



Research Article

EVALUATION OF IN VITRO ANTIOXIDANT POTENTIAL OF *MORINGA OLEIFERA* AND *AEGLE MARMELOS L.* AND THEIR NEPHROPROTECTIVE ACTIVITY

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ABSTRACT

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In the present study *Moringa oleifera* and *Aegle marmelos L.* were selected for their high medicinal value and wide range of pharmacological effects on the basis of earlier studies to evaluate their in vitro antioxidant potential and in vivo nephroprotectivity. Haemolytic assay were also done.

Results: Highest antioxidant activity was showed by methanol extracts of the plants *Moringa oleifera* (94.47%) and *Aegle marmelos L.* (93.01%) at its highest applied concentration (500 µg/ml). Standard drug ascorbic acid had showed 97.5% inhibition at the same highest concentration. The reductive ability of methanol extracts of both the plant *Moringa oleifera* (96.3) and *Aegle marmelos L.* (99.92) were found to be highest in its highest applied concentration (500 µg/ml). The plants bark extracts possess highest amount of phenolics in methanol extracts and the reducing power of the extract increased linearly with concentration. Extract of *Aegle marmelos L.* shows higher nephroprotectivity in compared to *Moringa oleifera* in in vivo studies.

Conclusions: The results of the present study showed that the crude methanolic extract of *Moringa oleifera* and *Aegle marmelos L.* contains high amount of phenolics and flavonoids and also showed good antioxidant activity and extract of *Aegle marmelos* shows higher nephroprotectivity. It might be an alternative to the synthetic antioxidants available in the market which can be used as nephro-protective agent.

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INTRODUCTION:

Moringa oleifera Lam. (Moringaceae) is a highly valued plant, distributed in many countries of the tropics and subtropics. In some parts of the world *M. oleifera* is referred to as the 'drumstick tree' or the 'horse radish tree', *oleifera* is a shrub and small deciduous tree of 2.5–10m in height. The plant is reported to possess wide range of pharmacological effects that include antitumor, antipyretic, antispasmodic, diuretic, antiulcer, hypotensive, hypolipidemic, Hepatoprotective, antifungal and antibacterial activities. In the present study we have selected the bark part of this two plants as not much work have done on the barks for both the plants Khasi *et al*, 2000. Preliminary phytochemical testing showed the presence of high amount of flavonoids and phenolics content. This has prompted us to study the free radical scavenging activity of methanol extract of bark of *Aegle marmelos* and *Moringa oleifera* Anwar *et al*, 2005; Bhattacharya *et al*, 1982.

MATERIALS AND METHODS

The bark part of *Moringa oleifera* (MO) and *Aegle marmelos* L. (AM) were obtained in the month of January-February 2014 from Morigaon district, located in Assam, North-east India.

CHEMICALS

Quercetin, rutin, aluminium chloride, 2,2-diphenyl-1-picryl-hydrazyl, trisodium citrate, triton x, were obtained from Sigma Chemical. cisplatin, silymarin, Ascorbic acid, Methanol, Acetone, Ferrous chloride and Ferric chloride were purchased from Merck. All other reagents were of analytical grade.

EXTRACT PREPARATION

Plant materials were properly cleaned, shade dried at room temperature and grinded to make fine powder. Extracts were prepared in three different solvent hexane (HX), ethyl acetate (EA) and methanol (ME) in soxhlet apparatus, which is then concentrated in rotary evaporator and stored at 4°C for further use.

PRELIMINARY PHYTOCHEMICAL ANALYSIS

The extracts were subjected to preliminary phytochemical, Harborne *et al*, 1992 testing of to detect for the presence of different chemical groups of compounds. Air-dried and powdered plant materials were screened for the presence of saponins, tannins, alkaloids, flavonoids, triterpenoids, steroids, glycosides, saponins Anna *et al* 2008, Dahot *et al* 1988, Wolve *et al* 2003

QUALITATIVE ACTIVITY ASSAY

PHYTOCHEMICAL

DETERMINATION OF TOTAL PHENOLIC CONTENT

Phenolic compounds are plant secondary metabolites produced either from phenylalanine or from its precursor shikimic acid Harborne *et al* 1992. The antioxidant potential of phenolic compounds had been shown in a number of *in-vitro* studies. They are capable of direct chain breaking antioxidant action by radical scavenging. A Folin-Ciocalteu colorimetric method was adopted to determine the total phenolics. Total phenolic content is expressed at 0.1 mg/g gallic acid equivalent using the following equation based on calibration curve, $y = 0.1216x$ where x =absorbance and y =gallic acid equivalent ($\mu\text{g/g}$) Ordon *et al* 2006.

DETERMINATION OF TOTAL FLAVONOID CONTENT

The aluminum chloride method was used for the determination of the total flavonoid, Ordon *et al* 2006 content of the extracts. Absorbance at 415 nm was recorded after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using known concentrations of quercetin. The concentrations of flavonoid content was be calculated as quercetin mg/g using the following equation based on calibration curve, $y = 0.0255x$ where, x was absorbance and y , was the quercetin equivalent ($\mu\text{g/g}$).

DETERMINATION OF TOTAL *IN-VITRO* ANTIOXIDANT ACTIVITY

FREE RADICAL SCAVENGING ACTIVITY (DPPH ASSAY)

The free radical scavenging activity for DPPH radicals by plant extract of MO and AM was measured by the following method Ordon *et al*, 2006. Assays were performed in 300 mL reaction mixtures, containing 200 mL of 0.1mM DPPH ethanol solution, 90 mL of 50mM Tris-HCl buffer (pH 7.4), 10 mL of ethanol (as solvent blank) or test plant extracts and ascorbic acid were used as positive controls. After 30 min of incubation at room temperature, absorbance (540 nm) of the reaction mixtures was taken by UV spectrophotometer. All determinations were performed in triplicate. The inhibitory effect of DPPH was calculated according to the following formula:

$$\text{DPPH Scavenged (\%)} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \times 100$$

REDUCTIVE ABILITY ASSAY

Reducing power assay Robert *et al*, 1999 method is based on the principle that substances which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. The reducing power of the extracts and standards increases with the increase in amount of sample and standard concentrations.

IN-VITRO HAEMOLYTIC ACTIVITY

Mammalian blood sample was collected by using 4% Trisodium citrate as anti-coagulating agent, blood sample was centrifuged at 3000 rpm for 10 min and the precipitate was collected containing the erythrocytes. Precipitated collected was wash with PBS pH (7.4) for three times, different concentrations of the extracts were added to the erythrocytes and incubate for 2 hour at 37°C , centrifuged at 3000 rpm for 10min and absorbance was taken at 415 nm where PBS was taken as the negative control, TritonX 100 was taken as the positive control.

IN-VIVO ANIMAL STUDY

STANDARDIZATION OF ANIMAL MODEL

Healthy adult wistar rats of either sex approximately of same age, weighing between 150-200 g respectively, were used for the study. The animals were group housed in polypropylene cages containing sterile paddy husk bedding under controlled conditions at $25 \pm 2^{\circ}\text{C}$, RH $50 \pm 5\%$ and 10/14 h of light/dark cycles. Food and water were provided at regular interval. The study was conducted after obtaining the approval of the Institutional Animal Ethics Committee (No: IAEC/PER/2016/2017-3).

ACUTE TOXICITY STUDIES

The acute oral toxicity of the plant extracts was estimated by following up- and-down stair case method in wister rat as per OECD TG 425 guidelines. Based on the toxicity study, the doses of plant extract were selected.

DRUGS AND SELECTION OF DOSES

Silymarin (Marck, India) used as standard drug and Cisplatin (Marck, India) used as nephrotoxic agent in the present study. The doses were selected on the basis of the relevant previous studies. Plant extracts were given orally as 100 and 200 mg/kg doses for

the treated group half and hour prior to the treatment of cisplatin toxicity (8 mg/kg, i.p) and silymarin (8mg/kg) was used as standard drug for the standard group and for the control group vehicle(distill water) was used prior to the cisplatin injection.

ESTIMATION OF THE NEPHROPROTECTIVE ACTIVITY OF THE CRUDE EXTRACTS

The crude plant extracts were assessed for their nephroprotective ability. Wistar Albino rat models were used for the *in vivo* study. In each group 6 numbers of animals were taken ($n=6$). Two different doses of the extracts, 100mg/Kg body weight and 200mg/ Kg body weight, were given daily orally. After half an hour, Cisplatin at 7.5mg/Kg body weight was injected Intraperitonally. The positive control, Silymarin taken at a concentration of 14.5mg/ Kg body weight was also given orally. Normal saline was given to the negative control group. The study was carried out for five days in which all the doses including the extract and cisplatin was repeated daily Chirino Y *et al*, 2004; Kunihiiko S *et al*, 2002. The animals were sacrificed on the 6th day. Blood were collected following the CPCSEA guidelines. Different biochemical estimation of the various markers of the kidney diseases like BUN test, Urea test, Uric acid and Serum Creatinine test were carried out to estimate the toxicity parameters of cisplatin and the plant extracts Suma M *et al*, 2000; Luciana M *et al*, 2003.

STATISTICAL ANALYSIS

Triplicate analyses were performed on a number of samples, established according to the total plant samples investigated and element analysed. For an assessment of the analytical relevance of the results, standard procedures of statistical calculation were used. All numeric data were expressed as mean $\pm$ standard deviation (SD). For statistical analysis, the commercially available software package graph pad prism (Windows) was used. Mean values did not differ significantly.

RESULTS

The present study focus on studying different phytochemical present in various extracts by different methods and identification of the phytochemicals that might be related to the

nephroprotective activity. Ethnomedicinal Plants used in different traditional medicine contains a high amount of different substances which can be used to treat different protective treatments. Secondary metabolites such as terpenoids that gives plants their odours and others metabolites (quinines and tannins) are responsible for plant flavour and some of the herbs and spices used by humans to season food yield useful medicinal compounds.



Moringa oleifera

Methanol extract

Alkaloid, reducing sugar, cardiac glycoside, phenolic, flavanoid

Ethylacetate extract

Alkaloid, phenolic, flavanoid, tannins

Hexan extract

Alkaloid, reducing sugar, cardiac glycoside, phenolic, flavanoid



Aegle marmelos

Methanol extract

Alkaloid, reducing sugar, cardiac glycoside, phenolic, flavanoid, tannins, coumerin

Ethylacetate extract

Alkaloid, cardiac glycoside, phenolic, flavanoid, tannins

Hexan extract

Alkaloid, cardiac glycoside, phenolic, flavanoid

TOTAL PHENOLIC AND FLAVONOID CONTENT

The Phenolic and flavonoid compounds are mainly known as powerful chain breaking antioxidants for their scavenging ability and which is due to their hydroxyl groups Ordon *et al* 2006, Rober *et al* 1999. The phenolic compounds may contribute directly to antioxidative action and are responsible for effective free radical scavenging Kocal *et al* 2008, Singh *et al* 2012. Flavonoids are the most important groups of plant secondary metabolites. Flavonoids has good antioxidant potential and effects of this secondary metabolites on human nutrition and health are highly

considerable. Phenols are group of compounds with potential antioxidant activity, which is believed to be mainly because of their redox properties and they play an important role in adsorbing and neutralizing free radicals. Total phenol and total flavonoid content of the methanol extract was significantly higher in both MO and AM extracts, i.e. 342.15 ± 0.54 , 238.11 ± 0.44 (μg Gallic acid/g of dry plant material) and 1.02 ± 0.15 , 3.65 ± 0.04 (μg quercetin/g of dry plant material) respectively. Total phenolic and flavonoid content of all the plant extracts were shown in table 1.

Table 1: Total phenolics and flavonoids content in different solvent extracts of *Moringa oleifera* and *Aegle marmelos*

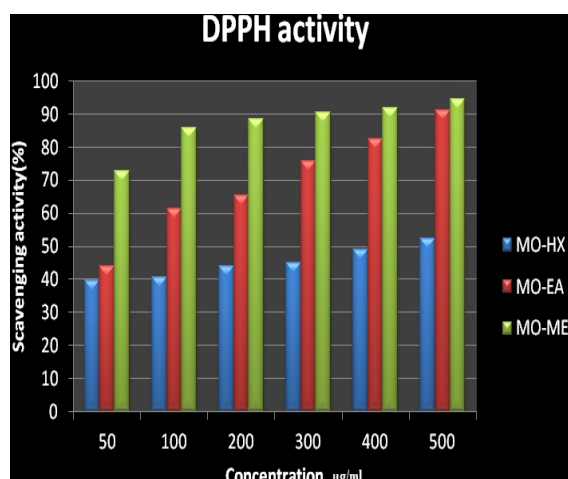
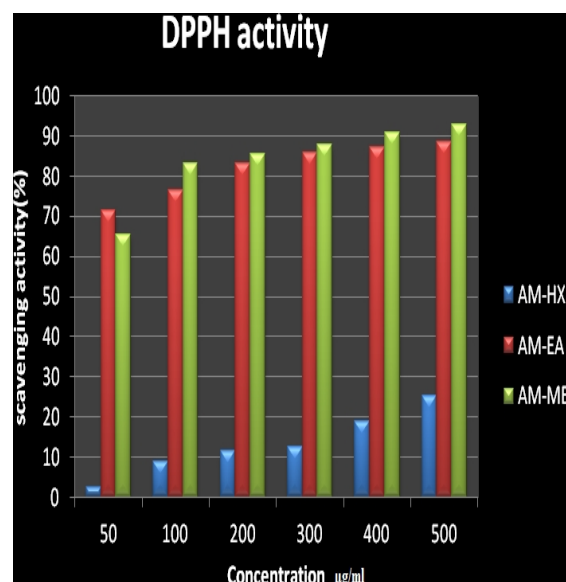
Extract	Total phenol (μg of GAE/g of extract)	Total Flavonoids(μg of QE/g of extract)
n-Hexane extract (MO-HX)	41.71 $\pm$ 1.0	1.02 $\pm$ 0.15
Ethyl acetate extract (MO-EA)	204.91 $\pm$ 2.39	0.39 $\pm$ 0.07
Methanol extract (MO-ME)	342.15 $\pm$ 0.5	0.18 $\pm$ 0.007
n-Hexane extract (AM-HX)	105.42 $\pm$ 0.35	3.65 $\pm$ 0.04
Ethyl acetate extract (AM-EA)	122.3 $\pm$ 1.76	0.2 $\pm$ 0.01
Methanol extract (AM-ME)	238.11 $\pm$ 0.44	0.09 $\pm$ 0.007

IN VITRO FREE RADICAL-SCAVENGING ACTIVITY

In the present study, evaluations of the antioxidant potential of the extract were done. Earlier reports have showed the relationship between a high phenolic content and antioxidant activity and this correlation was confirmed in this study. The percentage of total antioxidant activity of all the extracts of MO and AM was estimated and the results are presented below.

DPPH RADICAL SCAVENGING ASSAY

The antioxidant capacity of the extracts were measured against DPPH with increasing concentration of the solvent extracts of both the plants and ascorbic acid is used as the +ve control. The % of inhibition of the free radicals by the different extracts with increase in concentration of solvent extracts. The graph shown in fig 1, 2 is obtained by plotting the % of inhibition, calculated at 517nm against the concentration of solvent extracts.

Fig 1: DPPH scavenging activity of three extracts (HX, EA & ME) of *Moringa oleifera***Fig 2:** DPPH scavenging activity of three extracts (HX, EA & ME) of *Aegle marmelos*

REDUCTIVE ABILITY ASSAY

In the reducing power assay, the presence of antioxidants in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then monitored by measuring the formation of peari's Prussian blue at 700nm indicated an increase in reductive ability. Ascorbic acid is used as the +ve control. The graph shown in fig 3, 4 is obtained by plotting the reducing ability against the concentration of the solvent extracts.

In the present study, both the methanol extract of *Aegle marmelos* and *Moringa oleifera* showed good antioxidant activity thus based on the antioxidant assay methanol extract of MO and AM were Further evaluated for haemolytic assay and *in vivo* nephroprotective evaluation.

Fig 3: Reductive ability of the extracts (HX, EA & ME) of *Moringa oleifera*

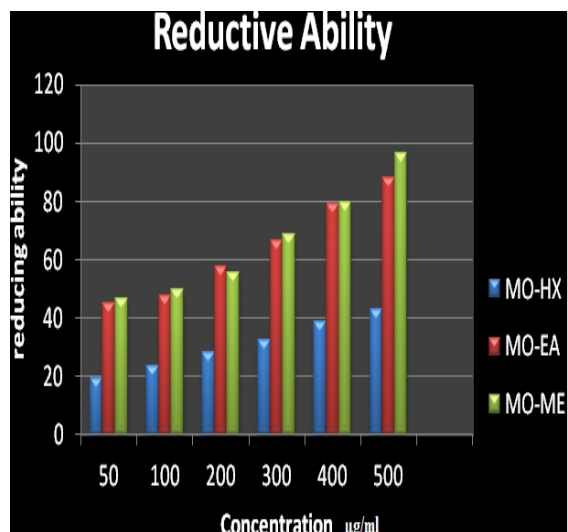
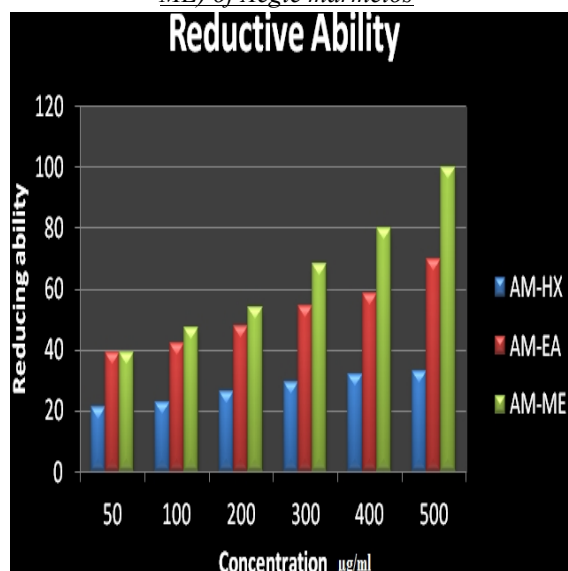


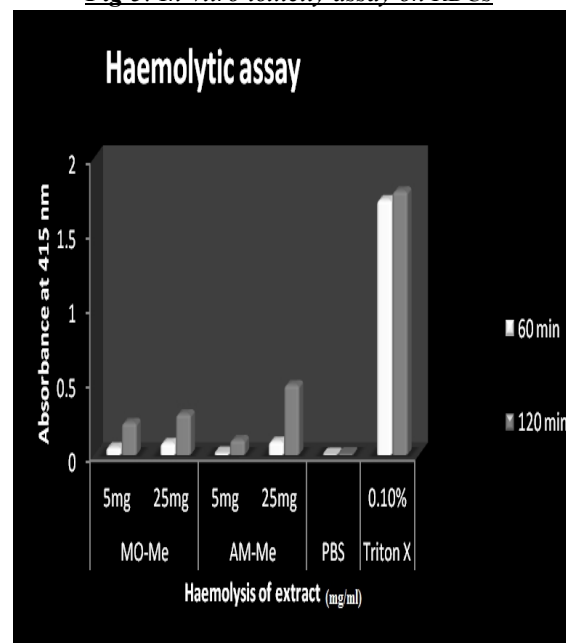
Fig 4: Reductive ability of the extracts (HX, EA & ME) of *Aegle marmelos*



IN-VITRO HAEMOLYTIC ACTIVITY

In the present study haemolytic activity was performed in mammalian blood cell and it was found that the methanol extract from MO and AM showed no toxicity on mammalian RBCs (almost same as positive control PBS) where as negative control Triton X showed high rate of haemolysis (fig 5).

Fig 5: In-vitro toxicity assay on RBCs



IN-VIVO ANIMAL STUDY

ACUTE TOXICITY STUDIES

Both the plant extracts were orally fed to wister rat to check the observations of behavioural change for 2000 mg/bdw of extract and all the tested sample didn't showed any toxic effect as different parameters studied didn't show any abnormal behavior as shown in table 2.

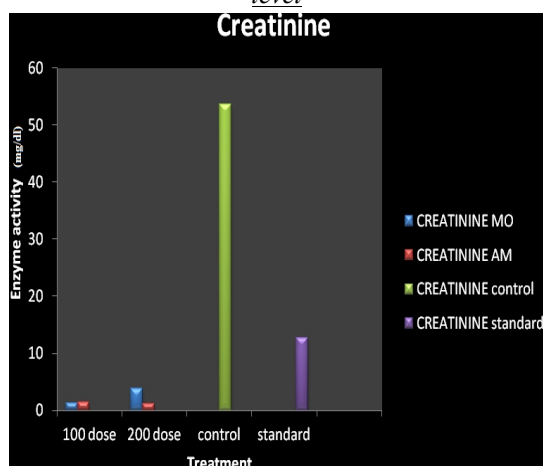
Table 2: Observation of behavioural change for 2000 mg/bdw of extract

Observation parameters	Control group		Treated groups	
	12 hrs	24 hrs	12 hrs	24 hrs
Eyes	Normal	Normal	Normal	Normal
Skin and fur	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal
Behavioural patterns	Normal	Normal	Normal	Normal
Salivation	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal
Diarrhea	Normal	Normal	Normal	Normal
Coma	Not observed	Not observed	Not observed	Not observed
Tremors	Not observed	Not observed	Not observed	Not observed

EFFECT OF PLANT EXTRACT ON CREATININE LEVEL

A significantly increased level of serum creatinine was observed in blood serum samples of the control in compare to the silymarin standard group (Fig. 6). However, supplementation with MO and AM extract at 100 and 200 mg/kg bdw dose significantly prevented further elevations of creatinine. Highest effects was observed in 200 mg/kg bdw extract of AM.

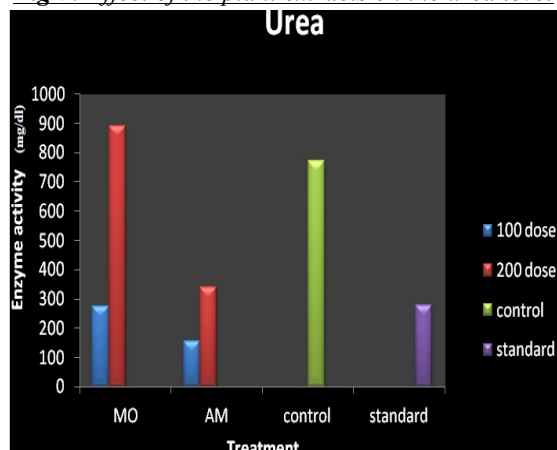
Fig 6: Effect of the plant extracts on the Creatinine level



EFFECT OF PLANT EXTRACT ON UREA LEVEL

A significantly increased level of serum creatinine was observed in blood serum samples of the control group in compare to the silymarin standard group (Fig. 7). However, supplementation with MO and AM extract at 100 and 200 mg/kg dose significantly prevented further elevations of urea level, with higher effects observed in 100 mg/ kg bdw extract of AM as compared to the control group.

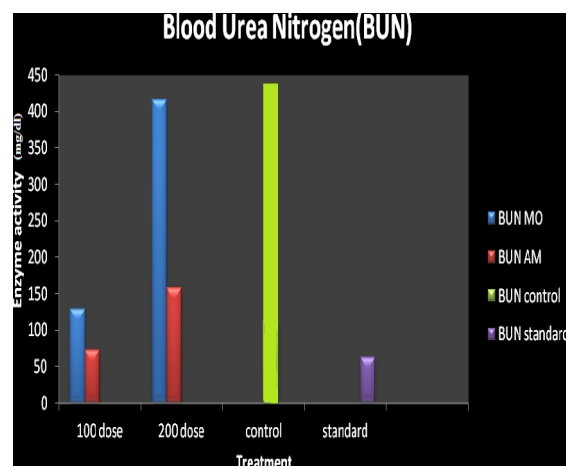
Fig 7: Effect of the plant extracts on the urea level



EFFECT OF PLANT EXTRACT ON BLOOD UREA NITROGEN LEVEL

A significantly increased level of serum creatinine was observed in blood serum samples of the control group in compare to the silymarin standard group (Fig 8). However, supplementation with MO and AM extract at 100 and 200 mg/ kg bdw dose significantly prevented further elevations of urea level, with higher effects observed in the 100 mg/kg extract of AM as compared to the control group.

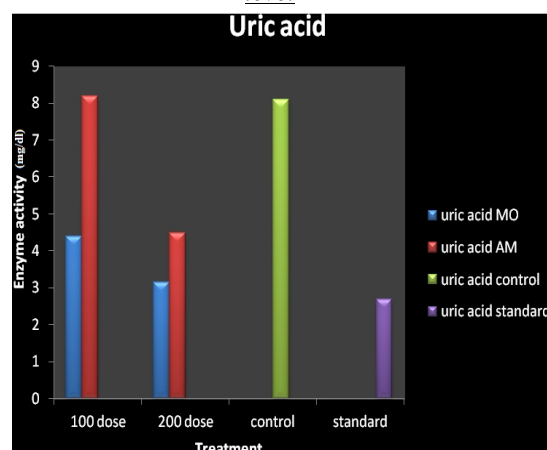
Fig 8: Effect of the plant extracts on the BUN level



EFFECT OF PLANT EXTRACT ON URIC ACID LEVEL

A significantly increased level of serum creatinine was observed in blood serum samples of the control group in compare to the silymarin standard group (Fig 9). However, supplementation with MO and AM extract at 100 and 200 mg/kg dose significantly prevented further elevations of uric acid level, with higher effects observed in the 200 mg/ kg bdw extract of MO as compared to the control group.

Fig 9: Effect of the plant extracts on the Uric acid level



DISCUSSION

The different plant bark crude extracts (HX, EA & ME) of the *Moringa oleifera* and *Aegle marmelos* L. tested for phytochemical analysis which showed the presence of many important phyto constituents. Alkaloids, cardiac glycosides, phenols and flavonoids were present in all of the bark extracts (HX, EA & ME) whereas saponins, steroids and anthraquinone were absent.

Quantitative analysis of the total phenolics and flavonoids present in the plant extracts of *Moringa oleifera* showed that methanol extract had contained highest amount of phenolics i.e. (342.15±0.54, GAE/g) and flavonoids content was found high in hexane extracts of the plant (1.02±0.15; QE/g) (table 1). The high content of phenolics in methanol extract might be responsible for the strong antioxidant activity. Among *Aegle marmelos* L. extracts methanol extract had contained highest amount of phenolics (238.11±0.44; GAE/g) and flavonoids content was found high in n-Hexane extracts (3.65±0.04; QE/g) (Table 1). Results of flavonoid and phenolic content showed that the methanol extracts of both the plants possess highest amount of phenolics and flavonoids, so methanol extracts of both the plants might be most effective and curative solvent extracts for further application *in-vivo* study. The antioxidant activity of *Moringa oleifera* and *Aegle marmelos* L. was determined by measuring the capacity to scavenge free radicals as well as reductive ability. Change in the original purple colour of DPPH into different shades of yellow colour after incubation with different extracts of the plant showed the reducing potentials of the extracts. Plant extracts and standard drug (ascorbic acid) reduced DPPH radicals with increasing concentrations. Highest antioxidant activity was shown by methanol extracts of both the plants *Moringa oleifera* (94.47%) and *Aegle marmelos* L. (93.01%) at its highest applied concentration (500 µg/ml). Standard drug ascorbic acid, 97.5% inhibition at the same highest concentration. The reductive ability of methanol extracts of both the plants *Moringa oleifera* (96.3%) and *Aegle marmelos* L. (99.92%) were found to be highest in its highest applied concentration (500 µg/ml). Thus the scavenging activity of methanol extract of both the plants were very promising as it showed almost equivalent activity to ascorbic acid. High phenolic content in methanol extract could be attributed to its strong antioxidant activity. The haemolytic activity of the RBCs shows no toxic effect of the plant

extracts of both MO and AM and thus we can use this plant extract for *in vivo* studies.

The *in-vivo* study for the behavioural experiment shows that the plant extract does show any abnormalities after the treatment of all the extracts and not. The *in vivo* study for the nephroprotective activity shows good protectivity against the cisplatin toxicity effect which may be correlated with the high phenolics and high antioxidant activity. The level of creatinine, urea, uric acid and blood urea nitrogen was higher in the cisplatin treated group whereas the biochemical markers are lowered by the methanolic crude extract of *Aegle marmelos* and *Moringa oleifera* in comparison to the control group. However urea, uric acid, creatinine and BUN level of *Aegle marmelos* L. treated dose shows higher protectivity against cisplatin induced nephrotoxicity than that of cisplatin and comparable to the standard drug silymarin.

CONCLUSION

In conclusion, result of the present study shows that the *in-vitro* antioxidant study of both the plant extracts shows significant scavenging free radical activity however the study shows that the methanol extracts of both the plants have huge potential as a source of natural antioxidant. Present investigation of the *in-vivo* nephroprotective study shows significant nephroprotective role of both the extracts of *Aegle marmelos* and *Moringa oleifera*. However the extract of *Aegle marmelos* shows higher nephroprotectivity in comparison to *Moringa oleifera*. It may be possible that compounds with high polarity such as phenolics and flavonoids are present in the plant extracts and with high antioxidant activity which may be possibly responsible for the nephroprotective activity. The present study also demonstrated that out of the two potent plant extracts, none of the plants exhibited any toxic activity towards the red blood cells as observed through the haemolysis test. Therefore the present study suggests that the plant *Aegle marmelos* might be a potential source of natural antioxidant and nephroprotective agent and it makes the plants suitable as drug candidature.

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