International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 3, Issue 2, 2011

Research Article

# ANTIOXIDANT ACTIVITY OF *TRIGONELLA FOENUM GRAECUM* USING VARIOUS *IN VITRO* AND *EX VIVO* MODELS

**N.SUBHASHINI,*[1] A.THANGATHIRUPATHI[1], N.LAVANYA[2]**

**[1] Department of Pharmacology, S.B.College of Pharmacy, Sivakasi, [2] Department of Pharmacology, National Institute of Siddha, Chennai, Tamilnadu, India- 600047 Email:drsubhas2006@rediffmail.com**

*Received: 04 Dec 2010, Revised and Accepted: 06 Jan 2011*

**ABSTRACT**

Free radicals are implicated for more than 80 diseases including diabetes mellitus, atherosclerosis, cataract, rheumatism, and other auto immune disease like aging. etc. in treatment of these diseases antioxidant therapy has gained an utmost importance. Current research is now directed towards finding naturally occurring antioxidant of plant origin. In Indian system of medicine Trigonella foenum graecum is an important medicinal plant and its leaves and a seed has been used in various ailments and as health tonic. To understand the mechanism of pharmacological actions, the in vitro antioxidant activity of ethanol (70%) extract of Trigonella foenum graecum (EETFG) was investigated for in vitro antioxidant assays which includes H- donor activity, nitric oxide scavenging, superoxide scavenging, reducing ability, hydroxyl radical, hydrogen peroxide scavenging, total phenolic content, total flavonoid content, total antioxidant activity by thiocyanate and phosphomolybdenum method, metal chelating, β-carotene bleaching, total peroxy radical assays. The pro oxidant activity was measured using bleomycin dependent DNA damage. Ex vivo models like lipid peroxidation and erythrocyte haemolysis were also studied. The various antioxidant activities were compared with suitable standard antioxidants such as ascorbic acid, butylated hydroxyl toluene, α- tocopherol, curcumin, quercetin, and trolox. In all the methods the extract offered strong antioxidant activity in a concentration dependent manner. The total phenolic content, flavonoid content and total antioxidant activity in EETFG were determined as µg pyrocatechol, quercetin and α- tocopherol equivalent/ mg respectively. The extract did not exhibit any pro oxidant activity when compared with ascorbic acid. The results clearly indicate that EETFG is effective against free radical mediated diseases.

**Keywords**: *Trigonella foenum graecum*, Reactive oxygen species, free Radical, Lipid peroxidation, Antioxidants.

## INTRODUCTION

Majority Of The diseases/disorders are mainly linked to oxidative stress due to free radicals[1]. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism[2, 3]. The most common reactive oxygen spices (ROS) include super oxide anion ($O_2$·), hydroxyl radical (OH·), hydrogen peroxide ($H_2O_2$) peroxyl radical radicals (ROO·). The nitrogen derived free radicals are nitric oxide (NO·) and peroxynitrite anion (ONOO·)[4]. ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and cardio vascular malfunction[5, 6]. In treatments of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS; any may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers[7, 8]. Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability[9, 10]. Poly phenol compounds such as flavonoids and phenolic groups widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti inflammatory, anti tumour. Etc.[11, 12] they were also suggested to be a potential iron chelator. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties[13].

In Indian system of medicine *Trigonella foenum graecum* is an important medicinal plant and its leaves and a seed has been used in various ailments and as health tonic. *Trigonella foenum graecum* (leguminosae) (Eng: fenugreek, Tamil: Vendayam) is a well known spicy agent which prevent ageing, labour pain, impart immunity, improve mental function and add vitality to the body and it is also used in nervous disorders, dyspepsia, inflammation, tumors, cholesterolemic, hyperglycemic, and ulcer[14]. Reports indicate that the pharmacological activities of *Trigonella foenum graecum* include anti diabetic, antifertility, antifungal, analgesic, anti-inflammatory, antipyretic, and immunomodulatory activities[15, 16]. EETFG contains alkaloids flavonoids, saponins, carbohydrates, proteins, and tannins. Therefore, the objectives of the present study were to investigate the in vitro antioxidant activity of EETFG through the H- donor activity, nitric oxide scavenging, superoxide scavenging, reducing ability, hydroxyl radical, hydrogen peroxide scavenging, total phenolic content, total flavonoid content, total antioxidant activity by thiocyanate and phosphomolybdenum method, metal chelating, β-carotene bleaching, total peroxyl radical assays[17, 18]. The pro oxidant activity was measured using bleomycin dependent DNA damage. *Ex vivo* models like lipid per oxidation and erythrocyte haemolysis in rats[19, 20, 21].

## MATERIALS AND METHODS

The seeds of *Trigonella foenum graecum* were collected from Coimbatore district, Coimbatore, Tamilnadu, India. The plant material were identified and authenticated by Dr. R. Gopalan, Director, Botanical Survey of India, Tamilnadu Agriculture University, and Coimbatore, India. (Ref. No.BSI/SC/5/23/06-07/Tech-304). The Voucher Specimen Is Available In The Herbarium File Of Our Department.

### Preparation of the extract

The dried seed were pulverized into fine powder using a grinder and sieved through No.22 mesh sieve and stored in an air tight container. About 750 ml of 70% ethanol was added to 75 g of powder and kept on a mechanical shaker for 72 h, the content was filtered and concentration under reduced pressure, under controlled temperature of 40 °C, to yield a dark oily residue. The concentrated extract was stored dry at 4°C in amber colored jars with Teflon lined caps. The percentage yield of the *Trigonella foenum graecum* ethanolic extract was found to be 4.1 % w/v.

### Drugs and chemicals

2,2 Diphenyl –1-picryl hydrazyl hydrate (DPPH), linoleic acid, ammonium molybdate, β-carotene were purchased from Himedia, Mumbai, 2deoxy 2-ribose, xanthine oxidase, quercetin, hypoxanthine, pyrocatechol were purchased from SRL, Mumbai, thiobarbituric acid, trichloroacetic acid, Folin ciocalteu reagent, were purchased from SD Fine Ltd, Mumbai, calf thymus DNA from

Genei chemicals, Bangalore, ferrozine, (2'-2' azobis (2-amidinopropane) dihydrochloride), Trolox from Sigma Aldrich, USA, 2,7 Dichloro flurescein diacetate from Fluka and Butylated hydroxyl toluene from Loba cheme. All other chemicals used in the study were of analytical grade procured from local suppliers

**Experimental animals**

*Wistar* albino rats of either sex (150-200 g) were used for the *ex vivo* study. They were housed in standard polypropylene cages and kept under controlled room temperature (24 ± 20°C, relative humidity 45-55%) in a 12 h light-dark cycle. The rats were given a standard laboratory diet and water *ad libitum*. The study was conducted after ethical clearance from the institutional animal ethics committee bearing the reference number 817/04/ac/CPCSEA.

**Phytochemical screening**

Preliminary phytochemical screening of the powdered seed was performed for the presence of alkaloids, phenolics, flavonoids, saponins, tannins, carbohydrates and proteins.

**In vitro antioxidant activity**

**DPPH radical scavenging assay[22]**

The hydrogen donating ability of extracts was examined in the presence of DPPH stable radical. One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentration and allowed to react at room temperature. After 30 minutes the absorbance values were measured at 517 nm. Ethanol (1.0 ml) plus plant extract solution (2.5ml) was used as a blank, DPPH solution (1.0ml, 0.3 mM) plus ethanol (2.5ml) served as negative control. The positive controls were those using the standard (Ascorbic acid) solutions.

**Nitric oxide radical scavenging assay[23, 31]**

Various concentrations of the EETFG and sodium nitroprusside (10 mM) in standard phosphate buffer solution (0.025 M, pH 7.4) in a final volume of 3 ml was incubated at 25°C for 150 min. Control experiments without the test compounds but with equivalent amount of buffer were prepared in the same manner as done for the test. There after, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess' reagent (1% sulphanilamide, 2% O-Phosphoric acid and 0.1% naphthyethylene diamine dihydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthyethylene diamine dihydrochloride was read at 546nm. The percentage inhibition was calculated. The experiment was done in triplicate using curcumin (50-800 µg/ ml) as positive control.

**Deoxyribose degradation assay[24]**

The decomposing effect of EETFG on hydroxyl radicals was determined by the assay of malondialdehyde chromogen formation due to 2-deoxy 2-ribose degradation. The assay mixture contained in a final volume of 1ml. 100 µl of 28 mM 2-deoxy 2-ribose dissolved in phosphate buffer, pH 7.4, 500 µl of the plant extract of various concentrations in buffer, 200 µl of 200 mM ferric chloride (1:1 v/v) and 1.04 mM EDTA and 100µl of 1.0 mM hydrogen peroxide and 100 µl of 1.0 µM ascorbic acid. After incubation of the test sample at 37°C for one hour the extent of free radical damage imposed on the substrate deoxyribose was measured using thiobarbituric acid (TBA) test. Percentage inhibition of deoxyribose degradation was calculated. Quercetin was used as standard.

**NBT reduction assay[25]**

A reaction mixture with a final volume of 3 ml per tube was prepared with 1.4 ml of 50 mM $KH_2PO_4$-KOH pH 7.4 containing 1 mM EDTA, 0.5 ml of 100µm hypoxanthine, 0.5 ml of 100µM NBT. The reaction was started by adding 0.066 units per tube of xanthine oxidase freshly diluted in 100 µl of phosphate buffer and 0.5 ml of test extract in saline. The xanthine oxidase was added last. The subsequent rate of NBT reduction was determined on the basis of spectrophotometric determinations of absorbance at 560 nm Ascorbic acid was used as standard. The results are expressed as the percentage inhibition of NBT reduction rate with respect to the reaction mixture without test compound (saline only).

**Reducing power ability**

Reducing power ability was measured by mixing 1.0 ml extract prepared with distilled water to 2.5ml of phosphate buffer (0.2m, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide and incubated at 50°C for 30 minutes. After that 2.5 ml of trichloroacetic acid (10%) were added to the mixture and centrifuged for 10 min at 3000 g, 2.5 ml from the upper part were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1%, ferric chloride. The absorbance was measured at 700 nm using UV-spectrophotometer. The reference solution was prepared as above, but contained water instead of the samples. Increased absorbance of the reaction mixture indicates increased reducing power. All experiments were done in triplicate using butylated hydroxyl toluene as positive control.

**Estimation of total phenolic component[26]**

Total soluble phenolics of the extract were determined with Folin-ciocalteu reagent using pyrocatechol as a standard following the method. One millilitre (1.0 ml) of extract solution in a test tube was added to 0.2 ml of Folin Ciocalteu reagent (1:2 in distilled water) and after 20 min 2.0 ml of purified water and 1.0 ml of sodium carbonate (15%) was added. Allowed to react for 30 min and then absorbance was measured at 765 nm. The concentration of total phenolic component in the extract was determined as microgram of pyrocatechol equivalent.

**Total flavonoid content[27]**

Total soluble flavonoid of the extract was determined with aluminium nitrate using quercetin as a standard. Plant extracts (1000 µg/ml) added to 1ml of 80 % ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10 % aluminium nitrate, 0.1 ml of 1 M potassium acetate and 4.3 ml of 80 % ethanol. The absorbance of the supernatant was measured at 415 nm after 40 min at room temperature. Total flavonoid concentration was calculated using quercetin as standard.

**Phosphomolybdate method**

The total antioxidant capacity of the extract was determined with phosphomolybdenum using α- tocopherol as the standard. An aliquot of 0.1ml of plant extract (100 µg) solution was combined with 1.0 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in UV spectrophotometer.

The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as equivalents of α-tocopherol.

**Bleomycin dependent DNA damage**

The reaction mixture contained 0.5 ml calf thymus DNA (10µg/ml), 50 µg of 1.0 ml bleomycin sulfate, and 1.0 ml of 5mM magnesium chloride, 1.0 ml of 50 mM ferric chlorides and 1.0 ml of different concentrations of EETFG. The mixture was incubated at 37°C for 1 h. The reaction was terminated by addition of 0.05 ml EDTA (0.1 M). The color was developed by adding 0.5 ml thiobarbituric acid (TBA) (1% w/v) and 0.5 ml HCl (25% v/v) followed by heating at 37 °C for 15 min. After centrifugation the extent of DNA damage was measured in a UV-spectrophotometer at 532 nm. Each determination was done in triplicate.

**Thiocyanate method[28]**

The peroxy radical was determined by thiocyanate method using α-tocopherol as standard. Increasing concentration of the samples (25-400 µg/ml) in 0.5 ml of distilled water was mixed with 2.5 ml of linoleic acid emulsion (0.02 M, in 0.04 M pH 7.0 phosphate buffer)

and 2 ml phosphate buffer (0.04M, pH 7) in a test tube and incubated in darkness at 37 °C. At intervals during incubation, the amount of peroxide formed was determined by reading absorbance of red color developed at 500 nm by the addition of 0.1 ml of 30% ammonium thiocyanate solution and 0.1ml of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture.

**Hydrogen peroxide scavenging activity**

Hydrogen peroxide solution (2 mM) was prepared with standard phosphate buffer (pH, 7.4). Extract samples (25 - 400 µg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of both plant extracts and standard (α- tocopherol) compound was determined.

**Metal chelating complex**

The ferrous level was monitored by measuring the formation of the ferrous ion-ferrozine complex. The reaction mixture containing 1.0 ml of different concentrations of EETFG (1.0 ml) were added to 0.1 ml of 2 mM ferrous chloride and 0.2 ml of 5 mM ferrozine to initiate the reaction and the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The positive controls were those using ascorbic acid and all tests and analysis were run in triplicate. The percentage chelating effect of Ferrozine-$Fe^{2+}$ complex formation was calculated.

**β-carotene linoleic acid assay**

A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml chloroform and 1.0 ml of this solution was then pipettes into a flask containing 20 mg of linoleic acid and 200 mg of Tween 40 emulsifier. Chloroform was completely evaporated using a vacuum evaporator. Aliquots of 5.0 ml of this emulsion were transferred into a series of tubes containing various concentration of EETFG (25 - 400 µg/ml) or α tocopherol for comparison. Optical density (OD) at 470 nm were taken for EETFG and standard immediately (t=0) and the end of 90 min (t = 90). The tubes were incubated at 50 °C in a water bath during the test. Measurement of OD was continued until the color of β-carotene disappeared in the control.

**Total peroxy radical trapping potential (TRAP) [29]**

A water soluble azo initiator 2, 2' azo bis (2-amidino propane) dihydrochloride (AAPH) produced the peroxyl radicals while a spectrophotometric analysis of 2, 7 dichlorofluresecin - diacetate (DCF) monitored the scavenging activity of the plant extracts. A 350 µl of 1 mM stock of DCF in ethanol was mixed with 1.75 ml of 0.01 N sodium hydroxide and allowed to stand for 20 min before the addition of 17.9 ml of 25 mM sodium phosphate buffer (pH 7.2). The reaction mixture contained 0.5 ml of various concentration of plant extract in methanol, 150 µl of activated DCF solution and 25 µl of AAPH (56 mM). The reaction was initiated with the addition of the AAPH. Absorbance was read at 490 nm. Trolox (6-hydroxy 2, 5, 7-8 tetra methyl chroman 2 carboxylic acids) was used as standard and all the determination was done in triplicate.

**Ex vivo studies**

**Assay of lipid peroxidation method[30]**

Lipid peroxidation induced by $Fe^{2+}$- ascorbate system in rat liver homogenate was estimated by TBA reaction method. The reaction mixture consisted of rat liver homogenate 0.1 ml (25% w/v) in Tris –HCL buffer (20 mM, pH 7.0), potassium chloride (30 mM), ferrous ammonium sulphate (0.16 mM), ascorbate (0.06 mM), and various concentrations of the EETFG in a final volume of 0.5 ml.

The reaction mixture was incubated for 1 h at 37 °C. After the incubation time, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS) (8.1%), 1.5ml TBA (0.8 %), and 1.5 ml glacial acetic acid (20%, pH 3.5). The Total volume was made upto 4 ml of distilled water and then kept in a water bath at 95-100° C for 1 h. after cooling, 1.0 ml of distilled water and 0.5 ml of n- butanol and pyridine mixtute (15: 1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4,000 g for 10 min. the organic layer was removed and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by comparing the OD of the treatments with that of control. Ascorbic acid was used as standard.

**Assay of erythrocyte hemolysis**

The blood was obtained from human and collected in heparinized tubes. Erythrocytes were separated from plasma and the Buffy coat was washed three times with 10 volumes of 0.15 M sodium chloride. During the last wash, the erythrocytes were centrifuged at 3000 rpm for 10 min to obtain a constantly packed cell preparation.

Erythrocyte hemolysis was mediated by peroxyl radicals in this assay system. A 0.2 ml of 10 % suspension of erythrocytes in phosphate buffered saline pH 7.4 (PBS) was added to the similar volume of 200 mM 2, 2' azobis (2 amidinopropane) dihydrochloride (AAPH) solution (in PBS) containing samples to be tested at different concentrations. The reaction mixture was shaken gently while being incubated at 37° C for 2 h. The reaction mixture was then removed, diluted with eight volumes of the PBS and centrifuged at 2000 g for 10 min. The absorbance of the supernatant was read at 540 nm (A). Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete hemolysis, and the absorbance (B) of the supernatant obtained after centrifugation was measured at 540 nm. The data were expressed as mean ± S.E.M. L-ascorbic acid was used as a positive control.

**Calculation of 50% inhibitory concentration ($IC_{50}$)**

The Concentration (mg/ ml) of the extract required to scavenge 50% of the radicals was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated using the formula.

$$I\% = \left[\frac{(A_c - A_s)}{Ac}\right]100$$

Where $A_c$ is the absorbance of the control and $A_s$ is the absorbance of the sample.

**Statistical analysis**

Tests were carried out in triplicate for 3-5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, $IC_{50}$, was graphically determined by a linear regression method using Ms Windows based graph pad instat (version 3) software. Results were expressed as graphically / mean ± standard deviation.

**RESULTS**

**Phytochemical screening**

Phytochemical screening of the plant extract revealed the presence of alkaloids, saponins, tannins, carbohydrates and protein.

**Hydrogen donating assay**

The radical scavenging activity of EETFG was determined from the reduction in the optical absorbance at 517 nm due to scavenging of stable DPPH free radical. Positive DPPH test suggests that the extract is a potential free radical scavenger. EETFG showed strong activity compared with the standard, ascorbic acid. Table 1 shows the $IC_{50}$ values of the sample and the standard.

**NO assay**

Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25° C for 150 min resulted in the generation of NO. The EETFG effectively reduced the generation of NO· Scavenging activity of the extract. The $IC_{50}$ was found to be 0.480 mg/ ml for EETFG and for standard, curcumin, it was found to be 0.076mg/ml (Table 1).

**Deoxyribose degradation**

The scavenging effect of EETFG on hydroxyl (OH) was quantified by measuring the effect on the 2- deoxy –ribose degradation produced by reacting $Fe^{3+}$ with ascorbate, in the presence of EDTA. The $IC_{50}$ value of EETFG was 0.56mg /ml and that of standard, quercetin was 0.112 mg / ml (Table 1).

**Superoxide radical scavenging activity**

EETFG suppressed the superoxide anion radicals generated from hypoxanthine / xanthine oxidase system. Inhibition of NBT reduction by superoxide in the presence of the test preparation increased with raise of their concentrations. All measurements were compared with control experiment. The result shows that EETFG had antioxidative activity similar to the positive control, BHT (Table 1).

**Reducing power**

The reducing ability of the extract served as a significant indicator of its potential antioxidant activity. EETFG and standard (BHT) were used at dose range of 50- 800 µg/ ml. the reducing power of EETFG increased concentration dependently. All concentration of the extract offered higher activities than control (Table 3).

**Total phenolic, flavonoid contents and total antioxidant capacity**

The content of total phenolics in EETFG was determined using folin ciocalteau assay, calculated from regression equation of calibration curve of pyrocatechol. Phenolic content of EETFG was found to be 40 g pyrocatechol equivalent / mg. the total flavonoid content of EETFG was found to be 88 g quercetin equivalent / mg. the total antioxidant capacity of EETFG was found to be 6 µg α- Tocopherol equivalent / mg.

**Bleomycin – dependent DNA damage**

The pro-oxidant activity of EETFG and the standard, ascorbic acid are represented in Table 4. EETFG and ascorbic acid were tested at concentrations ranging from 25 – 400 g /ml. EETFG decreased the absorbance and the bleomycin- $Fe^{3+}$ is not converted into bleomycin-$Fe^{2+}$, thereby preventing the DNA degradation suggesting that EETFG is devoid of pro- oxidant activity.

**Thiocyanate method**

The total antioxidant activity of the EETFG was determined and compared with that of standard, α- Tocopherol by thiocyanate method. EETFG exhibited effective antioxidant activity at all doses and increased concentration dependently and $IC_{50}$ value was found to be 0.265 mg/ ml. the standard, α- Tocopherol showed the $IC_{50}$ value of 0.933 mg/ ml (Table 1).

**Hydrogen peroxide scavenging assay**

EETFG was capable of scavenging $H_2O_2$ in an amount dependent manner. The scavenging ability of the extract and standard, α-Tocopherol are shown in Table 1. $H_2O_2$ scavenging activity of EETFG was closer to that of α- Tocopherol at doses of 100, 200 and 400 µg/ml.

**Ferrous chelating ability**

The ability to chelate ferrous ions also increased with an increase in EETFG concentration, which indicates that EETFG chelates the iron ions. The metal chelating effect of EETFG was lower than the standard, ascorbic acid. The values shown in Table 1 demonstrate the action of EETFG, as peroxidation protector.

**β- Carotene bleaching method**

The antioxidant activity EETFG and the standard drug α- Tocopherol were evaluated by β- Carotene bleaching method and the results are presented in Table 5. EETFG and α- Tocopherol were used in the concentration between 25 – 400 mg/ml and an increase in concentration of the extract and standard decreased the absorbance and this was due to the inhibition of bleaching of the color β-Carotene. The 50 % inhibition value for EETFG was 0.202 mg/ml and for α- Tocopherol was0.1 mg/ml. EETFG exhibited equivalent β-Carotene bleaching activity when compared with α- Tocopherol.

**Total radical antioxidant potential (TRAP)**

The peroxyl radical scavenging activity was determined for EETFG and the results were compared with trolox (Table 1). Addition of increasing concentration of EETFG to solution containing AAPH-derived peroxyl radical decreased the luminescence produced by DCF and the absorbance decreased in a linear fashion. EETFG and Trolox exhibited $IC_{50}$ values of 0.262 and 0.099 mg/ml respectively.

**Lipid peroxidation**

EETFG was effective in inhibiting the lipid peroxidation induced by $Fe^{2+}$- ascorbate system in rat liver homogenate. The MDA generated as a result of lipid peroxidation reacts with thiobarbituric acid and was found to be inhibited in the presence of the extract. The $IC_{50}$ value was found to be 0.241 mg/ml for EETFG while for standard ascorbic acid the $IC_{50}$ was found to be 0.081 mg/ml (Table 2).

**Erythrocyte haemolysis**

The peroxyl radical generated by AAPH on addition to erythrocyte suspension and its subsequent scavenging action produced by graded concentrations of EETFG and standard, ascorbic acid are given in Table 2. An increase in inhibition was noticed at all concentrations of the extract and ascorbic acid.

**Table 1: Antioxidant activity of *Trigonella foenum graecum* by different models**

| Drugs | *In vitro* methods $IC_{50}$ (Mg/Ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | DPPH | NO | · OH | $O_2$ | · $H_2O_2$ | ThioCyanate method | Metal chelating | TRAP |
| EETFG | 0.80±2.33 | 0.48±18.0 | 0.560±5.2 | 0.160±3.3 | 0.84 ± 4.41 | 0.265± 7.26 | 0.105±7.26 | 0.262 ± 1. |
| Ascorbic Acid | 0.003 ± 0.06 | | | 0.072 ±12.6 | | | 0.044 ±26.8 | |
| Quercetin | | | 0.112±11.2 | | | | | |
| curcumin | | 0.076 ±9.33 | | | | | | |
| α- Tocopherol | | | | | 0.066±1.66 | 0.09±3.33 | | |
| Trolox | | | | | | | | 0.099±2.33 |

Values are mean ± SD (n = 3)

**Table 2: Antioxidant activity of *Trigonella foenum graecum* by different models**

| Drugs | *Ex vivo* methods $IC_{50}$ (MG/ML) | |
|---|---|---|
| | lipid peroxidation | Erythrocyte haemolysis |
| EETFG | 0.241± 4.33 | 0.267 ± 5.26 |
| Ascorbic Acid | 0.081± 4.41 | 0.110± 0.66 |

Values are mean ± SD (n = 3)

**Table 3: Reducing power ability**

| Drug | Absorbance at 700 nm | | | | |
|---|---|---|---|---|---|
| | 50 µg/ ml | 100 µg/ ml | 200 µg/ ml | 400 µg/ ml | 800 µg/ ml |
| EETFG | 0.181± 0.005 | 0.221 ± 0.03 | 0.252 ± 0.002 | 0.274 ± 0.004 | 0.354 ± 0.01 |
| BHT | 0.092 ± 0.002 | 0.214 ± 0.004 | 0.314 ± 0.004 | 0.640 ± 0.001 | 1.092 ± 0.008 |

**Table 4: Pro-oxidant activity of different concentration of *Trigonella foenum graecum***

| Drug | Absorbance at 532 nm | | | | |
|---|---|---|---|---|---|
| | 25 µg/ ml | 50 µg/ ml | 100 µg/ ml | 200 µg/ ml | 400 µg/ ml |
| EETFG | 0.270 ± 0.003 | 0.210 ± 0.004 | 0.161 ± 0.010 | 0.102 ± 0.012 | 0.048 ± 0.02 |
| Ascorbic acid | 0.805 ± 0.016 | 0.655 ± 0.009 | 0.425 ± 0.008 | 0.150 ± 0.005 | 0.035 ± 0.001 |

**Table 5: β- Carotene bleaching inhibitory activity**

| Drug | Absorbance at 470 nm | | | | | | |
|---|---|---|---|---|---|---|---|
| | Time of Incubation(min) | 25 µg/ ml | 50 µg/ ml | 100 µg/ ml | 200 µg/ ml | 400 µg/ ml | $IC_{50}$ mg/ ml |
| EETFG | 0 | 0.137 | 0.165 | 0.226 | 0.333 | 0.373 | 0.202 ± 0.03 |
| | 90 | 057 | 0.078 | 0.133 | 0.232 | 0.254 | |
| Ascorbic Acid | 0 | 0.098 | 0.126 | 0.189 | 0.285 | 0.725 | 0.100 ± 0.01 |
| | 90 | 0.058 | 0.082 | 0.138 | 0.230 | 0.664 | |

Values are mean ± SD (n = 3)

**DISCUSSION**

Antioxidants exert their mode of action by suppressing the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements. DPPH is widely used to evaluate the free radical scavenging effect of natural antioxidant. DPPH is a stable free radical at room temperature, which produces a violet solution in ethanol. DPPH shows a strong absorption band at 517 nm in visible spectrum (deep violet color). As the electron became paired in the presence of free radical scavenging the absorption vanishes and the resulting discoloration stochiometrically coincides with the number of electrons taken up. The bleaching of DPPH absorption is representative of the capacity of the test drugs to scavenge free radicals independently. The bleaching of DPPH molecules can be correlated with the number of available hydroxyl groups. We can infer that, the very good activity of the extract may be probably due to the presence of substance with an available hydroxyl group.

NO is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecules in diverse biological systems including neuronal messenger, vasodilation, antimicrobial and antitumour activities. Scavengers of NO compete with oxygen leading to the reduced production of NO. Our finding suggests that the phenolic compounds present in the extract might be responsible for NO scavenging effect.

Hydroxyl radicals are very reactive and can be generated in biological cells through the Fenton reaction. Hydroxyl radicals scavenging activity was quantified by measuring inhibition of the degradation of the deoxyribose by free radicals. When EETFG and reference compound quercetin were added to the reaction mixture, they removed hydroxyl radicals from the sugar and prevented their degradation.

Superoxide anions serve as precursors of singlet oxygen and hydroxyl radicals. The superoxide anions generated by hypoxanthine / xanthine oxidase system reduced nitroblue tetrazolium (NBT) to form a chromophore (diformazan) that absorbs at 560 nm. The extract decreased the mean rate of absorption by inhibiting NBT reduction by the superoxide anion radicals[32].

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing ability is generally associated with the presence of reductones, which breaks the free radical chain by donating a hydrogen atom. The extract had reductive ability which increased with increasing concentrations of the extract.

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen, flavonoids are wide spread in all natural compounds and posses a broad spectrum of biological activities. The chemical composition of Trigonella foenum graecum indicates the presence of phenolic compounds including tannins and flavonoids, which are known to possess antioxidant activities. The high phenolic and flavonoid content in the EETFG may be responsible for its free radical scavenging activity.

The phosphomolybdenum method is based on the reduction of $M_o$ (VI) to $M_o$ (V) by the sample analyte and the subsequent formation of green phosphate $M_o$ (V) complex with a maximum absorption at 695 nm. The extract reduced molybdenum VI to a green colored phosphomolybdenum V complex. The antioxidant capacity was expressed as equivalents of α-tocopherol. This method is used to investigate the total antioxidant capacity of the extracts[33].

Damage to DNA in the presence of a bleomycin- Fe complex has been adopted as a sensitive and specific method to examine potential pro-oxidant agents. If the samples are able to reduce the bleomycin- $Fe^{3+}$ to bleomycin $Fe^{2+}$, DNA degradation in this system would be stimulated, resulting in a positive test for pro-oxidant activity. The extract decreased the absorbance and bleomycin- $Fe^{3+}$ is not converted into bloemycin $Fe^{2+}$ thereby preventing the DNA degradation. The results confirm that EETFG is devoid of pro-oxidant activity[34].

The thiocyanate method measures the amount of peroxides produced at the initial stage of lipid peroxidation which is depicted by a decrease in absorbance indicating increased level of antioxidant activity. The good antioxidant activity exhibited by EETFG might be attributed to the presence of flavonoid like phytochemicals.

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it can give rise to hydroxyl radical in the cells. Thus the removal of $H_2O_2$ is very important for antioxidant defence in cell or food systems. $H_2O_2$ can cross membranes and may oxidize a number of compounds. EETFG scavenged $H_2O_2$ which may be attributed to the presence of phenolics, which could donate electrons thereby neutralizing it into water[35].

Ferrozine can quantitatively form complexes with $Fe^{2+}$ but in the presence of ion chelating agents, the complex formation is disrupted resulting in a reduction in the red color of the complex measurement of the rate of reduction of the color, therefore allows estimation of the chelating activity of the coexisting chelator. The absorbance of $Fe^{2}$ - Ferrozine complex was linearly decreased dose dependent manner. The data obtained from results that the extracts of *Trigonella foenum graecum* demonstrate an effective capacity for iron binding, suggesting that its action as peroxidation protector may be related to its iron binding capacity. In this assay the extract and standard compound interfered with the formation of ferrous complex with the reagent ferrozine, suggesting that it has chelating activity and captures the ferrous ion before ferrozine. The ion chelating activity of the extract may be attributed due to the presence of endogenous chelating agents, mainly phenolics[36, 37].

The decoloration of β-carotene is widely used to measure the antioxidant activity of plant extracts, because β-carotene is extremely susceptible to free radical mediated oxidation of linoleic acid. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsatured β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the compound loses its yellow color. The EETFG inhibited β-carotene oxidation suggesting that the antioxidant activity could be related to the high levels of phenolic compounds.

TRAP assay is based upon the potential of antioxidants in extract to scavenge peroxyl radicals generated by thermal decomposition of a water soluble azo initiator AAPH. EETFG decreased the absorbance upon increasing concentrations of the sample, which is similar to that of the standard, Trolox.

Oxidative stress can lead to peroxidatin of cellular lipids and can be measured by the determining the levels of thiobarbituric acid reactive substances. Quantification of MDA, one of the products of lipid peroxidation, with TBA at low pH and high temperature (100$^{o}$ C) resulted in the formation of a red complex, which is measured at 532 nm. EETFG inhibits the rate of lipid peroxidation by a reduction in the red color complex formed reflecting its anti- lipid per oxidative potential.

The azo compound generates free radicals by its unimolecular thermal decomposition. The rate of generation of peroxyl radicals can be easily controlled and measured by adjusting the concentration of AAPH. Therefore, the haemolysis induced by AAPH clearly demonstrates the oxidative erythrocytes membrane damage by peroxyl radical attack from the outside of the membrane. The EETFG inhibited the erythrocyte haemolysis induced by AAPH in a concentration dependent manner.

## CONCLUSION

Based on the various *in vitro* and *ex vivo* assays, it can be concluded that the EETFG posses string antioxidant activity, evidenced by the free radical scavenging property, iron chelating, and reducing power property, which may be due to the presence of phenolic components in the extract. Overall, the plant extract is a source of natural antioxidant that can be important in disease prevention, health preservation and promotion of longevity promoter.

## REFERENCES

1. Benzie IFF. Evolution of dietary antioxidants. Comp Biochem Physiol Part *A* 2003; 136: 113-126.
2. Fang Y, Yang S, Wu G., Free radicals, antioxidants and nutrition. Nutrition 2002; 18: 872-879.
3. Manavalan K, Ramasamy C. Physical pharmaceutics. 2nd ed. Chennai: Vignesh publishers. p. 286 (2001)
4. Nagendrappa CG. An appreciation of free radical chemistry- 3. Free radicals in diseases and health. Resonance 2005; 10(2): 65-73.
5. Nordberg J, Arner ESJ. Reactive oxygen species, antioxidants and mammalian thioredoxin system. Free Rad Biol Med 2001; 31(11): 1287-1312.
6. Ray G, Husain SA. Oxidant, antioxidants and carcinogenesis. Indian J Exp Biol 2002; 40: 1213-1232.
7. Halliwell B, Gutteridge JMC. Free radical in biology and medicine. 3rd ed. London: Oxford. p. 36-40, 311-12 (1999)
8. Daniel RS, Mathew BC, Devi KS. Antioxidant effect of two flavonoids from the bark of *Ficus bengalensis Linn.* In hyperlipidemic rats. Indian J Exp Bio 1998; 36; 902-906.
9. Agarwal A, Prabakaran SA. Mechanism, measurement and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol 2005; 43: 963-974.
10. Chaurasia S, Tripathi P, Tripathi YB. Antioxidant and Anti-inflammatory property of Sandhika: A compound herbal drug. Indian J Exp Biol 1995; 33: 428-432.
11. Irshad M, Chaudhuri PS. Oxidant antioxidant system: Role and significance in human body. Indian J Exp Biol 2002; 40: 1233-1239.
12. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005; 53: 1841-1856.
13. Lee J, Koo N, Min DB. Reactive oxygen species, aging, and antioxidative nutraceuticals. Comprehensive Reviews in Food Science and Safety 2004; 3: 21-33.
14. Nadkarni KM. The Indian material medica with ayurvedic unani and home remedies. 3rd ed. Bombay: Popular Prakashan. p.1240-1243 (1954)
15. Bin-Hafeez B, Haque R, Parvez S, Pandey S, Sayeed I, Raisuddin S. Immunomodulatory effects of fenugreek (*Trigonella foeneum-graecum L.*) extract in mice. Int Immunopharmacol 2003; 3(2): 257-265.
16. Ahmadiani A, Javan M, Semnanian S, Barat E, Kamalinejad M. Anti-inflammatory and antipyretic effects of *Trigonella foenum-graecum* leaves extract in the rat. J Ethnopharmacol 2001; 75(2-3): 283-286.
17. Gutierrez RMP, Luna HH, Garrido SH. Antioxidant activity of *Tagetes erecta* essential oil. J Chil Chem Soc 2006; 51(2): 883-886.
18. Shirwaikar A, Rajendran K, Kumar CD. *In vitro* antioxidant studies of *Annona squamosa* Linn leaves. Indian J Exp Bio 2004; 42: 803-807.
19. Ng TB, Liu F, Lu Y, Cheng CHK, Wang Z. Antioxidant activity of compounds from the medicinal herb *Aster tataricus.* Comp Biochem Physiol Part C 2003; 136: 109-15.
20. Oktay M, Gulcin I, Kufrevioglu I. Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Labenson-Wiss U-Technol 2003; 36: 263-271.
21. Jayaprakasha GK, Jena BS, Negi PS, Sakariah KK. Evaluation of antioxidant activities and antimutagenicity of turmeric oil: a byproduct from curcumin production. Z Naturforsch 2002; 57: 828-835.
22. Gomes AJ, Lunardi CN, Gonzalez S, Tedesco AC. The antioxidant action of *Polypodium leucotomos* extract and kojic acid: reactions with reactive oxygen species. Braz J Med Biol Res 2001; 34: 1487-1494.
23. Yildirim A, Oktay M, Bilaloglu V. The antioxidant activity of the leaves of *Cydonia vulgaris*. Turk J Med Sci 2001; 31: 23-27.
24. Mongkolsilp, Pongbupakit I, Sae-Lee N, Sitthithaworm W. Radical scavenging activity and total phenolic content of medicinal plants used in primary health care. SWU J Pharm Sci 2004; 9(1): 32-35.
25. Huang S, Kuo JC. Concentrations and antioxidative activity of anserine and carnosine in poultry meat extracts treated with demineralization and papain. Proc Natl Sci Counc ROC (B) 2000; 24(4): 193-201.
26. Panovska TK, Kulevanova S, Stefova M. *In vitro* antioxidant activity of some *Teucruim* species (Lamiaceae). Acta Pharm 2005; 55: 207- 214.
27. Govindarajan R, Vijayakumar M, Rao CV, Shirwaikar A, Rawat AKS, Mehrotra S, et al. Antioxidant potential of *Anogeissus latifolia.* Biol Pharm Bull 2004; 27(8): 1266-1269.
28. McCune LM, Johns T. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the North American boreal forest. J Ethnopharmacol 2002; 82: 197-205.
29. Valkonen M, Kuusi T. Spectrophotometric assay for total peroxyl radical trapping antioxidant potential in human serum. J Lipid Res 1997; 38: 823-833.

30. Gulcin I, Oktay M, Kufrevioglu I, Aslan A. Determination of antioxidant activity of lichen *Cetraria islandica*. J Ethnopharmacol 2002; 79: 325-329.
31. Sreejayan, Rao MNA. Nitric oxide scavenging by curcuminoids, Pharma Pharmacol 1997; 49: 105-107.
32. Guzman S, Gato A, Calleja JM. Anti-inflammatory, analgesic and free radical scavenging activities of the marine micro algae *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. Phytother Res 2001; 15: 224-230.
33. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical Biochem1999; 269(2): 337-41.
34. Elzaawely AA, Xvan TD, Tawata S. Antioxidant and antibacterial activities of *Ramex japonicus* HOUFF. Aerial parts. Biol Pharm Bull 2005; 28(1): 2225-2230.
35. Haraguchi H, Inoue J, Tamura Y, Mizutani K. Antioxidative components of *Psoralea corylifolia*. Phytother Res 2002; 16: 539-544.
36. Orech FO, Akenga T, Ochora J, Friis H, Aagaard-Hansen J. Potential Toxicity of some traditional leafy vegetables consumed in Nyang`oma division, Western Kenya. Afri J Food Nutri Sci 2005; 5(1): 1-13.
37. Senevirathne M, Kim S, Siriwardhana N, Ha J, Lee K, Jeon Y. Antioxidant potential of *Ecklonia cava* on reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition. Food Sci Tech Int 2006; 12(1): 27-38.