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PHYOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *Martynia annua FOR* IMMUNOMODULATORY POTENTIAL

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Abstract

Ethnopharmaco logical relevance

Martynia annua L (Martyniaceae) is a well-known aquatic plant which has been used for the treatment of several disorders including skin disease, cough, inflammation, fever and many other disorders.

Aim of the study

To explore the immunomodulatory activity of extract of (MEMA) and (MEAC) of the plant.

Materials and methods

The immunomodulatory activity of MEMA and MEAC was evaluated using various *in vivo models* including the total and differential leukocyte count (TLC and DLC), nitroblue-tetrazolium reduction (NBT) test, neutrophil adhesion test, phagocytic response and delayed type hypersensitivity (DTH) reaction. Sheep red blood cells (SRBC, 5 × 109 cells/ml) were used to immunize the animals. NNRE and NNSE at the doses of 100 and 300 mg/kg were administrated.

Result

The TLC and lymphocyte count increased significantly but the neutrophil count was decreased for MEMA and MEAC treated groups compared to the control. A dose-dependent potentiation of DTH reaction induced by SRBC was observed from the extracts. The percentage of neutrophil adhesion to the nylon fiber was increased in MEMA treated groups (63.22 and 62.91%)

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compared to the MEAC treated group (54.86 and 54.23%). A potential phagocytic response was seen on treatment of the extracts, and significant changes were observed in the formation of formazone crystals.

Conclusion

This finding suggests that the extract of rhizome and seed *Martynia annua* stimulate defense system by modulating several immunological parameters.

INTRODUCION

Natural products and folklore medicines are the main contributors of the leads in the design and development of therapeutic agents. Several plant derived compounds have been identified over the years for their immunomodulatory characteristics [(1)]. Numerous illnesses can be alternatively treated by immunomodulation using medicinal plants, instead of chemotherapy. The discovery and isolation of more specific immunomodulatory agents from plant origin possesses potential to counteract the side effects and high cost of synthetic compounds. This highlights the significance of medicinal plants as producers of immunomodulatory molecules of very varied chemistries with possible uses in animal and human health [(2)]. The challenges encountered by the application of plant derived immunomodulators need to be addressed. Though, the path from traditional medicines to western pharmaceutical practices is not always easy. The inconsistency of responses of phytomedical practices can be clarified in terms of the typically strong reliance of plant secondary metabolite profiles on environmental signals that can disturb reproducibility of results with extracts. This can be decreased if the principles of standardization of extracts and enriched fractions are thoroughly applied [(3)].

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.

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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.

Drugs

Cyclophosphamide (Endoxan from Cadila Healthcare Limited) 50 mg/kg.b.w was used intraperitoneally [(5)]. Methanol extract of *Martynia annua* (MEAC), was used at a dose of 150 and 300 mg/kg p.o. [(6)] was used at a dose of 800 and 1200 mg/kg, p.o. [(7)]. Cyclophosphamide and plant extracts doses and dosing schedules were based on published report.

Test animals

Healthy mice (25-30 g) of either sex were selected for the study. They were kept in the Animal House of Faculty of Pharmacy, College of Pharmacy, SSSUTMS, Sehore in colony cages at an ambient temperature of 25 ± 2 °C and relative humidity 45–55 % with 12 h light/dark cycles after initial acclimatization for about 1 week. They had free access to standard rodent pellet diet and water ad libitum. The experimental protocol and animal house has been approved by the institutional ethical committee with approval no. COP/Pharm/Ph.D./CPCSEA/12/06.

Antigenic materials

For the present study, the antigenic material used was sheep RBCs (SRBC). Fresh blood was collected from sheep sacrificed in the local slaughter house. It was mixed with Alsever's solution in 1:1 proportion and was stored at 4ºC in the refrigerator.

Table 1. Composition of Alsever's Solution

Contents	**% W/ V**
Glucose	2.05
Sodium Chloride	0.42
Sodium Citrate	0.80

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Citric Acid	0.55

During the experimentation, from the above stock solution (i.e. SRBCs, stored in Alsever's solution), an enough quantity of blood was taken and was allowed to stand at room temperature. It was washed three times with pyrogen free normal saline (0.9% w/v NaCl). Using Neubauer's chamber, the RBC count of this suspension was determined by hemocytometer. The known amount of RBCs (0.5x109 cells/ml/100g) was injected intraperitoneally to the mice as an antigenic challenge.

Immunosuppressant

In the present study cyclophosphamide (CP) was used as immunosuppressing agent [8].

Dosing schedule

Animals were divided into six groups (I-VI). Each group comprised of a minimum of six animals. Group I (control) animals received normal saline for 7th consecutive days; group II (CP) animals were injected with a single dose of CP on 6th day of initiation of experiment. Group III (MEMA 1) animals in MEBC treatment and group III, IV (MEAC 1 and MEAC 2) animals in MEAC treatment, received plant extract treatment for 7th consecutive days. Group V, VI animals (MEBC 1+ CP and MEBC 2 + CP), in MEBC treatment and V, VI animals in MEAC treatment (MEAC 1+ CP, and MEAC 2 + CP), were given plant extract treatment for 7th days along single injection of CP on 6th day of initiation of experiment. For humoral response animals of all groups were challenged with 0.2 ml of 10% SRBC *i. p.* on the 5th day. It was perform using the procedure of Bin-Hafeez et al. (2001) with some modifications. Cell Mediated Immunity was assayed by footpad reaction method. On the 7th day, SRBC was injected in right hind paw of animals of all groups. While 0.9% saline was injected into the left hind paw of the mice of all groups. Blood parameter was assessed in the blood, withdrawal from tail veins. The mice were decapitated under ether anesthesia 24 hr. after the last dose, for body weight determination.

Table 2. Dosing Schedule of MEBC Treatment

GROUP	TREATMENT	TREATMENT SCHEDULE
I	Control	1 to 7th day Normal saline
II	Cyclophasphamide (CP)	1 to 7th day saline, SRBC on 5th day, CP on 6th day

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III	MEBC1	1 to 7th day MEBC1
IV	MEBC2	1 to 7th day MEBC2
V	MEBC1+CP	1 to 7th day MEBC1, SRBC on 5th day, CP on 6th day
VI	MEBC2+CP	1 to 7th day MEBC2, SRBC on 5th day, CP on 6th day

Table 3. Dosing Schedule of MEAC Treatment

GROUP	TREATMENT	TREATMENT SCHEDULE
I	Control	1 to 7th day Normal saline
II	Cyclophasphamide (CP)	1 to 7th day saline, SRBC on 5th day, CP on 6th day
III	MEAC1	1 to 7th day MEAC1
IV	MEAC2	1 to 7th day MEAC2
V	MEAC1+CP	1 to 7th day MEAC1, SRBC on 5th day, CP on 6th day
VI	MEAC2+CP	1 to 7th day MEAC2, SRBC on 5th day, CP on 6th day

Immunization schedule

All the above groups' mice were antigenically challenged with SRBC (0.5x109 cells/ml/100 g) on the 5th day intraperitoneally [9].

Humoral immune response model

By using the method of Bin-Hafeez et al. (2001) with some modification, measurement of antibody titer by hemagglutination reaction was performed. Anesthetic ether was used to anaesthetize mice. With the help of a fine capillary gently inserted into the lower angle of the eye at 45°, the blood was obtained from retro-orbital plexus. The blood was collected into the vial and centrifuged for separating serum. The mice serum was used for analysis of hemagglutination titer. Microtitration plate having 96 cups was used for carrying out titration. Each cup was filled with 25±l µl of normal saline. 25±l µl of serum obtained from mice blood was added to 1stcup and was mixed with 25±l µl of normal saline present in microtitration plate. By this method, two-fold serial dilutions of serum were prepared. To each cup 25±l µl of 1% v/v SRBC was added. The plate was incubated at 37oC for one hr. and then was observed for agglutination. The antibody titer was expressed in terms of maximum dilution, which gave positive hemagglutination reaction [10, 11].

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Cellular immune response model

Footpad reaction test was done for cell-mediated immune response. On 7th day, after measuring the volume of footpad of both legs, SRBC (0.025x109 cells) was injected in right paw and 0.025 ml of saline was injected into the left paw of animals of all groups. On 8thday after 24 hours, the paw volume was measured again to check the increase or decrease in volume. The increase in paw volume was considered as an index of cell-mediated immunity (delayed type hypersensitivity) [12, 13].

Blood Parameters

For the detection of blood parameters, blood withdrawn from the above antigenically challenged mice were used to check hematological parameters (hemoglobin, RBCs, and WBCs) [14].

Relative organ weight Determination

For relative organ weight determination, animals of all groups were sacrificed 24 hr after the last dose. Relative organ weight (organ weight/100g of body weight) of the liver, kidney, and spleen were determined for each animal [15].

Assessment of antioxidant parameters

In all group animals, Spleen was collected after the scarification and washed immediately with cold saline to remove blood. Spleen tissues of mice were homogenized (10%) in phosphate buffer (pH 7.4). The homogenate was centrifuged at 12000g for 20 min at 4°C to obtain the supernatant, and it was used for the estimation of LPO, reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD).

Assay of TBARS

Lipid peroxidation is a free radical settled event. The primary products of such damage are a complex mixture of peroxides that then break down to produce carbonyl compounds. The MDA (malondialdehyde) is one such carbonyl compound, which forms a characteristic chromogenic adduct with two molecules of TBA. The colorimetric reaction of TBA with MDA, a secondary product of lipid peroxidation, has been widely accepted for measuring lipid peroxidation. The total protein that was present in the homogenate was estimated by following the method that was

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described by Lowry *et al.* [16] The TBARS assay was performed according to earlier reported method[17]. 1 ml of homogenate was combined with 2 ml of TCA-TBA, HCl reagent and mixed thoroughly the solution was then heated on a boiling water bath for 15 min. then the mixture was cooled and centrifuged for 15 min. The supernatant absorbance was read at 535 nm against a blank solution. TBARS activity was determined using a molar extinction coefficient of 1.56×105 M^{-1} cm^{-1}. The units of TBARS activity expressed in terms of nmoles MDA/mg protein.

Assay of Glutathione

This spectrophotometric procedure was based on the method of Ellman. DTNB [5, 5'-dithiobis-(2-nitrobenzoic acid)] is reduced by –SH groups to form one mole of 2-nitro-5- mercaptobenzoic acid per mole of –SH. The GSH activity unit was expressed in terms of µg/mg protein [18].

Assay of SOD

The assay of SOD was carried out, based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol as described by McCord with some modification. The total protein that was present in the homogenate was estimated by the method that was described by Lowry *et al.* [19]. The units of the SOD activity which were determined were expressed in terms of Units /mg protein [20].

Assay of Catalase

Catalase activity was determined using Aebi's method with some modifications. In the UV range, H_2O_2 shows a continuous increase in the absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in the absorbance at 240 nm. The difference in absorbance (ΔA) per unit time is a measure of the catalase activity. The molar extinction coefficient of H_2O_2, 43.6 M^{-1} cm^{-1} was used to determine the catalase activity. The units of the CAT activity which were determined were expressed in terms of nmol H_2O_2/mg protein [21].

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Determination of TNF-α, and IL-6 level

The concentrations of TNF- α and IL-6 in the mice serum were determined using specific quantitative sandwich ELISA kits according to the instruction of the manufacturer purchased from Pierce Biotechnology, Rockford, IL, USA [(22)].

Statistical Analysis

All the results were expressed as means ±SEM. Data was analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey-Karmer multiple comparison tests to determine significant differences in data of various groups. P values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Haemagglutinating antibody (HA) titer

The HA titer was used to assess humoral immune response. The effect of *Martynia annua* extract on humoral immune response showed that the administration of both the plant different doses in group 3rd and 4th group's animals produced a significant dose-related increased in H.A titer value when compared to control group animals (Table 2). While in negative control group animals, cyclophosphamide treatment produced a significant ($P<0.001$) decreased in titer value when compared to control group animals. When cyclophosphamide treatment was given along with different dose of both plants in group 5th and 6th groups animals, significant ($P<0.001$) recovery of immunosuppressive effect of cyclophosphamide (CP) was observed by increasing the titer value as compared to CP treated group. Hence, the plant extract showed the protective effect over humoral immunity.

Delayed type hypersensitivity (DTH) reactions

The effect of test extract on DTH response showed that the test extract in both plant different doses (group 3rd and 4th animals) produced a significant ($P<0.001$) dose-related increased in DTH reactivity in mice when compared to control animals (Table-3). It showed the stimulatory effect of test extracts on T-cells. Potentiation of DTH response was also observed in cyclophosphamide-treated animals because it has damaged short lived suppressor T-cells in the immune system. When comparing MEBC (150mg/kg) treatment along with CP (group 5th)

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animals, to cyclophosphamide treated group, elevation in DTH reactivity was found but it was not statistically significant, while in group 6th when plant extract treatment at higher dose (MEBC 300 mg/kg) was given with CP a significant (P<0.001) elevation in DTH response was found as compared to CP alone treated group. On the other side when treatment of MEAC different doses along with CP was given in group 5th and 6th group animals no significant elevation in DTH reactivity was observed as compared to cyclophosphamide alone treated group. Thus, alteration of DTH reactivity in mice in response to T-cell dependent antigen (SRBCs) revealed the stimulatory effect of MEBC and MEAC extracts on T cells.

Effect on Relative organ weight

The effect of MEBC and MEAC different doses (group 3rd and 4th group animal) on relative organ weight showed no significant relative weight difference on liver, kidney and spleen of the animals of different group when compared to control group (Table-4). But CP injection in group 2nd animals caused a significant reduction in relative organ weight of spleen as compared to control group animals. No significant recovery of spleen weight was observed in plant extract and CP treated animals (group 5th and 6th group) in both plants.

Effect on Hematological parameters

Effect of plant extract on hematological parameters showed that MEBC significantly ($p<0.05$) increased the white blood cell count at the dose level 150 mg/kg and 300 mg/kg as compared to control group animals. While MEAC treatment also showed significantly increased in white blood cell count at the dose level 800 mg/kg ($p<0.05$) and 1200 mg/kg ($p<0.01$) as compared to control group. But CP injection caused a significant ($p<0.001$) reduction in white blood cell count as compared to normal control group animals (Table-5). Combined treatment of CP and MEBC (150 mg/kg) showed significant ($p<0.05$), and MEBC (300 mg/kg) showed significant ($p<0.001$) restoration of bone marrow activity as compared with cyclophosphamide alone treated mice. While combined treatment of CP and MEAC (800mg/kg) showed significant ($p<0.01$) and MEAC (1200mg/kg) showed significant ($p<0.0001$) restoration of bone marrow activity as compared to cyclophosphamide treated group. But no significant effect was observed in RBC and H_b count in various groups of animals in plant extract treatment (both plants) when compared to normal control group animals, and no significant protective effect was observed in RBC and

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H_b count in plant extract and CP treated groups in both plants when compared to CP alone treated animals.

Effect of MEBC and MEAC on antioxidant enzymes

The oxidative stress marker study revealed (Table- 6) that the administration of CP significantly increased ($p<0.001$) the level of LPO, decreased the activity of SOD ($p<0.001$), CAT ($p<0.001$) and reduced the content of GSH ($p<0.001$) as compared to control group animals. While CP treatment along with different doses of extract significantly decreased the LPO ($p<0.001$) level as compared to CP exposed group, and a significant elevation in CAT ($p<0.001$), GSH and SOD were observed in comparison to the CP-treated group in both plants. When compared to MEBC and MEAC extract effects, on each enzyme, the overall result justified that the MEBC was more effective antioxidant than the MEAC.

Effect of test drugs on pro-inflammatory cytokine

Plant extract effects on pro-inflammatory cytokines level showed that the secretion of TNF-α and IL-6 significantly decreased ($P<0.001$) in the negative control group when compared to normal control groups animals. While TNF-α and IL-6 level were up-regulated significantly in MEBC different dose treatment, MEBC (150 mg/kg) showed significant ($p<0.001$) and MEBC (300 mg/kg) showed significant ($p<0.001$) up-regulation as compared to normal control animals. Co-administration of CP and MEBC different dose showed significant ($P<0.001$) increased in cytokines level as compared to CP alone treated group. While in case of MEAC different dose treatment increased was found in TNF-α and IL-6 level but it was not statistically significant when compared to control groups animals and upon co-administration of CP and MEAC different dose, increased was found in TNF-α and IL-6 cytokines level but also it was not statistically significant when compared CP exposed group.

Table 4: Effect of Methanol Extract of *Martynia annua* (MEMA) on Humoral Immune Response.

Group	**Treatment**	**Mean antibody titer** a **(in terms of rank of cups of titer plate) ± S.E.M.**
Group I	Control (Normal saline)	9.25 ± 0.30

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Group II	Normal saline + CP	$3.17^{b} \pm 0.16$
Group III	MEBC 150 mg/kg	$11.2^{b} \pm 0.30$
Group IV	MEBC 300 mg/kg	$13.1^{b} \pm 0.20$
Group V	MEBC 150 mg/kg + CP	$6.83^{c} \pm 0.30$
Group VI	MEBC 300 mg/kg + CP	$8.5^{c} \pm 0.22$

[a]Values are expressed as mean ± S.E.M. of 6 mice, [b]*P*<0.001 Statistical significance versus Group I, [c]*P*<0.001 Statistical significance versus Group II.

Table 5: Effect of Methanol Extract of *Martynia annua* (MA) Extract on Humoral Immune Response.

Group	**Treatment**	**Mean antibody titer [a] (in terms of rank of cups of titer plate) ± S.E.M.**
Group I	Control (Normal saline)	9.25 ± 0.30
Group II	Normal saline + CP	$3.17^{b} \pm 0.16$
Group III	MEAC 800 mg/kg	$10.5^{c} \pm 0.22$
Group IV	MEAC 1200 mg/kg	$11.25^{b} \pm 0.25$
Group V	MEAC 800 mg/kg + CP	$5.3^{d} \pm 0.21$
Group VI	MEAC 1200 mg/kg + CP	$7.6^{d} \pm 0.20$

[a]Values are expressed as mean ± S.E.M. of 6 mice, [b]*P*<0.001 Statistical significance versus Group I, [c]*P*<0.01 Statistical significance versus Group I, d*P*<0.001 Statistical significance versus Group II.

Table 6: Effect of Methanol Extract of *Martynia annua* on Delayed Type Hypersensitivity Response.

Group	**Treatment**	**Mean of right food pad thickness a (mm) ± S.E.M**
Group I	Control-Normal saline	0.80 ± 0.05
Group II	Normal saline + CP	1.078b ± 0.03
Group III	MEBC 150 mg/kg	1.16b ± 0.02
Group IV	MEBC 300 mg/kg	1.195b ± 0.03

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Group V	MEBC 150 mg/kg + CP	1.097 ns± 0.02
Group VI	MEBC 300 mg/kg + CP	1.37 c ± 0.01

[a]Values are expressed as mean ± S.E.M of 6 mice, [b]*P*<0.001 Statistical significance versus Group I, [c]*P*<0.001 Statistical significance versus Group II.

Table 7. Effect of Methanol Extract of *Martynia annua* Extract on Delayed Type Hypersensitivity Response.

Group	**Treatment**	**Mean of right food pad thickness** a **(mm) ± S.E.M.**
Group I	Control-Normal saline	0.80 ± 0.05
Group II	Normal saline + CP	1.078[b] ± 0.03
Group III	MEAC 800 mg/kg	1.15[c] ± 0.01
Group IV	MEAC 1200 mg/kg	1.147[c] ±0.08
Group V	MEAC 800 mg/kg + CP	1.09 [ns] ± 0.03
Group VI	MEAC 1200 mg/kg + CP	1.11[ns] ± 0.04

[a]Values are expressed as mean ± S.E.M. of 6 mice, [b]*P*<0.01 Statistical significance versus Group I, [c]*P*<0.001 Statistical significance versus Group I.

Table 8: Effect of *Martynia annua* Extract on Relative Organ Weight.

Group	**Treatment**	**Relative organ weight[a] (g) ± S.E.M.**		
		Liver	**Kidney**	**Spleen**
Group I	Control (Normal saline)	5.14±0.01	1.367±0.45	0.622±0.05
Group II	Normal saline + CP	4.82[ns] ±0.17	1.115[ns] ±0.26	0.334[b] ±0.06
Group III	MEBC 150 mg/kg	5.21[ns] ±0.05	1.225[ns] ±0.27	0.511[ns] ±0.01
Group IV	MEBC 300 mg/kg	5.25[ns]±0.06	1.391[ns]±0.46	0.620[ns] ±0.08
Group V	MEBC 150 mg/kg + CP	4.85[ns]±0.1	1.31[ns] ±0.35	0.452[ns] ±0.02
Group VI	MEBC 300 mg/kg + CP	4.92[ns] ±0.12	1.30[ns] ±0.02	0.552[ns] ±0.05

[a]Values are expressed as mean ± S.E.M. of 6 mice, [b]*P*<0.01 Statistical significance versus Group I.

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Table 9: Effect of *Martynia annua* Extract on Relative Organ Weight.

Group	Treatment	Relative organ weight[a] (g) ± S.E.M.		
		Liver	Kidney	Spleen
Group I	Control (Normal saline)	5.14±0.01	1.367±0.45	0.622±0.05
Group II	Normal saline + CP	4.82^{ns} ±0.17	1.115 ^{ns} ±0.26	0.334^{b} ±0.06
Group III	MEAC 800 mg/kg	5.04^{ns} ±0.74	1.325^{ns} ±0.02	0.500^{ns} ±0.01
Group IV	MEAC 1200 mg/kg	5.12^{ns} ±0.06	1.330^{ns} ±0.26	0.611 ns ±0.02
Group V	MEAC 800 mg/kg + CP	4.89^{ns} ±0.11	1.352^{ns} ±0.25	0.451 ns ±0.07
Group VI	MEAC 1200 mg/kg + CP	4.90^{ns}±0.07	1.34^{ns} ±0.53	0.511ns ±0.10

[a]Values are expressed as mean ± S.E.M of 6 mice, [b]P<0.05 Statistical significance versus Group I.

Table 10: Effect of *Martynia annua* Extract on Hematological Parameters [a]

Group	Treatment	RBC ($\times 10^6$/mm^3)	WBC ($\times$ 103/mm^3)	Hb (g/dl)
I	Control-Normal saline	9.26±0.60	6.26±0.40	13.76±0.48
II	Normal saline + CP	9.22 ±0.28^{ns}	1.52±0.29^{b}	12.52 ±0.78^{ns}
III	MEBC 150 mg/kg	9.64±0.34^{ns}	7.98±0.46^{c}	13.71 ± 0.92^{ns}
IV	MEBC 300 mg/kg	9.59±0.47^{ns}	8.21±0.38^{c}	14.01 ± 0.51^{ns}
V	MEBC 150 mg/kg + CP	9.35±0.44^{ns}	3.52±0.43^{d}	12.67 ± 0.28^{ns}
VI	MEBC 300 mg/kg + CP	9.40±0.31^{ns}	4.31±0.29^{e}	12.73 ± 0.31^{ns}

[a]Values are expressed as mean ± S.EM. for 6 mice, [b]P<0.001 Statistical significance versus Group I, [c]P<0.05 Statistical significance versus Group I, [d]P<0.05 Statistical significance versus Group II, [e]P<0.001 Statistical significance versus Group II RBC(million/mm^3), WBC(thousand/mm^3), hemoglobin(g/dl).

Table 11: Effect of Methanol Extract of *Martynia annua* on Hematological Parameters [a]

Group	Treatment	RBC ($\times 10^6$/mm^3)	WBC ($\times$ 103/mm^3)	Hb (g/dl)
I	Control-Normal saline	9.26±0.60	6.26±0.40	13.76±0.48
II	Normal saline + CP	9.22±0.28ns	1.52±0.29b	12.52±0.78 ns

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III	MEAC 800 mg/kg	9.53±0.14 ns	7.65±0.24c	13.36±0.92 ns
IV	MEAC 1200 mg/kg	9.47±0.38 ns	7.93±0.40d	13.65±0.51 ns
V	MEAC 800 mg/kg + CP	9.24±0.21 ns	3.21±0.15 e	12.33±0.28 ns
VI	MEAC 1200 mg/kg + CP	9.29±0.57 ns	3.99±0.30f	12.39±0.31 ns

[a]Values are expressed as mean ± S.E.M. for 6 mice, [b]*P*<0.001 Statistical significance versus Group I, [c]*P*<0.05 Statistical significance versus Group I, [d]*P*<0.01 Statistical significance versus Group I, [e]*P*<0.01 Statistical significance versus Group II, [f]*P*<0.001 Statistical significance versus Group II, RBC (million/mm3), WBC (thousand/mm^3), Hemoglobin (g/dl).

Table 12. Effect of *Martynia annua* Extract on Oxidative Stress Parameters [a]

Group	**Treatment**	**LPO (nmol MDA/mg protein)**	**GSH (µg/mg protein)**	**SOD (units/mg protein)**	**CAT (nmol H_2O_2 /mg protein)**
I	Control (Normal saline)	3.79±0.04	3.26±0.02	3.34±0.02	28.21±0.09
II	Normal saline + CP	7.38 ±0.01[b]	1.87 ±0.05[b]	2.70 ±0.03[b]	19.83 ±0.03[b]
III	MEBC 150 mg/kg	4.13 ±0.08[b]	2.72 ±0.09[b]	2.93 ±0.01[b]	26.82 ±0.02[b]
IV	MEBC 300 mg/kg	4.30 ±0.01[b]	2.95 ±0.04[d]	3.11 ±0.0[f]	27.78 ±0.05[b]
V	MEBC 150 mg/kg + CP	5.95 ±0.06[c]	2.19 ±0.03[e]	3.17 ±0.05[c]	23.84 ±0.05[c]
VI	MEBC 300 mg/kg + CP	6.31 ±0.06[c]	2.52 ±0.03[c]	3.22 ±0.08[c]	24.14 ±0.03[c]

MEBC= Methanol extract of *Martynia annua* bark, CP=cyclophosphamide, LPO=lipid peroxidation, SOD=superoxide dismutase, GSH=reduced glutathione, CAT=catalase.
[a]Values are mean ± S.E.M. of 6 mice, [b]P<0.001 Statistical significance versus Group I, [c]*P*<0.001 Statistical significance versus Group II, [d]*P*<0.01 Statistical significance versus Group I, [e]*P*<0.01 Statistical significance versus Group II, [f]*P*<0.05 Statistical significance versus Group I.

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Table 13. Effect of *Martynia annua* Extract on Oxidative Stress Parameters [a]

Group	Treatment	LPO (nmol MDA/mg protein)	GSH (µg/mg protein)	SOD (units/mg protein)	CAT (nmol H_2O_2 /mg protein)
I	Control (Normal saline)	3.79±0.04	3.26±0.02	3.34±0.02	28.21± 0.09
II	Normal saline + CP	7.38±0.01[b]	1.87±0.05[b]	2.70±0.03[b]	19.83±0.03[b]
III	MEAC 150 mg/kg	3.24±0.04[b]	2.31±0.03[b]	2.85±0.06[b]	26.23±0.09[b]
IV	MEAC 300 mg/kg	3.97±0.03[c]	2.49±0.04[b]	3.01±0.06[b]	27.12±0.04[b]
V	MEAC 150 mg/kg + CP	4.10±0.01[d]	2.11±0.05[d]	3.07±0.03[d]	23.21±0.23[d]
VI	MEAC 300 mg/kg + CP	4.33±0.06[d]	2.27±0.02[d]	3.10±0.04[d]	23.94±0.05[d]

MEAC= methanol extract of *Martynia annua* leaves, CP=cyclophosphamide, LPO=lipid peroxidation, SOD=superoxide dismutase, GSH=reduced glutathione, CAT=catalase, [a]Values are mean ± S.E.M. of 6 mice, [b]P<0.001 Statistical significance versus Group I, [c]*P*<0.05 Statistical significance versus Group I, [d]*P*<0.001 Statistical significance versus Group II.

Table 14. Effect of *Martynia annua* Extract on Proinflammatory Cytokines (pg/ml)

Group	Treatment	TNF-α	IL-6
Group I	Control (Normal saline)	23.4±0.13	34.5±0.1
Group II	Normal saline + CP	9±0.21b	12±0.35b
Group III	MEBC 150 mg/kg	26.98±0.45b	35.87±0.05b
Group IV	MEBC 300 mg/kg	28.0±0.82b	36.04±0.07b
Group V	MEBC 150 mg/kg + CP	14.43±0.55c	15.73±.0.21c
Group VI	MEBC 300 mg/kg + CP	17.97±0.71c	17.09±0.07c

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Data were expressed as the mean ± S.E.M., n = 6 animals per group. Concentration was expressed in pg/ml. b*P*<0.001 Statistical significance versus Group I, c*P*<0.001 Statistical significance versus Group II.

DISCUSSION

The shade-dried roots of *M. annua* (0.75 Kg) were extracted with petroleum ether (40-60°C) followed by chloroform, acetone and ethyl alcohol. The extracts were concentrated in vacuum to remove the solvent. The concentrated acetone extract was fractionated into hexane and dichloromethane soluble fractions. The hexane extract did not yield any compound. The dichloromethane extract was concentrated under vacuum to give a semi-solid (1.5 g) which was made into slurry with silica gel (4 g). The extract was chromatographed over a column of silica gel (100 g) packed in hexane. The column was eluted with a) hexane, b) hexane: dichloromethane mixtures with increasing amounts of dichloromethane, c) dichloromethane. Fractions of 100 mL were collected each time, distilled off the solvent and the resulting residues were examined on TLC by using different solvent systems and similar fractions were mixed together. The identification of the compound was done by spectroscopic techniques like UV-Visible, IR spectroscopy, NMR spectroscopy, HPLC and GC-MS. To predict the antiviral property of the isolated compound was docked against HIV-1 protease. In addition to anti-viral properties.

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