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In Vitro Antioxidant Activity of Methanolic Extract of *Trigonella foenum-graecum*

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ABSTRACT

Trigonella foenum-graecum is an annual herb found in India. Locally known as Methi. The literature of *Trigonella foenum-graecum* revealed that less reports or studies were done on pharmacognostical and antioxidant studies on this plant. Keeping this in point of view, the present work was undertaken to know the antioxidant potential and to establish the pharmacognostical standards. The whole plant of *Trigonella foenum-graecum* was extracted with methanol and it was subjected to preliminary phytochemical and antioxidant studies. Steroids, tannins, carbohydrates, alkaloids, glycosides, etc., are found to be present in *Trigonella foenum-graecum.* Phytochemical analysis and isolation of active constituents has given information regarding the antioxidant activity which was carried out by using methods like reducing activity assay, iron chelation, total antioxidant activity and lipid peroxidation. The total antioxidant activity was found to be is less when compared to remaining methods. Presence of tannins, plant has shown good antioxidant property and experiment results suggested that *Trigonella foenum-graecum* has potential antioxidant properties.

INTRODUCTION

Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from the metabolism of oxygen. ROS, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as byproducts of biological reactions or from exogenous factors. *In vivo*, some of these ROS play positive roles in cell physiology; however, they may also cause great damage to cell membranes and DNA, inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity, and DNA mutations leading to cancer, degenerative, and other diseases.

Mammalian cells possess elaborate defense mechanisms for radical detoxification. Key metabolic steps are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), which destroy toxic peroxides. In addition to antioxidant enzymes, non-enzymatic molecules, including thioredoxin, thiols, and disulfide-bonding play important roles in antioxidant defense systems. Some of the compounds are of an exogenous nature and are obtained from food, such as α-tocopherol, β-carotene, and ascorbic acid, and such micronutrient elements as zinc and selenium. If cellular constituents do not effectively scavenge free radicals, they lead to disease conditions.

Antioxidant-based drugs/formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer have appeared during the last three decades. This has attracted a great deal of research interest in natural antioxidants. Subsequently, a worldwide trend towards the use of natural phytochemicals present in berry crops, tea, herbs, oilseeds, beans, fruits, and vegetables has increased.

Several herbs and spices have been reported to exhibit antioxidant activity, including rosemary, sage, thyme, nutmeg, turmeric, white pepper, chili pepper, ginger, and several Chinese medicinal plant extracts. The majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, β-carotene, and α-tocopherol are known to possess antioxidant potential. A direct relationship between antioxidant activity and phenolic content of plant extracts has been reported. Epidemiological studies have shown that the consumption of foods and beverages rich in phenolic content can reduce the risk of heart disease.

Many Indian medicinal plants are considered potential sources of antioxidant compounds. In some cases, their active constituents are known. *Terminalia chebula, T.bellerica, T. muelleri,* and *Phyllanthus emblica* all of which have antioxidant activity, showed high content of phenolics like gallic acid. Conversely, the antioxidant activity of *Hemidesmus indicus, Cichorium intybus, Withania somnifera, Ocimum sanctum, Mangifera indica,* and *Punica granatum,* as determined by several methods, has been poorly documented .There is an increased quest to obtained natural antioxidants with broad-spectrum actions. The majority of the rich diversity of Indian medicinal plants is yet to be scientifically evaluated for such properties. Furthermore, the relationship between phenolic content and antioxidant activity is largely not examined in Indian medicinal plants.

In this study, we investigated the crude methanolic extract of *Trigonella foenum-graecum* Plant for their potential antioxidant activity by the reducing activity Assay, iron chelation method, total antioxidant activity, lipid peroxidation test**.**

Aim:

The survey of literature revealed that only few studies have done on phytochemical studies so we planned for these studies and also to evaluate antioxidant activity.

Objective:

The objective of present work is to extract the plant by using methanol as solvent and to evaluate phytochemical constituents and antioxidant activity.

Oxidative stress:

Oxidative stress is defined as an imbalance between reactive oxygen species generating and scavenging systems, antioxidants. The term "oxidative stress" was first coined by Sies (1985).

Oxidative stress is defined as a state of higher cellular levels of reactive oxygen species (ROS, e.g.: O_2,$^{\bullet}$OH, OH, NO and ONOO) than the cellular antioxidant defense. Generation of ROS is generated during aerobic metabolism, i.e. mitochondrial oxidations and other monoamine oxidations. Under normal circumstances, ROS are eliminated by cellular enzymatic (superoxide dismutase, SOD; glutathione peroxidase, GSHPx and catalase, CAT) and non-enzymatic (GSH

and uric acid) antioxidant defenses and dietary antioxidants such as Vit A, E and C, β-carotene, quinines and flavones. If ROS are not effectively eliminated, they can cause the oxidative cell injury, i.e. peroxidation of cell membrane phospholipid esterifies EPUFAs, proteins (receptors and enzymes) and DNA (Mahadik et al., 2001).

Oxidative stress resulting from increased production of free radicals and reactive oxygen species and a decrease in antioxidant defense leads to damage of biological macromolecules and disruption of normal metabolism and physiology.

Over the past decade, there has been substantial interest in oxidative stress and its potential role in epilepsy, development of diabetic complications, atherosclerosis and associated cardiovascular disease. Consequences of oxidative stress are damage to DNA, lipids, proteins, resulting in the disruption of cellular homeostasis.

Free radicals:

Free radicals are chemical species possessing an unpaired electron that can be considered as fragments of molecules, which are generally very reactive. Free radicals are very reactive because they actively seek out electrons from other compounds. The reactions they cause can result in things like heart disease and cancer. Some free radical reactions are beneficial like the immune response that destroys viruses. Uncontrolled free radical reactions damage the body and may lead to diseases including cancer (Gutteridge*et al.*, 1990).

Free radicals can be formed in three ways

1) By the hemolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one paired electrons.

2) By the loss of a single molecule from a normal molecule.

3) By the addition of a single electron to a normal molecule.

The processes by which free radicals or ions are formed are illustrated below :

Radical formation by electron transfer:

$$A + e \rightarrow A^{-\bullet}$$

Radical formation by hemolytic fission :

$$X:Y \rightarrow X^{\bullet} + Y^{\bullet}$$

Ion formation by heterolytic fission :

$$X:Y \rightarrow X:^{-} + Y^{+}$$

Types of free radicals:

1. Singlet oxygen

Oxygen in the air we breathe is in its "ground" state and is symbolized by the abbreviation $^3O_2$. Ground-state oxygen is in the triplet state (indicated by the superscripted "3" in $^3O_2$)– its two unpaired electrons have parallel spins (↑↑), a characteristic that, does not allow them to react with most molecules. Thus, ground-state or triplet oxygen is not very reactive.

If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it forms the singlet state. Singlet oxygen, $^1O_2$*, has a pair of electrons with opposite spins; though not a free radical it is highly reactive.

$\cdot$**O—O**$\cdot$ triplet oxygen (↑↑) (ground state)

↓

O—O: singlet oxygen (↑↓) (highly reactive).

This reaction can also be represented as:

$$^3\mathbf{O_2} + \text{energy} \rightarrow {}^1\mathbf{O_2}^*$$

Singlet oxygen has a long lifetime for an energetically excited molecule, and must transfer its excess energy to another molecule in order to relax to the triplet state.

2. Superoxide

Superoxide radical is formed by the monovalent reduction of triplet oxygen. It is shown with a negative sign ($O_2^{\cdot -}$), indicating that it carries a negative charge of -1(due to an extra electron, e^-, it gained).

·O—O· triplet oxygen (ground state)

↓ **monovalent reduction**

·O—O: superoxide

This reaction can also be written in this form:

$$^3O_2 + e^- \rightarrow O_2^{\cdot -}$$

Superoxide can act both as an oxidant (by accepting electrons) or as a reductant (by donating electrons). Superoxide is not particularly reactive in biological systems and does not by itself cause much oxidative damage. It is a precursor to other oxidizing agents, including singlet oxygen, peroxynitrite, and other highly reactive molecules.

Under biological conditions, the main reaction of superoxide is to react with itself to produce hydrogen peroxide and oxygen, a reaction known as "dismutation".

Superoxide dismutation can be spontaneous or can be catalyzed by the enzyme superoxide dismutase ("SOD").

$$\text{ii) } ^2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + {}^3O_2$$

Superoxide is also important in the production of the highly reactive hydroxyl radical (HO·). In this process, superoxide acts as a reducing agent. This is because superoxide donates one electron to reduce the metal ions (for example: ferric iron, Fe^{3+}) that act as catalyst to convert hydrogen peroxide (H_2O_2) into the hydroxyl radical (HO·).

$$\text{iii) } O_2^{\cdot -} + Fe^{3+} \rightarrow {}^3O_2 + Fe^{2+}$$

This reduced metal then catalyzes the breaking of the oxygen bond of hydrogen peroxide to produce a hydroxyl radical **(HO·)** and a hydroxide ion **(HO^-):**

iv) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^-$

Superoxide can react with the hydroxyl radical (HO·) to form singlet oxygen **($^1O_2$*)**

v) $O_2\cdot^- + HO\cdot \rightarrow {}^1O_2* + HO^-$

Superoxide can also react with nitric oxide (NO·) to produce peroxynitrate (OONO·), another highly reactive oxidizing molecule.

3. Hydrogen peroxide

Superoxide ($O_2\cdot^-$) can undergo monovalent reduction to produce peroxide (O_2^{-2}),

An activated form of oxygen that carries a negative charge of -2.

·O—O: Superoxide

↓ monovalent reduction

H:O—O:H Hydrogen peroxide

4. Hydroxyl radical

Hydrogen peroxide, in the presence of metal ions, is converted to a hydroxyl radical (HO·) and a hydroxide ion (HO^-). A metal ion is required for the breaking of the oxygen-oxygen bond of peroxide. This reaction is called the **Fenton reaction.**

H:O—O:H Hydrogen peroxide

↓ Metal ion

HO· : Hydroxyl radical and

HO : Hydroxide ion

This reaction can also be written (with iron as the metal):

$$\text{i)}\quad Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^-$$

A hydroxyl radical can also react with superoxide to produce singlet oxygen and a hydroxide ion:

$$\text{ii)}\ O_2\cdot^- + HO\cdot \rightarrow {}^1O_2{}^* + HO^-$$

$$\text{iii)}\ HO\cdot + R \rightarrow HOR\cdot$$

$$\text{iv)}\ HOR\cdot + Fe^{3+} \rightarrow HOR + Fe^{2+} + H^+$$

$$\text{v)}\ HOR\cdot + {}^3O_2 \rightarrow HOR\cdot + O_2\cdot^- + H^+$$

Two adduct radicals can also react with each other, forming a stable, cross linked-but oxidized-product, with water as a byproduct.

$$\text{vi)}\ HOR\cdot + HOR\cdot \rightarrow R\text{—}R + 2H_2O$$

The hydroxyl radical can also oxidize the organic substrate by abstracting an electron from it.

$$\text{vii)}\ HO\cdot + R \rightarrow R\cdot + H_2O$$

The resulting oxidized substrate is again itself a radical, and can react with other molecules in a chain reaction.

$$\text{viii)}\ ROO\cdot + RH \rightarrow ROOH + R\cdot$$

This type of chain reaction is common in the oxidative damage of fatty acids and other lipids and demonstrates why radicals such as the hydroxyl radical can cause so much damage than one might have expected.

Sources of Oxygen-Derived Free Radicals:

Potentially damaging free radicals are produced largely within the cells. There are a large number of physiologic and pathologic sources of oxygen free radicals and related oxidants.

They are even produced in small amounts during normal cellular processes. Besides intracellular and extracellular sources for generation of free radicals, exogenous agents such as photochemical smog, ozone, pesticides, xenobiotics and ionizing radiation are well known to generate free radicals (Karlra *et al.*, 1994).

The following are the various sources for the generation of free radicals in the biological system.

1) Auto-oxidation of reduced flavin, thiols, and small molecules such as hydroquinones, catecholamines, and tetrahydro proteins.

2) Soluble enzymes such as xanthine oxidase.

3) Mitochondrial sources: The leaks in mitochondrial electron transport systems allow O_2 to accept single electron forming O_2.

4) Endoplasmic reticulum and nuclear membrane electron transport system.

5) Peroxisomes potent sources of cellular H_2O_2.

6) Respiratory burst: Various types of stimulants such as opsonized bacteria, viruses, immunoglobulins and macrophages which take up large amounts of oxygen and convert into superoxide anion.

Why brain is more vulnerable to oxidative stress:

Oxidative stress affects predominantly the brain (Halliwell, 1992; Mahadik and Mukharjee, 1996). This preferential brain vulnerability is due to its higher oxidative stress than other organs since it generates very high levels of ROS due to its very high aerobic metabolism and blood perfusion and it has a relatively poor enzymatic antioxidant defense; it is enriched in lipids (O'Brien and Sampson 1965) that are preferentially susceptible to oxidative damage (Bieleski et al., 1983); the damaged neuronal DNA in the adult brain can not be effectively repaired since there is no replication. In addition to these factors, brain is subjected to increased levels of ROS from the environmental factors such as radiation, a variety of smokes and pollution due to higher reperfusion. Depending on the degree of oxidative stress and the developmental time, oxidative neuronal injury it may cause abnormal neurodevelopment, neurodegeneration or neuronal

membrane impairment i.e. a specific breakdown of membrane phospholipids may results into altered membrane receptor mediated phospholipids regulated signal transduction.

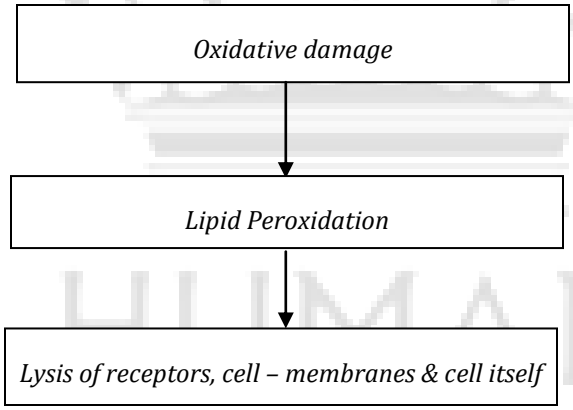
The major targets of oxidative activity in biological systems:

- Lipids
- Proteins and Carbohydrate
- DNA

Free radicals disrupt the equilibrium of biological systems by damaging their major constituent molecules, leading eventually to cell death.

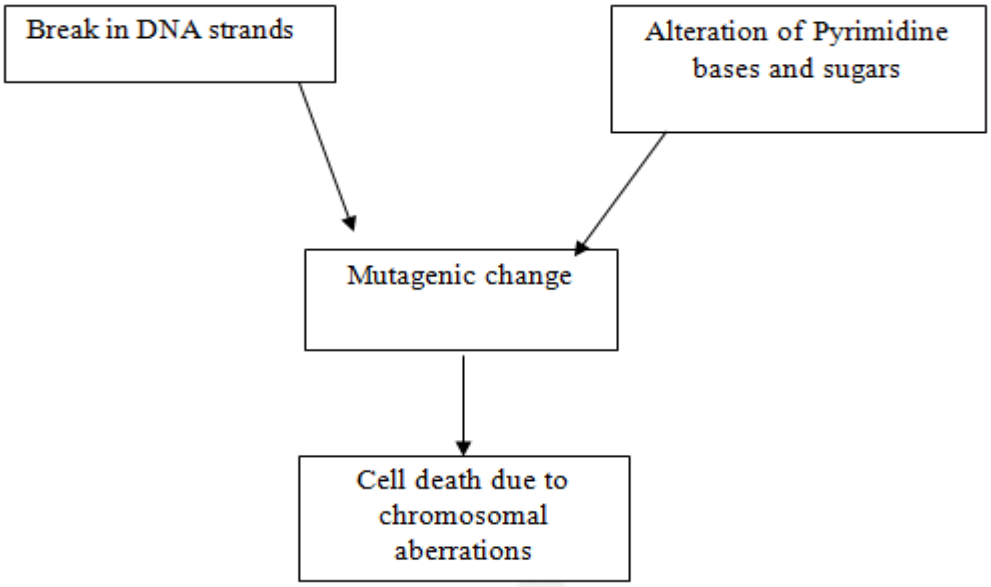
Free radical target to lipid:

Cell membranes are lipids and are vulnerable to oxidative damage.



Free Radical attack on DNA:

Free radical induced damage to DNA may cause destruction of bases and deoxyribose sugar or single and double strand breaks. These events have been implicated as a cause in mutagenesis, carcinogenesis and cell death (Aust *et al.*, 1985).



Selected Human Diseases and Free radicals Implicated

S.No	Disease	Free radicals implicated	Damage inflicted
1	Epilepsy (Sudha *et al.*, 2001)	OFR	Oxidative stress
2.	Cancer (Dreher*et al.*, 1996)	OH^-	Oxidative DNA damage
3	Myocardial damage (Chanph*et al.*, 1996)	OFR	Myocardial reperfusion
4	Ischemic hepatitis (Poli*et al.*, 1993)	OFR	Acute lethal damage of hepatocytes
5	Inflammation (Wniro*et al.*, 1993)	O_2, H_2O_2	Mediates tissue destruction
6	Parkinson's disease (Evens, 1992)	OFR	Mediates neuronal loss

OFR : Oxygen Free Radicals ROS : Reactive Oxygen Species

ANTIOXIDANTS:

There has been a great deal of research carried out recently on a whole range of antioxidants and the conclusions in general show that these substances have a profound effect on aging and the degenerative disease of this modern age (Ronaghy *et al.*, 1974).

Action of antioxidants: (Halliwell, 1989)

- They may reduce the energy of the free radical
- preventive (suppress radical formation)
- radical scavengers (break chain propagation)
- repair (repair damage and reconstitute membranes)

The interaction between free radicals and antioxidants:

Antioxidants are powerful electron donors and react preferentially with free radicals before more important target molecules are damaged. In doing so the antioxidant is sacrificed (oxidized) and must be regenerated or relapsed. By definition, the antioxidant radical is relatively unreactive as to attack further molecules (Halliwell, 1989).

In the human body a complex combination of enzymatic and non-enzymatic function to minimize the stress induced by ROS. These antioxidants may be classified as endogenous antioxidants, those which are have physiological origin and exogenous antioxidants are those which can not be produced by the human body but may protect against pro-oxidant forces when administered as supplements.

Endogenous/enzymatic antioxidants:

1) Superoxide dismutase:

Although superoxide is produced at a relatively high rate by cells during normal metabolism, its low intracellular level is maintained by either spontaneous dismutation and or catalytic breakdown by enzyme superoxide dismutase (SOD). This enzyme was first reported by McCord and Fridovich, (1968). It is found to be present in the cytosol extracellular and in the mitochondria (Freeman and Capro, 1982).

2) Catalase:

This cytoplasmic heme enzyme is a tetrameric protein which catalyzes the reduction of H2O2 in millimolar concentrations.

3) Glutathione peroxidase:

This enzyme is important for the detoxification not only of H2O2 but also of organic peroxides. This enzyme is located mainly in the cytosol of eukaryotic cells and also in the mitochondria. Unlike catalase it is more effective at low concentrations of H2O2.

Non-enzymatic antioxidants:

1) Vitamin C:

Ascorbic acid is one of the strongest reductants, radical scavengers and reduces stable oxygen, oxygen and thiol radicals and acts as a primary defense against radicals in the blood. Ascorbic acid acts as a synergist with tocopherol for the reduction of lipid peroxy radicals within the lipid compartment by reacting with tocoperoxy radical and regenerating active tocopherol. Ascorbate appears to belong to the first line of defense. Ascorbate should prove very helpful in degenerative processes caused by oxidative stress (Stocker et al., 1990).

2) Vitamin E:

Tocopherols belong to a class of phenolic antioxidants which can inhibit autooxidation by scavenging free radicals and by reacting with singlet oxygen. Vitamin E is well accepted as

nature's most effective lipid soluble, chain-breaking antioxidant, which protects cell membranes from peroxidative damage. Vitamin E has a significant role in preventing or minimizing peroxidative damage in biological systems.

It is stored in adipose tissue and is thought to stabilize the lipid portions of cell membranes. Vitamin E protects unsaturated acids from oxidation. Other functions attributed to Vitamin E are enhancement of Vitamin A utilization, inhibition of prostaglandins production and stimulation of an essential factor in steroid metabolism (American Medical Association, 1995).

3) Vitamin A (beta-carotene):

Vitamin A and carotenoids can both accept and donate electrons. Carotenoids can also quench singlet oxygen. Thus both sets of compounds can theoretically participate in biological antioxidant network. Vitamin A, is transported and stored in specific retinoid binding proteins. Vitamin A functions synergistically with tocopherol to prevent lipid peroxidation (Frankel, 1989).

4) Selenium:

Selenium is an essential trace element and is an integral part of the enzyme system glutathione peroxidase; this enzyme protects intracellular structures against oxidative damage.

Glutathione peroxidase enzyme removes hydrogen peroxide and other peroxides with reducing power of glutathione. In conjunction with other enzymes, it prevents the metal ions leading to formation of the highly destructive hydroxyl radical. Activity of glutathione peroxidase may be promoted by selenium supplements.

Selenium is increasingly recognized as a versatile anti-carcinogenic agent. It appears to operate by several mechanisms other than the action of glutathione peroxidase (Schrauzer, 1992).

5) Copper:

Copper is an essential component of a number of proteins (e.g. throcuprien, hepatocuprein) and enzymes (e.g. lysylhydroxylase, dopamine beta-hydroxylase). This mineral is thought to act as a

catalyst in the storage and release of iron to form hemoglobin (American Medical Association, 1995).

Its ability to catalyze the oxidation of ferrous ions to the ferric state (ferroxidase activity) makes an important antioxidant *in-vivo* (Scholes, 1983).

6) Manganese:

Manganese shows a free radical scavenging activity. The chain breaking antioxidant Capacity of manganese seems to be related to the rapid quenching of peroxy radicals (Coassin and Urisini, 1992).

7) Zinc:

Zinc has been shown to have an antioxidant role in defined chemical systems. Two mechanisms of activity, sulfhydroxyl groups against oxidation and inhibition of the production of reactive oxygens by transition of the production of reactive oxygens by transition metals. Administration of pharmacological doses of zinc in-vivo has a protective effect against general and liver specific pro-oxidants. Dietary zinc deficiency causes increased susceptibility to oxidative damage (Nikie, 1991).

Botanical Review:

Fenugreek Family

About family:

Fabaceae or Leguminosae is a large and economically important family of flowering plants, which is commonly known as the legume family, pea family, bean family or pulse family. The name 'Fabaceae' comes from the defunct genus *Faba*, now included into *Vicia*. Leguminosae is an older name still considered valid and refers to the typical fruit of these plants, which are called legumes.

Fabaceae is the third largest family of flowering plants, with 730 genera and over 19,400 species, according to the Royal Botanical Gardens. The largest genera are *Astragalus* with more than

2,000 species, *Acacia* with more than 900 species, and *Indigofera* with around 700 species. Other large genera include *Crotalaria* with 600 species and *Mimosa* with 500 species.

The species of this family are found throughout the world, growing in many different environments and climates. A number are important agricultural plants, including: *Glycine max* (soybean), *Phaseolus* (beans), *Pisumsativum* (pea), *Cicerarietinum* (chickpeas), *Medicago sativa* (alfalfa), *Arachishypogaea* (peanut), *Ceratoniasiliqua* (carob), and *Glycyrrhizaglabra* (licorice), which are among the best known members of Fabaceae. A number of species are also weedy pests in different parts of the world, including: *Cytisusscoparius* (broom) and *Puerarialobata* (kudzu), and a number of *Lupinus* species.

The Fabaceae comprise three subfamilies (with distribution and some representative species):

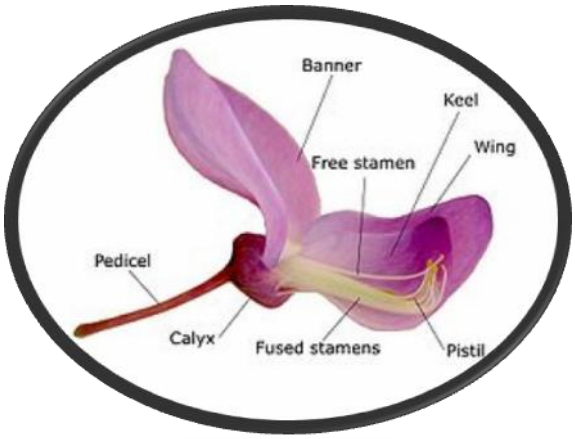
Mimosoideae: 80 genera and 3,200 species. Mostly tropical and warm temperate Asia and America. *Mimosa, Acacia*.

Caesalpinioideae: 170 genera and 2,000 species, cosmopolitan. *Caesalpinia, Senna, Bauhinia, Amherstia*.

Plants have indeterminate inflorescences, which are sometimes reduced to a single flower. The flowers have a short hypanthium and a single carpel with a short gynophore, and after fertilization produce fruits that are legumes.

The leaves are usually alternate and compound. Most often they are even- or odd- (e.g. Caragana and Robinia respectively), often trifoliate (e.g. Trifolium, Medicago) and rarely palmately compound (e.g. Lupinus), commonly bipinnate (e.g. Acacia, Mimosa). They always have stipules, which can be leaf-like (e.g. Pisum), thorn-like (e.g. Robinia) or be rather inconspicuous. Leaf margins are, serrate. Both the leaves and the leaflets often have wrinkled pulvini to permit nastic movements. In some species, leaflets have evolved into tendrils (e.g. Vicia).

Many species have leaves with structures that attract ants that protect the plant from herbivore insects (a form of mutualism). Extrafloral nectaries are common among the Mimosoideae and the Caesalpinioideae and are also found in some Faboideae (e.g. *Vicia sativa*). In some Acacia, the modified hollow stipules are inhabited by ants.



Flowers of methi

The flowers always have five generally fused sepals and five free petals. They are generally hermaphrodite, and have a short hypanthium, usually cup shaped. There are normally ten stamens and one elongated superior ovary, with a curved style. They are usually arranged in indeterminate inflorescences.

Fruits :

The ovary most typically develops into a legume. A legume is a simple dry fruit that usually dehisces (opens along a seam) on two sides. A common name for this type of fruit is a "pod", Roots

Roots:

Root Nodules: These bacteria, known as rhizobia, have the ability to take nitrogen gas (N_2) out of the air and convert it to a form of nitrogen that is usable to the host plant(NO_3^- or NH_3) This process is called nitrogen fixation. The legume, acting as a host, and rhizobia, acting as a provider of usable nitrate, form a symbiotic relationship.

Description of *Trigonella foenum-graecum*:

Fenugreek is grown as a green leafy vegetable and for its seeds in different parts of the country. In India, it is used both as herb as well as a spice. Fenugreek is said to have several medicinal values. This plant is cultivated as a semi-arid crop. In India, fenugreek is more popular as a spice. Fenugreek basically has light green leaves that are 30 to 60 cm tall. This plant also has

slender beaked pods around 10 to 15 cm long. India is considered to be one of the major producers and exporters of Fenugreek seeds. Fenugreek holds a significant position in terms of production among all other spices that are grown in this country. Fenugreek is mainly exported to Saudi Arabia, Japan, Malaysia the USA, the United Kingdom, Singapore and Sri Lanka.

Whole plant of (*Trigonella foenum-graecum*

Taxonomy

kingdom: Plantae

(unranked): Angiosperms

(unranked): Eudicots

(unranked):Rosids

Order: Fabales

Family: Fabaceae

Genus: *Trigonella*

Species: *T. foenum-graecum*

Binomial name*Trigonella foenum-graecum*

USES:

It is useful in the carminative, smallpox, dysentery, cough stomach upset, and treating swelling (inflammation) of the upper air passages or throat, appetite, for lowering blood sugar levels, for softening the stool, for obesity. It also has been used as a gargle to relieve sore throat, and as an external dressing for swelling (local inflammation) and also useful in the making of tonic. Mainly it is used as vegetable in India.

Chemical Review:

By performing all chemical identification tests it is confirmed to contain alkaloids, proteins, steroids, glycosides, flavonoids, tannins, and phenolic compounds.

M. Rehmann and Bhatiya et al.,2005 said that Fenugreek extract modulates the cyclophosphamide induced toxicity and also the extract is used as a dietary and medicinal herb showed protective effect not only on lipid peroxidation but also on the enzymatic antioxidant.

Bin-Hafeez et al.,2003 proved that **T.foenum-graceum**extract produces immunomodulatory effect, anti-inflammatory, antipyretic, hypoglycemic and immunomodulatory activities in mice.

Carlsen et al., 2005 said that Fenugreek seed extract prevented the lipid peroxidation and haemolysis in RBC and also raise the antioxidant levels and to lower the lipid peroxidation in liver of ethanol intoxicated and diabetic rats.

Ozcan et al., 2005 said that Fenugreek seed extract exhibits scavenging of hydroxyl radicals and inhibition of hydrogen peroxide induced lipid peroxidation in rat. The hydroxyl radical scavenging activity of the extract was evaluated by pulse radiolysis and deoxiribose system. The antimutagenic activity of the extract was recorded by following the inhibition of c-radiation induced strand break formation in plasmid PBR322 DNA. The results indicate the extract of fenugreek seeds contains antioxidant and protect cellular structures from oxidative damage.

Amr Amin et al., 2005 It showed potential protective effect of Fenugreek leaves against 7, 12-dimethylbenz (a) anthracene (DMBA)-induced breast cancer in rats at 200 mg/kg b.wt. Fenugreek leaves extract significantly inhibited the DMBA-induced mammary hyperplasia and

decreased its incidence. Epidemiological studies also implicate apoptosis as a mechanism that might mediate the Fenugreek's antibreast cancer protective effects.

Yang,G et al., 1998 said that *T. foenum- graecum* has also been shown to have stimulatory effects on macrophages.

Sameer et al., 2003 said that **Fenugreek** as an antihyperglycemic herb in humans as well as laboratory animals, cholesterol reducing effect and a stimulatory effect on the specific as well as non specific immune function in mice.

The soluble dietary fibre (SDF) fraction of *Trigonella Foenum-Graecum* has shown to reduce post praindian elevation in blood glucose level of type II model diabetic rats by delaying the digestion of sucrose.

Narender et al., 2006 said that the aqueous extract of fenugreek seeds showed significant ulcer protective effects. The fenugreek seeds also prevented the rise in lipid peroxidation induced by ethanol presumably by enhancing antioxidant potential of the gastric mucosa thereby lowering mucosal injury, and also he said that the soluble gel fraction derived from the seeds was more effective than omeprazole in preventing lesion formation. These observations show that fenugreek seeds possess antiulcer potential.

Pandiansuja et al., 2001said that Fenugreek leaves extracts showed significantAnti - inflammatory and Antipyretic effects.

Research envisaged:

The flora of India is both abundant and rich in every kind and description of plants well known. An account of tremendous variability is climate and general features. The country has versatile emporium of medicinal and other plants. This is particularly the case with the great chain of Himalaya Mountains and Western Ghats of Nilgiris. The Western Ghats of Nilgiris and Himalayas are more or less a treasure house of natural wealth; particularly medicinal herbs that take a loins share in the rich Material Medica of Ayurveda. A large number of Indian medical plants have been screen by the scientist of various disciplines viz, Agriculture , Botany, Chemistry, Pharmacology, Toxicology, and Clinical sciences. The present day research on indigenous drug and medicinal plants has made rapid stride depending on vast amount of

literature based on compilation, cataloging of claims from the folklore, Ancient test of Indian system of medicine (Ayurveda), Siddha, Unani, Anthropological, Chemical correlation of various species and field survey.

The good amount of phytochemical work was carried out. The perusal ethnomedical information reveals that a plant *Trigonella foenum-graecum* seeds possess anti-inflammatory (skin), reducing blood sugar levels, etc, Hence it is thought prudent to evaluate the leaves of *Trigonella foenum-graecum* for antioxidant activity.

PLAN OF WORK: Collection and identification of plant and Extraction and phytochemical screening of crude extract Estimation of Antioxidant activity of Methanolic crude extract by following methods:

- Reducing activity
- Iron chelation method
- Total antioxidant activity
- Lipid peroxidation test

Qualitative phytochemical screening of *Trigonella foenum-graceum* aerial parts extract

CONSTITUENT	RESULTS
Alkaloids	
Mayer's test	+++
Dragendorff's test	-
Wagner's test	++
Hager's test	+++
Carbohydrates	
Molisch's test	+++
Fehling's test	-
Benedict's test	++
Barfoed's test	+

Bial'sorcinol test	-
Aniline acetate test	-
Phloroglucinol test	-
Tannic acid test for starch	+++
Glycosides	
Cardiac glycosides	
Legal's test	+++
Test for deoxy sugars (Keller Killian test)	++
Test for anthraquinone glycosides	
Borntrager's test	+++
Modified Borntrager's test	++
Phenolic compounds	
Ferric chloride test	++
Shinoda test	++
Lead acetate test	++
Proteins and amino acids	
Millon's test	+++
Biuret test	-
Ninhydrine test	+
Test for proteins containing sulfur	++
+++Extreme, ++ Moderate, + Mild, - Negative.	

EXPERIMENTAL PROCEDURES AND RESULTS

Collection of Plant material and Authentication:

Whole plant of *Trigonella foenum-graecum* was collected from local market, Kadapa district, Andhra Pradesh, India in the month of January 2011. The plant was authenticated by Dr. A. Madhusudhana Reddy, Taxonomist, Yogi Vemana University, Kadapa, Andhra Pradesh, India.

A voucher specimen of the collected plant was deposited in laboratory of phytochemistry and pharmacognosy, Fathima Institute of Pharmacy, Kadapa, India for future reference. The whole plant was cleaned and shade-dried for a week, powdered mechanically and passed through sieve no 10/44 and stored in air tight containers.

Extraction:

The fresh whole plant of *Trigonella foenum-graecum* was chopped and dried in shade. The dried mass is blended into fine powder by frequent sieving. This powder was extracted by soxhlet process with methanol (100%). After extraction, the content was concentrated at maintained conditions and dried in a desiccator to get corresponding extract.

Pharmacognostical studies:

Microscopical studies:

The microscopic examination of leaf, stem of *Trigonella foenum-graecum* were carried out. The transverse section of *Trigonella foenum-graecum* were taken and treated with chloral hydrate. The cleared sections were mounted in glycerine. The sections were also treated with phloroglucinol and HCl in the ratio of 1:1 to study the lignified tissues.Similarlymicroscopical examination of powder material of *Trigonella foenum-graecum.*

Leaf anatomy:

Trigonella foenum-graecum has long stalked leaves up to 5 cm long stipules triangular, lanceolate, leaflets about 2.5 cms long, obovate to oblanceolate.

Qualitative phytochemical screening of *Trigonella foenum-graceum* aerial parts extract

CONSTITUENT	RESULTS
Alkaloids	
Mayer's test	+++
Dragendorff's test	-
Wagner's test	++
Hager's test	+++
Carbohydrates	
Molisch's test	+++
Fehling's test	-
Benedict's test	++
Barfoed's test	+
Bial'sorcinol test	-
Aniline acetate test	-
Phloroglucinol test	-
Tannic acid test for starch	+++
Glycosides **Cardiac glycosides** Legal's test Test for deoxy sugars (Keller Killian test) **Test for anthraquinone glycosides** Borntrager's test	 +++ ++ +++
Modified Borntrager's test	++
Phenolic compounds Ferric chloride test Shinoda test Lead acetate test	 ++ ++ ++
Proteins and amino acids Millon's test Biuret test Ninhydrine test Test for proteins containing sulfur	 +++ - + ++
+++Extreme, ++ Moderate, + Mild, - Negative.	

Qualitative phytochemical studies:

The methanolic extract was subjected to qualitative chemical screening to detect the presence of various plant constituents.

I. Detection of Alkaloids :

Small portion of the solvent free extract was treated separately with few drops are diluted hydrochloric acid ad filtered. Filtrates are tested with alkaloid reagents.

- **Dragendroff's test (solution of potassium bismuth iodide):**

The filtrate was treated with dragendroff's reagent. The formation of orange brown precipitate indicates the presence of alkaloids.

- **Wagner's test** (solution of iodine in potassium iodide) :

The filtrate was treated with Wagner's reagent. The formation of orange reddish brown precipitate indicates the presence of alkaloids.

- **Hager's test** (saturated picric acid solution) :

The filtrate was treated with Hager's reagent. The formation of yellow colored precipitate indicates the presence of alkaloids.

II. Detection of carbohydrates:

Small quantity of extract was dissolved in 5 ml distilled water and filtered. The filtrate was tested for the presence of carbohydrates.

- **Molisch's test**(solution of alpha-naphthol in alcohol) :

To the filtrate, Molisch reagent and a few drops of concentrated sulphuric acid was added through the sides of the test tube without shaking. The formation of violet ring at the junction indicates the presence of carbohydrates.

- **Fehling's test** (solution of copper sulphate, sodium tartrate and sodium hydroxide) **:**

Small amount of extract is hydrolyzed with dilute hydrochloric acid neutralized with alkali and heated with Fehling's solutions. Formation of red precipitate indicates the presence of reducing sugars.

- **Benedict's test** (solution of copper sulphate, sodium citrate and sodium carbonate) :

To small amount of extract added Benedict's solution and heated on water bath. Formation of orange red precipitate indicates presence of reducing sugars.

III. Detection of Glycosides :

- **Libermann-Burchard test:** The extract in chloroform was treated with few drops of acetic anhydride, few drops of concentrated sulphuric acid was added along the sides of the test tube. Red, pink or violet color at the junction of the liquids indicates the presence of steroids/triterpenoids and their glycosides.
- **Salkowski test**:

The extract in chloroform was treated with a few drops of concentrated sulphuric acid, shaken well and allowed to stand. The formation of yellow colored layer indicates the presence of triterpenes and formation of reddish brown colored layer indicates the presence of steroids.

- **Borntrager's test:**

Small amount of extract was treated sulphuric acid, heated to boiled for about five minutes. The mixture was cooled and then shaken with an equal volume of immiscible organic solvents such as chloroform, then the organic layer is added to about half of its volume of ammonia layer indicates the presence of anthraquinone glycosides.

- **Detection of saponins:** A small quantity of extract was taken in a test tube and few ml of water was added and shaken, saponins will produce froth, which is stable for 15 minutes.

IV. Detection of phenolic compounds:

- **Ferric chloride test :**

1 ml of 5% neutral ferric chloride reagent was added to extracts. Phenolic compounds produce blue, green or violet colors.

- **Shinoda test (**magnesium – hydrochloric acid) :

To the test extract in alcohol, magnesium turnings were added, followed by hydrochloric acid Flavanoids produce magenta, crimson red color.

- **Lead acetate test :**

To the test solution, a few drops of 10% lead acetate solution were added. The formation of white precipitate indicates the presence of phenolic compounds.

- **Alkaline reagent test :**

To the test solution, add a few drops of sodium hydroxide solution. The formation of an intense yellow color, which turns to colorless on addition of a few drops of dilute acid, indicates the presence of flavonoids.

- **Vanillin hydrochloride acid test :**

The test solution was treated with a few drops of vanillin hydrochloric acid reagent. The formation of pinkish red color indicates the presence of phenolic compounds.

- **Gelatin test :**

To the solution add 1% gelatin solution containing sodium chloride and heated to boil. The formation of white precipitate indicates the presence of tannins.

V. Detecting of Proteins and Amino acids:

- **Millon'stest :**

To the test solution, 2 ml of millons's reagent is added and heated to boil. The formation of white precipitate, which turns to red upon heain, indicates the presence of proteins/amino acids.

- **Biuret test :**

Treat the test solution with 1 ml of 10% sodium hydroxide solution and heat. Add a drop of copper sulphate solution. The formation of purple violet color indicates the presence of proteins.

- **NinhydrinTest :**

To the test solution, add a few drops of 0.5% ninhydrin reagent and boil for few minutes. The violet / blue color formation indicates the presence of amino acids.

VI. Detection of Coumarin glycosides:

Small quantity of the extract is dissolved in few ml of methanol. This was made alkaline using aqueous sodium hydroxide. Blue or green fluorescence indicates the presence of coumarin glycosides.

FREE RADICAL SCAVENGING STUDIES

1) Reducing Activity Assay:

The reducing power of the aqueous extract was carried out by adapting the method of Oyaizu (1986).About 2.5ml of different concentrations of the plant extract (100-500µg/ml) were mixed

with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50^0C for 20min, then rapidly cooled, mixed with 2.5ml of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 min. About 2.5ml of the supernatant was taken and 2.5ml distilled water and 0.5ml of 0.1% ferric chloride was added to it, mixed well and allowed to stand for10 min. The absorbance was measured at 700 nm. See Figure 3.

Concentrations	**Absorbance of Methanolic extract of *Trigonella foenum-graecum***	**Absorbance of Ascorbic acid (Positive control)**
100 µgm/ml	0.030433±0.000208	0.013867±0.000306
200 µgm/ml	0.1411±0.001044	0.212367±0.000208
300 µgm/ml	0.194733±0.000208	0.341267±0.00028
400 µgm/ml	0.267167±0.000321	0.397833±0.000252
500 µgm/ml	0.310133±0.000208	0.432167±0.000306

2) Iron Chelation Method:

To 1ml of each extract was treated with an equivalent amount of reaction mixture which contains 1 ml of 0.05% O-Phenanthroline in methanol, 2ml ferric chloride (200mM).The treated compound was incubated at ambient temperature for 10min and the absorbance of same was measured at 510nm . See figure 4

Concentrations	**Absorbance of Methanolic extract of *Trigonella foenum-graecum***	**Absorbance of Ascorbic acid (Positive control)**
100 µgm/ml	0.024533±0.00035	0.030533±0.001002
200 µgm/ml	0.0416±0.000819	0.044267±0.001007
300 µgm/ml	0.045267±0.001007	0.052467±0.00115
400 µgm/ml	0.174267±0.00105	0.2042±0.0011
500 µgm/ml	0.2244±0.001353	0.306533±0.001429

3) TOTAL ANTIOXIDANT ACTIVITY:

The total antioxidant activity was eluted using the method described by Prieto et al (1999). Ascorbic acid was used as the standard antioxidant drug. 3ml of the extract/standard drug (0.1, 0.3,1 and 3 mg/ml) was placed in a test tube 0.3 ml of reagent solution (0.6M sulphuric Acid, 28mM Sodium Phosphate, 4Mm Ammonium molybdate) was then added and the resulting mixture was incubated at 95^0C for 90 min, after the mixture has cooled to room temperature, the absorbance of the each solution was measured using UV-Visible spectrophotometer at 695nm against blank. See figure 5.

The total antioxidant capacity was expressed as ascorbic acid equivalent using the Graph pad for Window version 4.02(Graph pad software, San Diego, CA, USA).

Concentrations	**Absorbance of Methanolic extract of *Trigonella foenum-graecum***	**Absorbance of Ascorbic acid (Positive control)**
100 µgm/ml	0.11915±0.000495	0.258233±0.001305
200 µgm/ml	0.1635±0.0015	0.3042±0.000755
300 µgm/ml	0.2275±0.0025	0.3324±0.001015
400 µgm/ml	0.352295±0.002074	0.433567±0.00095
500 µgm/ml	0.470067±0.063353	0.7322±0.0007552

4) LIPID PEROXIDATION TEST:

Phosphatidylcholine (20mg) in 2ml chloroform was dried under vacuum in a rotary evaporator to give a thin homogenous film and further dispersed in normal saline (5ml) with a vortex mixer. Lipid peroxidation was initiated by adding 0.05mM ascorbic acid into a mixture containing 0.1ml liposome, 150mM Potassium chloride, 0.2mM ferric chloride, extract (2-100µg/ml) .The reaction mixture as incubated at 37^0C for 40 min.After incubation, the reaction was stopped by adding 1ml ice-cold 0.25 M sodium hydroxide containing 20% TCA (w/v), and 0.05%BHT (w/v). After incubating in a boiling water bath for 20 min, the samples were collected to room temperature.

The Pink chromogen was extracted with 1ml of Methanol. The absorbance was read at 532nm. See figure 6

Concentrations	Absorbance of Methanolic extract of *Trigonella foenum-graecum*
100 µgm/ml	0.011933±0.000208
200 µgm/ml	0.034333±0.000252
300 µgm/ml	0.051133±0.000208
400 µgm/ml	0.0725±0.0006
500 µgm/ml	0.099433±0.001332

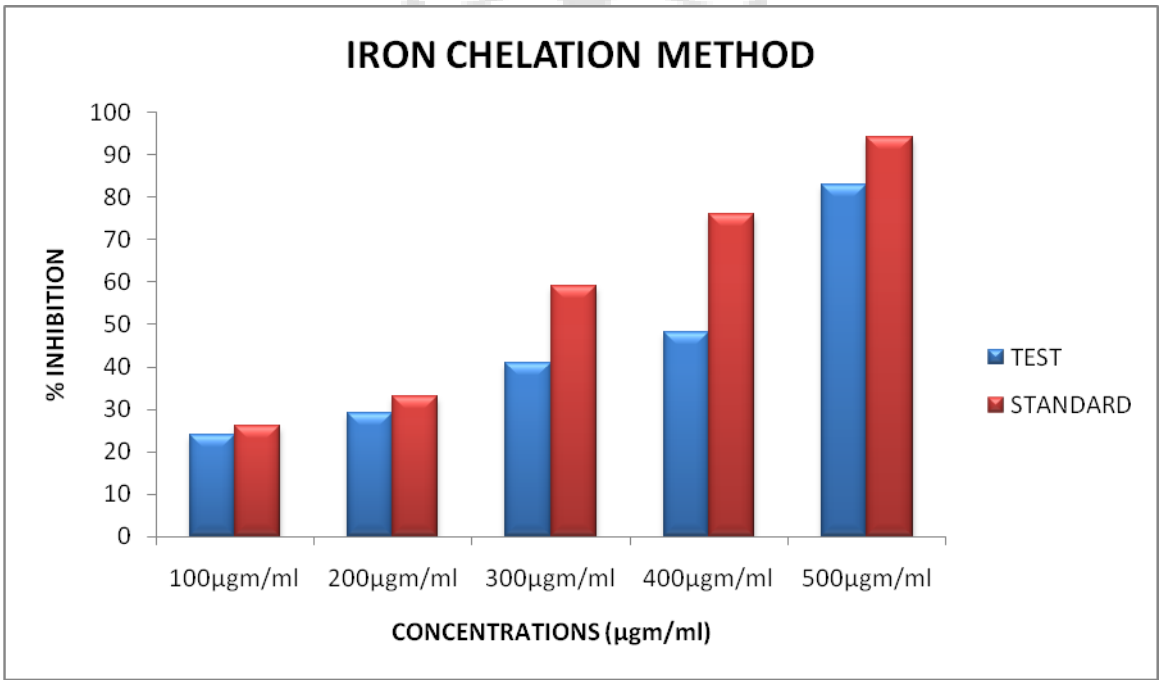


Figure -4

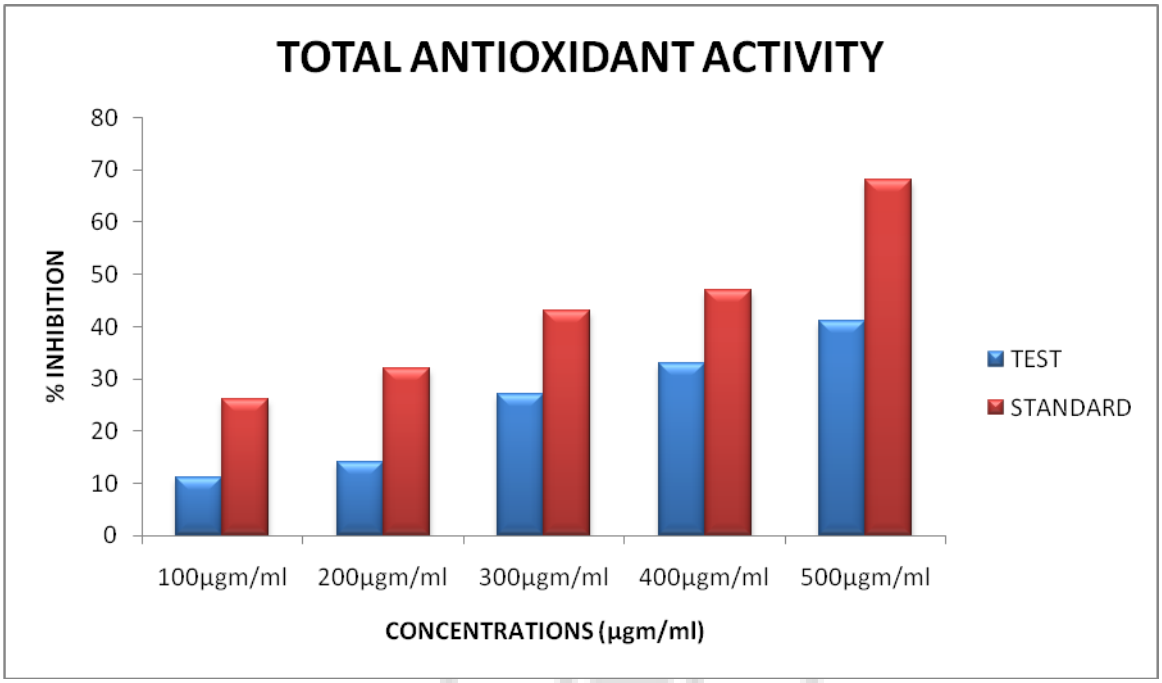


Figure -5

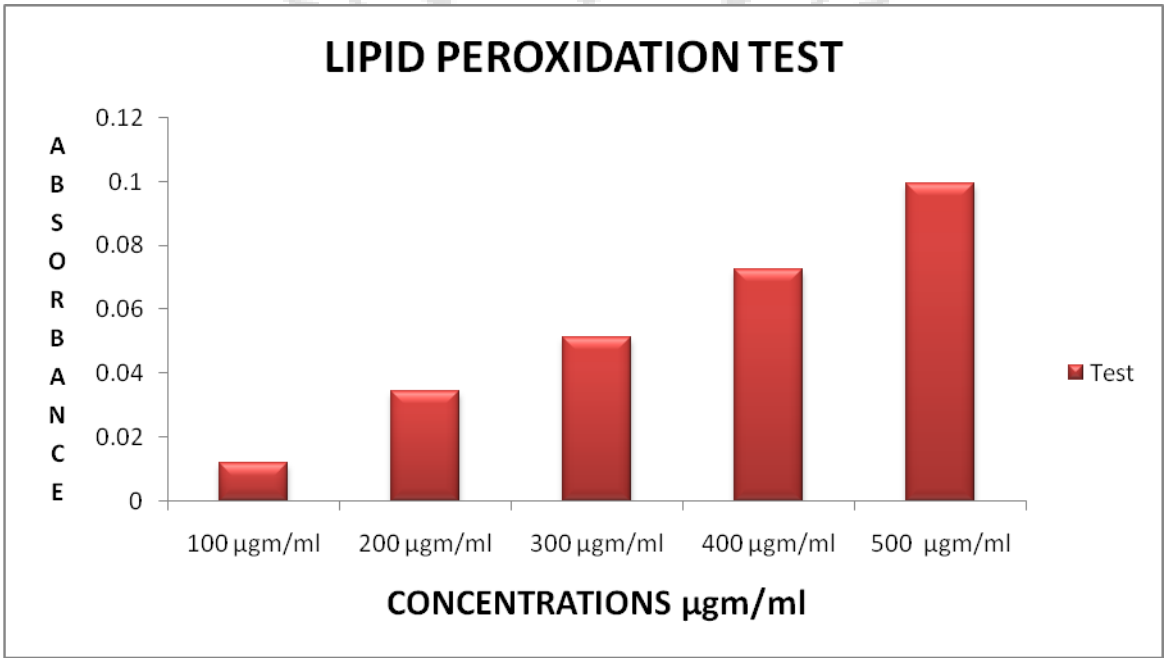


Figure- 6

DISCUSSION AND CONCLUSION

Trigonella foenum-graecum is an annual herb which is used as edible in India. Locally known as Methi. Trigonella has not been investigated for phytochemical investigations to any great extent. Very few reports are available recording these aspects. This little known plant is being investigated in phytochemistry and pharmacognosy laboratories by earlier workers for past few years. The perusal of literature of *Trigonella foenum-graecum* also revealed that, less reports or studies were done on pharmacognostical and antioxidant studies on this plant. Keeping this in view, the present work was undertaken to assess the antioxidant and also to establish the pharmacognostical standards. The fresh whole plant of *Trigonella foenum-graecum* was extracted with methanol by using soxhlet extractor. This extract was subjected to preliminary phytochemical and antioxidant studies. Steroids, tannins, carbohydrates, alkaloids, glycosides, etc., are found to be present in *Trigonella foenum-graecum* extracts by performing various tests. However, the detailed phytochemical analysis and isolation of active constituents may give clear information regarding the antioxidant activity. In this study,

- Figure -3 indicates that, the minimum inhibitory concentration (IC_{50}) of *Trigonella foenum-graecum* for Reducing activity assay is 400µgm/ml.
- Figure -4 indicates that, the minimum IC_{50} of *Trigonella foenum-graecum* for Iron chelation method is 400µgm/ml.
- Figure -5 indicates that, the minimum IC_{50} of *Trigonella foenum-graecum* for Total antioxidant activity is less when compared to the Reducing activity assay and Iron chelation method.
- Figure-6 indicates that, *Trigonella foenum-graecum* has quality of Inhibition of Lipid peroxidation property.

Due to presence of tannins, plant shows good antioxidant property. The experiment results suggest that *Trigonella foenum-graecum* has potential antioxidant properties.

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