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Determination of antioxidant activity of *Trigonella foenum-graecum* Linn. in mice

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Abstract

The objective of this study is to determine the *in vivo* effect of methanolic extracts of leaves and seeds of *Trigonella foenum-graecum* Linn. in blood plasma of mercuric chloride induced oxidative stress in mice, with respect to the levels of different antioxidant enzyme systems like catalase, Super oxide dismutase (SOD) and glutathione reductase enzyme activities. DPPH assay of the methanolic extracts of the seeds and leaves were conducted and it was seen that the free radical scavenging activity of seed was better than that of methanolic extract of leaves as seen by a lower $E.C._{50}$ value obtained for seed as compared to a higher $E.C._{50}$ value for the leaves. It was seen that there was an enhanced lipid peroxidation and antioxidant enzyme levels were significantly decreased in blood plasma of mercuric chloride treated mice. Subsequent oral administration of methanolic extract of *Trigonella foenum-graecum* Linn. seeds and leaves for seven days, demonstrated decreased lipid peroxidation in blood plasma of mice almost to the normal control range. Antioxidant enzyme levels post extract feeding also increased in contrast to decrease in their levels on mercuric chloride administration. However, the seeds were found to have a better antioxidant potential than the leaves *in vivo*.

Key words: Antioxidant activity, Oxidative stress, DPPH, $E.C._{50}$, Mercuric chloride, *Trigonella foenum-graecum* Linn., Lipid peroxidation, catalase, superoxide dismutase

Introduction

Oxidative stress, characterized by a disturbance in the fine balance between proxidant and antioxidants, can be induced by a variety of factors like UV exposure, microbial invasion and herbicide use in plants or by use of certain chemicals and drugs in animals. It is marked by an increase in the production of free radicals, mainly Reactive Oxygen Species (ROS), like hydrogen peroxide and superoxide. ROS are generally produced in the course of animal metabolism, but the endogenous antioxidant system neutralizes its toxic effects.Many cellular biomolecules are prone to extensive damage in the form of protein and nucleic acid (DNA) damage, depending on tissue/species, endogenous antioxidant content and the ability to induce the required response in an antioxidant system. Oxidative stress disturbs the balance between these systems, leading to widespread cellular damage and leading to a variety of diseases like cancer, arthritis and accelerating the ageing process.

Antioxidant systems native to a biological system can be enzymatic like superoxide dismutases, glutathione peroxidases and catalase or can be non-enzymatic like ascorbate, tocopherol, glutathione and flavanoids. Antioxidants decrease singlet oxygen concentration, bind metal ion catalysts and are involved in chain breaking to prevent continued hydrogen removal from substrates and monitoring excessive formation of free radicals.

Fenugreek or *Trigonella foenum-graecum* Linn. is a member of legume family and grows indigenously along Mediterranean coast in Europe. It has been studied extensively for its ethnomedicinal value or as a traditional medicine for the treatment of diabetes, a state characterized

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by significant oxidative stress. Because of its strong antioxidant properties, it is a principal ingredient in Ayurvedic formulations. It has also been seen to act as a tonic for liver and pancreas and is also helpful in lowering (LDL) cholesterol. Fenugreek also contains an amino acid, hydroxyisoleucine that stimulates pancreas to release insulin aiding in lowering blood sugar levels.

Hence, an effort has been made to study the antioxidant potential of *Trigonella foenum-graecum* Linn. in a mammalian system, Wistar mice in our case. Oxidative stress was induced with a suitable dose of mercuric chloride for seven days intravenously. Previously prepared methanolic extracts of the leaves and seeds were administered orally for next seven days. Blood enzymatic parameters at every stage of the experiment were determined to help us estimate the efficacy of fenugreek as an effective antioxidant *in vivo.*

Materials and Methods

Preparation of plant extracts

Fenugreek leaves were obtained from the local nursery and seeds at the grocer's. They were oven dried and powdered and soaked in 80% methanol for 48-72 hours in a rotatory shaker. It was passed through No. 1 Whatman paper using high pressure pump. The filterate obtained was further concentrated under reduced pressure at 40°C and stored at 4ºC under further use.

Free radical scavenging assay using DPPH *assay*

Method elucidated by Chan *et al*. (2007) was used where 1.0 ml of different dilutions of extract was added to 2.0 ml of DPPH solution prepared by dissolving 6.0mg DPPH in 100ml methanol. Ascorbic acid was used as standard. The mixture is allowed to stand for 30 minutes and the absorbance was measured at 517nm. % scavenging activity of the extracts was calculated in the following way:

$$\% \text{ scavenging activity} = [\text{Absorbance}_{control} - \text{Absorbance}_{sample} / \text{Absorbance}_{control}] \times 100.$$

where control is containing all reagents except test compound.

Experimental animal model

Male Wistar mice were chosen as the experimental animal system. Twenty mice were divided into four groups of five mice each as follows:

Group 1 (n=5): Control

Group 2 (n=5): Experimental control

Group 3 (n=5): Experimental plus methanolic leaf extract treated

Group 4 (n=5): Experimental plus methanolic seed extract treated

Method of Singh *et al*. (2008) was used. In groups 2 to 4, oxidative stress was induced with the help of Mercuric chloride administered intravenously at a dose of 0.5mg/kg body weight for a period of seven days. From the eighth day onwards plant extracts were fed to the corresponding groups at a dose of 30mg/kg weight through the oral route. Group 1 acts as control that is without any treatment *i.e.,* normal standard feed.

Collection of blood and plasma isolation

One milliliter of blood sample was collected by retro-orbital method at 14th day of treatment from all the above mentioned groups. 0.1 ml of blood sample was diluted 10 times with saline (0.9% w/v NaCl) pH 7.4 for the estimation of antioxidant enzyme levels in mice. Blood plasma was separated by centrifugation at 3,000 rpm for 10 minutes. All samples were stored in cold at 4°C.

Estimation of biochemical parameters

Assay of lipid peroxidation

Lipid peroxidation was estimated by the method Niehans and Samuelson (1968). Blank test tube was prepared by adding 1.0 ml phosphate buffer and 2.0 ml TBA mixture. For each test tube of 0.3 ml homogenate, 0.7 ml phosphate buffer (pH 7.4) was added along with 2.0 ml TBA mixture. All test tubes were incubated in water bath set at 60° C for 15 minutes. After incubation, the test tube sample were cooled and centrifuged for 10 minutes. The supernatant was collected and read at 535nm.

Calculation

n mole MDA released/mg protein= $\Delta OD \times 10^4 / 1.56 \times 0.3x$ mg/ml protein content

Assay of superoxide dismutase

The SOD activity was estimated by the method of Marklund and Marklund (1974). A blank was prepared by adding 2.85ml of tris-succinate buffer to 150µl ddw. in a test tube. For reference, 2.85ml tris-succinate buffer was added along with 50ml ddw. and 100µl pyrogallol. Test tubes for each 50µl of mice plasma were prepared by adding 2.85ml tris-succinate buffer along with 100µl pyrogallol solution. The OD was taken at 412nm of time span for 3 minutes at 30 sec. interval.

Calculation

$$\text{Specific activity} = \frac{(\Delta OD \text{ per min.}_{REF} - \Delta OD \text{ per min.}_{EX}) \times \text{reaction volume}}{\Delta OD_{REF.} / 2 \times \text{Sample volume} \times \text{protein volume (mg/ml)}}$$

Assay of catalase

The catalase activity was estimated by the method of Beers and Sizer (1952). 2.9ml phosphate buffer (pH 7.0) was taken in a test tube and 100µl of H_2O_2 was added to prepare blank. Then, for each 100µl diluted blood of treated group, tests were prepared having 2.8 ml phosphate buffer and 100µl H_2O_2. The absorbance was measured at 240nm for time spans of 3 minutes at every 30 sec. interval.

Calculation

Specific activity (U/mg) = ΔOD/ml (homogenate)/min. × 1000÷ 43.6× mg/ml protein

Assay of glutathione reductase

Glutathione reductase activity was measured by the method of Carberg and Mannervic (1985). The reaction mixture consisted of 1.5ml of potassium phosphate buffer (pH 7.0) containing 2.0mM EDTA,0.15ml 2.0mM NADPH,0.2ml 20 mM oxidised glutathione and distilled water was added to make up the final volume to 3.0ml. The reaction was started by adding the 0.1ml of sample in the linearity range. The absorbance was measured at 340 nm for one minute at 15 sec. intervals. Control lacking enzyme was run simultaneously.

Calculation

One unit of GR activity is expressed as the amount of NADPH formed in one minute by one ml of enzyme preparation. Calculation of the enzyme activity has been done by using the molar extinction coefficient of NADPH as 6.22x1000.

Assay of xanthine oxidase

Assay of xanthine oxidase was carried out essentially according to the method described by Roussos, 1967. The assay mixture, in final volume of 3.0 ml, consisted of 0.30 ml Tris-HCL buffer,50mM pH 7.4; 0.30 ml $CuSO_4$, 10mM;0.05 ml, xanthine , 2.58 mM per ml. in 0.05 M glycine buffer, pH 7.4. 0.1 ml of blood plasma and water was added to make up the volume. Change in absorbance was recorded at 290 nm at 15 seconds interval for one minute. Suitable control was run simultaneously.

Calculation

One unit of activity has been defined as change in absorbance at 290 nm in one minute by one milliliter enzyme preparation.

Protein content estimation

Protein content of all blood samples used were determined by using the method of Lowry *et al*. (1951).

Results and Discussion

DPPH *assay of plant extracts*

DPPH assay of the plant extracts were carried out according to the protocol mentioned. The results were expressed as percentage scavenging activity of the free radical. The $E.C._{50}$ value that is their half maximal effective concentration or the concentration that shows 50% radical scavenging activity, of the plant extracts were also determined graphically and results expressed in the form of a bar chart (Acharya *et al*., 2010). The results were depicted graphically for methanolic extracts of fenugreek plant leaf and seed in Figure 1.

Stock solutions of methanolic leaf and seed extracts were used. Ascorbic acid (1.0mg/ml) was used as standard. The % free radical scavenging activity is tabulated in Table 1.

Table 1. % scavenging activity (% S. A.) of plant extracts

VOLUME (ml)	CONCENTRATION (mg/ml)	ASCORBIC ACID (STD.)EAN ± S.D MEAN ± S.D	FENUGREEK LEAF (METHANOLIC) MEAN± S.D	FENUGREEK SEED (METHANOLIC)
% S. A.	% S. A.	% S. A.		
0.2	0.2	94.04±0.08	20.9±1.45	23.61±3.20
0.4	0.4	93.89±0.13	27.23±3.11	43.06±3.29
0.6	0.6	93.6±0.10	36.37±3.90	64.35±5.98
0.8	0.8	93.95±0.21	44.04±4.33	69.87±4.07
1	1.0	93.74±0.04	57.29±5.38	74.16±2.33

All values are reported as Mean± S.D of a minimum of two replicates

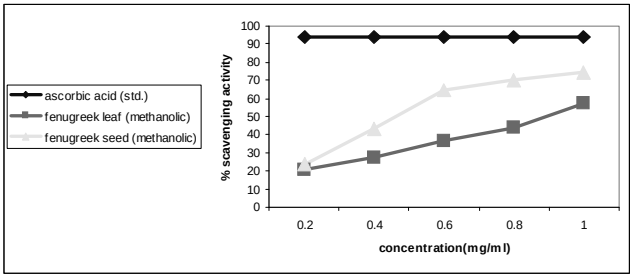

Figure 1a. DPPH assay of plant extracts

Comparing the E.C.$_{50}$ values of the samples tested, it was seen that fenugreek seed showed the maxixum scavenging activity, and a better antioxidant potential than leaf extract tested, as it had the lowest E.C.$_{50}$ value. A smaller E.C.$_{50}$ corresponds to a greater antioxidant acitivity (Azlim *et al.*, 2010).

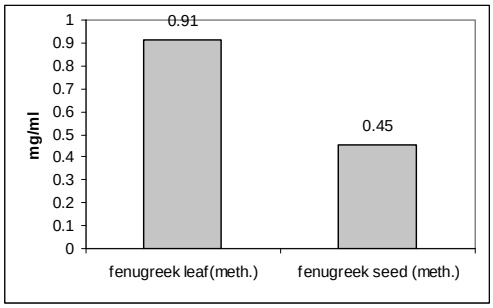

Figure 1b. E.C.$_{50}$ of plant extracts

Estimation of biochemical parameters

Extent of lipid peroxidation

Lipid peroxidation drastically increased on treatment with mercuric chloride, this being the major source of free radicals (Ercal *et al.*, 2001). In this study, there was a significant increase in the extent of lipid peroxidation in the mercuric chloride treated group (+40.95% as compared to the control group). Fenugreek seed extracts demonstrated a maxixum decrease in extent of lipid peroxidation by +5.15% as compared to a +10.73% increase with Fenugreek Leaf Methanolic Extract (FLME). It is given in Table 2 and Figure 2.

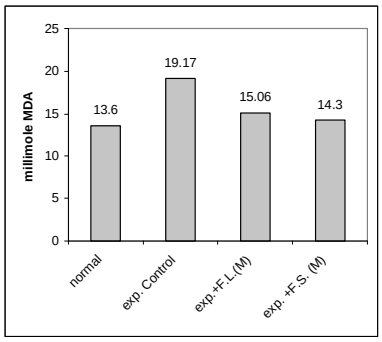

Figure 2. Lipid peroxidation assay

Table 2. Lipid peroxidation in control, experimental control and experimental groups

S.No.	Groups	Millimole MDA released	% increase or decrease
1.	NORMAL	13.6±1.2	
2.	EXP.CONTROL	19.17±0.61	+40.95%
3.	EXP. + F.L.(M)	15.06±0.47	+10.73%
4.	EXP. + F.S. (M)	14.3±0.50	+5.15%

All values are reported as MEAN ± SD of minimum three replicates.

Estimation of catalase activity

Statistically decreased catalase activity was seen for the mercuric chloride treated group with 52.17% decrease in activity as compared to the normal. Maxixum enzyme activity recovery was seen in group that was fed methanolic extract of fenugreek seed (13.04% as compared to normal). This is reported in Table 3 and Figure 3.

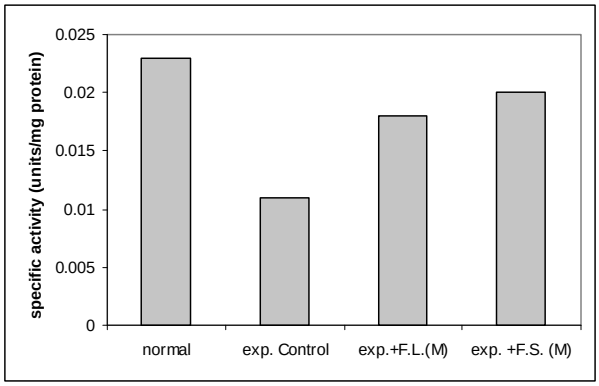

Figure 3. Catalase assay of plant extracts

Table 3. Catalase activity for control, experimental controland experimental groups

S. No.	Groups	S. ACTIVITY (units/mg prt)	% increase or decrease
1.	NORMAL	0.023±0.002	
2.	EXP.CONTROL	0.011±0.002	-52.17%
3.	EXP.+ F.L.(M)	0.018±0.001	-21.74%
4.	EXP. + F.S. (M)	0.020±0.001	-13.04%

All values are reported as MEAN ± SD of minimum three replicates

Estimation of super oxide dismutase activity

There was a 42.71% drop in the enzyme activity in the mercuric chloride treated group as compared to normal. Fenugreek leaf extracts showed a substantial enzyme activity recovery by 19.09% from normal for mthanolic extracts.

Extracts of fenugreek seeds showed an appreciable recovery, nearing control range (12.06% from normal for mthanolic extract). This details are given in Table 4 and Figure 4.

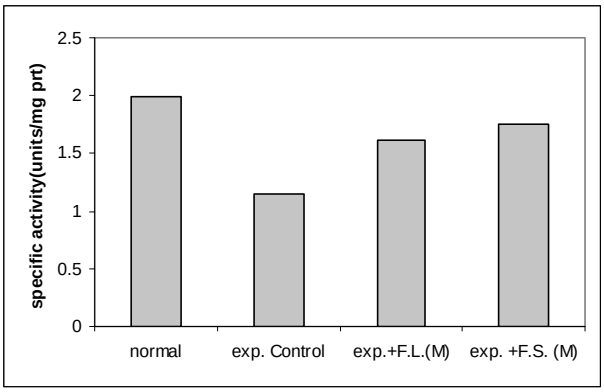

Figure 4. SOD assay for extracts

Table 4. SOD activity for control, experimental control and experimental groups

S. No.	Groups	S. ACTIVITY (units/mg protein)	% increase or decrease
1.	NORMAL	1.99±0.07	
2.	EXP.CONTROL	1.14±0.11	-42.17%
3.	EXP.+ F.L.(M)	1.61±0.02	-19.09%
4.	EXP. + F.S. (M)	1.75±0.03	-12.06%

All values are reported as MEAN ± SD of minimum three replicates.

Estimation of glutathione reductase activity

A significant decrease in the levels of the enzyme activity was seen in the mercuric chloride treated group with a decrease of 65.38% from normal. The response of extracts of fenugreek leaf was also appreciable (26.925% from normal for methanolic extracts.) Maxixum recovery was seen by extract of fenugreek seeds, reporting a 19.23% from normal enzyme recovery (Table 5 and Figure 5).

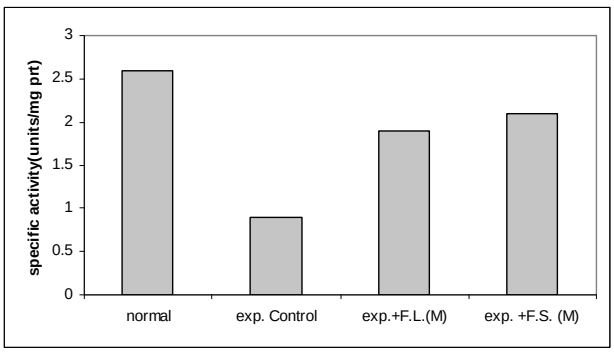

Figure 5. GR activity of plant extracts

Table 5. GR activity of control, experimental control and experimental group

S. No.	Groups	S. ACTIVITY (units/mg protein)	% increase or decrease
1.	NORMAL	2.6±0.17	
2.	EXP.CONTROL	0.9±0.05	-65.38%
3.	EXP.+ F.L.(M)	1.9±0.08	-26.92%
4.	EXP. + F.S. (M)	2.1±0.05	-19.23%

All values are reported as MEAN ± SD of minimum three replicates

Estimation of xanthine oxidase activity

There was an enormous increase in the level of this enzyme's activity in the mercury chloride treated group by 48.38% as compared to normal. Fenugreek leaf extracts showed promising results, with methanolic extract decreasing it to 19.3% from normal. Phenomenal results were shown by fenugreek seed extracts that brought down the level of enzyme activity almost upto the control range (Table 6 and Figure 6).

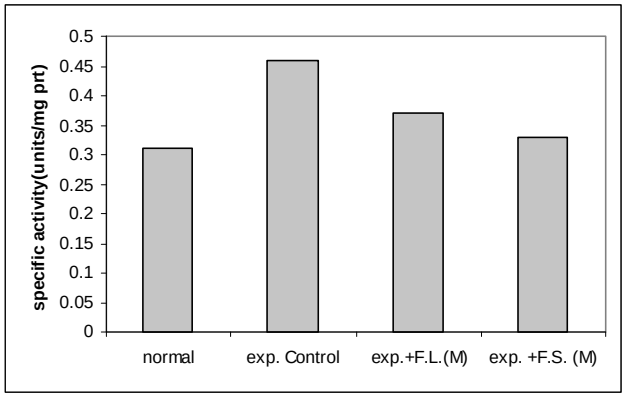

Figure 6. Xanthine oxidase assay

Table 6. Xanthine oxidase activity in control, experimental control and experimental group

S. No.	Groups	S. ACTIVITY (units/mg protein)	% increase or decrease
1.	NORMAL	0.31±0.01	
2.	EXP.CONTROL	0.46±0.017	+48.38%
3.	EXP.+ F.L.(M)	0.37±0.017	+19.3%
4.	EXP. + F.S. (M)	0.33±0.017	+6.4%

All values are reported as MEAN ± SD of minimum three replicates

Summary and Conclusion

In this study, mice were treated with a suitable dose of mercury chloride. Mercury has been seen to induce damage to the living system, mainly by producing a status of oxidative stress.

It was seen that mercuric chloride administration to the experimental group for seven days altered the oxidant/ antioxidant status in the plasma of mice, mainly in terms of levels of antioxidant enzyme systems. Statistically increased levels of lipid peroxidation, leading to production of peroxides which are a source of ROS, and xanthine oxidase activity were elevated. Antioxidant enzyme levels were also significantly decreased, mainly those of catalase, SOD and glutathione reductase, in some cases by demonstrating a 50% decrease.

The methanolic extracts of fenugreek leaf and fenugreek seeds were administered to the mercury chloride treated mice at a suitable concentration for seven days and their plasma enzyme parameters were determined to check the antioxidant levels after the oral feeding of these extracts. The plant extracts significantly increased the levels of antioxidant enzymes, although to different extents. Methanolic fenugreek seed extracts, significantly increased the enzymatic parameters and brought them close to the normal range, for all the enzymes assayed. Fenugreek leaf extracts also statistically improved their levels but to a lesser extent than fenugreek seed extract, according to its bioavailability and antioxidant potential *in vivo*. Methanolic seed extracts feeding seemed to decrease the levels of lipid peroxidation and xanthine oxidase activity, better than methanolic leaf extracts. Fenugreek seed extracts showed the maximum level of restoration by bringing the levels close to the normal range, followed by fenugreek leaf extracts.

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