPO levels and increased the GSH levels and SOD and catalase activities, but had no impact on the altered blood glucose levels. Similarly, metformin though controlled the blood glucose levels properly was not able to reduce the lipid peroxidation levels effectively.

However, the aqueous as well as ethanolic extract of *Martynia annua* not only regulated the blood glucose levels effectively but also was able to control the oxidative stress levels effectively. However, they did so it in different doses and hence concluding it can be said that the ethanolic extract exhibits good anti-diabetic and anti-oxidant activity.

CONCLUSION

The physico-chemical parameters are useful in judging the purity and quality of the drug. The acetone extracts of *M. annua* showed the presence of 19 different types of flavonoids with 8 different R_f values ranging from 0.20 to 0.97. Among the 19 different types of alkaloids, two alkaloids with R_f values 0.67 and 0.89 were unique to the root of *M. annua* and Rf values 0.72 and 0.97 were unique to the leaves of *M. annua*. The acetone extracts of *M. annua* showed the presence of 25 different types of flavonoids with 9 different Rf values ranging from 0.06 to 0.96. Among the 25 different types of flavonoids observed, flavonoid with Rf value 0.67 was unique to the *M. annua* root, *M. annua* stem and *M. annua* seed. The acetone extracts of *M. annua* showed the presence of 63 different types of tannins with 19 different Rf values ranging from 0.02 to 0.97. Among the 19 different types of tannins observed, tannins with Rf value 0.48 showed its presence in the aerial parts of *M. annua* root and *M. annua* seed. The tannins with Rf values 0.62, 0.86 and 0.91 were unique to *M. annua* root, *M. annua* stem and *M. annua* leaves. The acetone extracts of *B. garcini* and *M. annua* showed the presence of 76 different types of steroids with 27 different R_f values ranging from 0.04 to 0.96.

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