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Influence of Gamma Radiation on the Antioxidant Capability of Fenugreek.

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ABSTRACT

In the present investigation the free radical scavenging activity of methanol extract of Fenugreek (*Trigonella foenum graecum*) was assessed in series of assays under the effect of eight different doses of gamma radiation**.** The effect of eight doses of gamma radiation (100, 200, 300, 400, 500, 600, 700, and 800 Gy) on the antioxidant activity of fenugreek extract was examined. Antioxidants are chemical substances that reduce or prevent oxidation. The free radical scavenging activity of fenugreek was determined by its capability to scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, superoxide radical, hydroxyl radical, and ABTS [2,2^{-}-azino-bis (3-ethyl-benzothiazoline-6-sulphonic acid)] radical. Also, hydrogen peroxide scavenging activity, inhibition of lipid peroxidation, chelating power, and ferric reducing antioxidant power (FRAP) of fenugreek extract were studied. The results showed that the methanol extract of fenugreek possessed varied free radical scavenging activities in all adopted assays depending on gamma radiation dose. The free radical scavenging activity increased gradually with increasing irradiation dose up to 800 Gy. The scavenging activity of plant extract may be attributed to its polyphenolic content and other phytochemical constituents. The present results suggest that fenugreek extract could be a potential source of natural antioxidant that could have great importance as therapeutic agents.

Keywords: Gamma radiation, free radicals, antioxidants, DPPH, ABTS, FRAP, fenugreek

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INTRODUCTION

Free radicals are highly reactive unstable molecules that have an unpaired electron in their outer shell (Awah et al., 2010). Hydroxyl and superoxide radicals are formed during a variety of biochemical reactions (Patel et al., 2012).

Free radicals react with the different components of cells including protein, DNA and lipids. These reactions lead to malfunction of mitochondria, damage of cell membrane and finally cause apoptosis. Free radicals have been implicated in the etiology of various diseases including heart disease, neurodegenerative diseases, cancer, and hepatitis (Halliwell, 2001).

Production of antioxidants from natural sources has been considered important way for treatment of human diseases (Mosquera et al., 2007). Modern physicians are increasing their use of pure natural antioxidants extracted from plants to treat many important common diseases due to their proven ability to restrain specific enzymes, to stimulate a number of hormones and neurotransmitters, and to scavenge free radicals (Asif, 2015).

The amount of natural antioxidants in plants can be boosted by elicitation. Elicitors can be grouped into two categories: (i) biotic elicitors e.g. microbes, and (ii) abiotic elicitors e.g. irradiation. It is important to highlight that limited information exists on the potential and extent of enhancing production of secondary metabolites using physical elicitors such as gamma radiation, even though many of the biological effects of gamma radiation have received considerable attention (Kim et al., 2005).

Gamma rays belong to ionizing radiation and are the most energetic form of such electromagnetic radiation, having the energy level from around 10 kilo electron volts (KeV) to several hundred KeV. Thus, they are more penetrating than other types of radiation (Kovacs and Keresztes, 2002). The use of gamma irradiation as elicitor could enable the mass extraction of economically valuable secondary metabolites such as flavonoids which possesses important medicinal properties and are extensively used as a food supplements in many countries. Secondary metabolites and natural antioxidants can scavenge surplus free radicals under irradiation stress. Secondary metabolites improve the ability of plants to self-protection by inhibiting formation of cellular membrane lipid peroxidation product which called malondialdehyde (MDA) and by retaining membrane permeability (Zhou, 2008).

Gamma irradiations are often applied on plants for developing varieties which are agriculturally and economically important and comprise high productivity and efficiency potential. Therefore, the present investigation aimed to study the effect of gamma radiation on free radical scavenging activity of fenugreek as a medicinal plant.

MATERIALS AND METHODS

Plant material:

The experimental plant involved in this investigation was *Trigonella foenum graecum* (Fenugreek, family Fabaceae). Pure seeds were obtained from Egyptian Ministry of Agriculture.

Seeds radiation:

Seeds of *Trigonella foenum graecum* were exposed to eight various doses of gamma radiation (100, 200, 300, 400, 500, 600, 700, and 800 Gy) using cobalt-60 source in Egyptian Atomic Energy Authority (EAEA), Nasr City, Egypt.

Growth of Plant:

Seeds were germinated according to El-Shora (2001). They were cleaned by keeping in 0.5 % sodium hypochlorite for 24 hr. The seeds were then matured between paper towels, moistened with distilled water in sterilized plastic trays and were covered and incubated in dark at 25^{o}C for 7 days.

Preparation of plant extract:

The leaves of fenugreek are collected, air dried at room temperature and reduced in fine powder by milling. The produced powder was subjected to extraction with 80 % methanol. The resulting extract was concentrated with rotary evaporator and stored at 4^{o}C until used.

DPPH radical scavenging activity:

The scavenging activity of DPPH free radical by fenugreek extract was carried out according to Awah et al., (2010) with some modifications. Two ml of plant extract was mixed with one ml of 0.5 mM DPPH in methanol. The mixture was shaken and left to stand in the dark at room temperature for 15 min. A mixture of 2ml plant extract with 1ml methanol was prepared as blank. Also, 1ml of 0.5 mM DPPH was added to 2ml methanol as negative control, whereas ascorbic acid was adopted as positive control. The absorbance of the mixture was measured spectrophotometrically at 518 nm against the blank. The DPPH radical inhibition was calculated from the following equation:

$$\textbf{Inhibition (\%)} = [(A_c - A_s) / A_c] \times 100 \quad \{\text{equation .1.}\}$$

Where $\mathbf{A_c}$ is the absorbance of the control & $\mathbf{A_s}$ is the absorbance of the tested sample.

Superoxide radical ($O_2^{\cdot-}$) scavenging activity:

The method used here is based on the capability of the fenugreek plant to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to Awah et al., (2010) with some modifications. The reaction mixture of 3ml contained 0.1 M phosphate buffer (pH 7.5), 15 mM methionine, 5 µM riboflavin, 75 µM ethylene diamine tetracatate (EDTA), 100 µM nitroblue tetrazolium (NBT), and 1ml of the plant extract. The tubes were left in the front of a fluorescent light, and the absorbance was measured at 560 nm after 15 min.

It should be noted that the whole reaction was enclosed in a box covered with aluminum foil. The inhibition of superoxide anion was calculated according to the equation, 1.

Hydroxyl radical ($OH^{\cdot-}$) scavenging activity:

Hydroxyl radical ($OH^{\cdot-}$) scavenging activity of fenugreek extract was measured according to the method of Halliwell et al., (1987). The reaction solution consisted of 0.5ml of the extract, 1mM $FeCl_3$, 1mM EDTA, 20mM H_2O_2, 1mM L-ascorbic acid and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 hr. at 37^{o}C. Then, add 1ml of 2.8% (w/v) trichloroacetic acid and 1ml of 1% (w/v) 2-thiobarbituric acid, and heated in boiling water bath for 15 min. The color development was measured at 532 nm against blank containing phosphate buffer.

Inhibition of lipid peroxidation:

The assay principle is that a secondary product of lipid peroxidation called malondialdhyde (MDA) reacts with thiobarbituric acid (TBA) in acidic medium to give a pink color pigment at 97^{o}C at pH 2-3 and the absorbance was read at 532 nm (Heath and Packer, 1968).

A fresh sample (0.5 gm.) was homogenized in 10 ml of 0.1% (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged at 5000 rpm for 15 min. After that 1.0 ml aliquot of the supernatant was mixed with 0.4 ml of 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA were added. The mixture was heated at 95^{o}C for 30 min and then cooled in an ice bath. After centrifugation at 5000 rpm for 10 min, the absorbance of the supernatant was recorded at 532 nm. One unit is defined as µmol of MDA formed g^{-1}fresh weight.

ABTS [2,2$^{\cdot}$-azino-bis (3-ethyl-benzothiazoline-6-sulphonic acid)] radical cation scavenging assay:

Free radical scavenging activity of the extract was also determined by ABTS radical cation decolorization assay (Pellegrini et al., 1999). ABTS radical cation was generated by mixing 20mM solution with

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70mM potassium peroxodisulphate and allowing it to stand in dark at room temperature for 24 hr. before use. 0.5ml of plant extract was mixed with 0.5ml of ABTS reagent and record absorbance at 734 nm after 10 min. The inhibition of ABTS radical cation was calculated according to the equation, 1.

Hydrogen peroxide (H_2O_2) scavenging activity:

A solution of hydrogen peroxide (2mM) was prepared in phosphate buffer (pH 7.4), and 0.5ml of plant extract was added according to Guo et al., (2001).. The absorbance was measured spectrophotometrically at 230 nm after 15 min against a blank solution containing phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging activity was calculated using the following formula;

$$H_2O_2 \text{ scavenging activity (\%)} = (1 - A_s/A_c) \times 100 \text{ \{equation .2.\}}$$

Where $\mathbf{A_c}$ is the absorbance of control, and $\mathbf{A_s}$ is the absorbance of sample.

Chelating power:

Chelating power was determined using the method of Guo et al., (2001). One ml of plant extract was added to 0.02ml of 2mM $FeCl_3$ solution and 0.04ml of 5mM ferrozine. The mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance was measured at 562 nm against blank (1ml of methanol, 0.02ml of 2ml $FeCl_3$ solution and 0.04ml of 5mM ferrozine).

The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using equation, 2.

Ferric reducing antioxidant power (FRAP) assay:

FRAP assay was carried out according to (Benzie and Strain, 1996) .The stock solution of 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCL, 20mM $FeCl_3$, and 0.3M acetate buffer (pH 3.6) was prepared. The FRAP reagent contained 2.5ml TPTZ solution, 2.5ml ferric chloride solution, and 2.5ml acetate buffer. It was freshly prepared and warmed to 37^{o}C. FRAP reagent; (900µl) was mixed with 90µl water and 10µl extract. The reaction mixture was then incubated at 37^{o}C for 30 min, followed by measuring absorbance at 595 nm.

An intense blue color complex was formed when ferric-tripyridyl-triazine (Fe^{3+}-TPTZ) complex was reduced to ferrous (Fe^{2+}) form. The absorbance at 540 nm was measured.

RESULTS AND DISCUSSION

All over the world, the medicinal plants have become famous regarding their radical scavenging potential which is important in treating chronic diseases (Tiwari and Tripathi, 2007).

The reactive oxygen species (ROS) are generated in biological systems. These radicals play an important role in the pathogenesis of various diseases through causing serious damage to tissues and biomolecules (Catala, 2009).

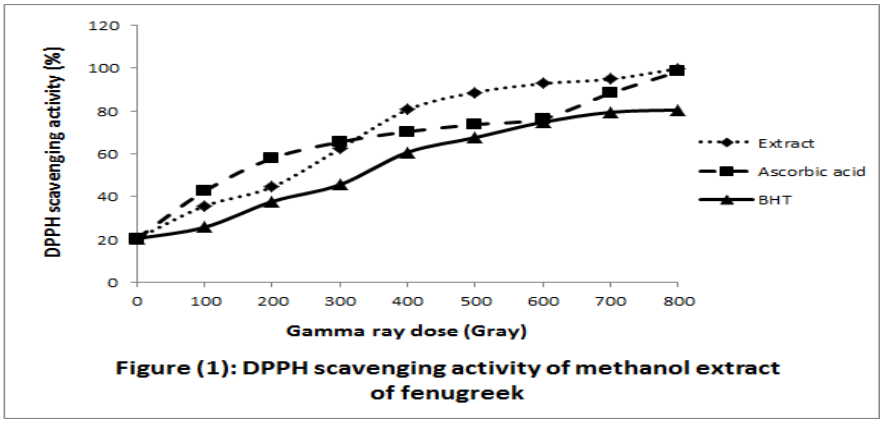

Figure (1): DPPH scavenging activity of methanol extract of fenugreek

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The methanolic extract of fenugreek expressed a potent DPPH radical scavenging potential (Fig. 1). Adding the plant extract to DPPH solution resulted in a rapid decline in the absorbance at 518 nm revealing appreciable scavenging capability.

The antioxidant compounds in methanolic extract of fenugreek neutralized the free radical character of DPPH. This occurs through transferring hydrogen atoms or electrons to the free radical $DPPH^{\cdot}$ (Naik et al., 2003) leading to changing the purple color to yellow color.

$$(DPP^{\cdot}) + (H\text{-}A) \longrightarrow DPPH + (A^{\cdot})$$

Purple antioxidant yellow

The extent of color change of DPPH is dependent on the structure of bioactive compounds in the plant extract. It was reported that the hydroxyl groups of flavonoids are favorable for this reactions (El-Sayed, 2009). The degree of color change is an indicator for the scavenging potential of fenugreek extract in term of donating ability of hydrogen atoms (Mosquera et al., 2007).

Superoxide anion ($O_2^{\cdot-}$) radical is harmful to the components of living cells (Muller et al., 2007). Superoxide has been observed to directly initiate lipid peroxidation (Nabavi et al., 2008)

The present results reveal that fenugreek extract is efficient scavenger of $O_2^{\cdot-}$ radical (Fig. 2). The radical scavenging capability of the fenugreek extract is possibly dependent on the number and the location of hydroxyl groups in the phenolic compounds present in the extract (Khanduja et al., 2006).

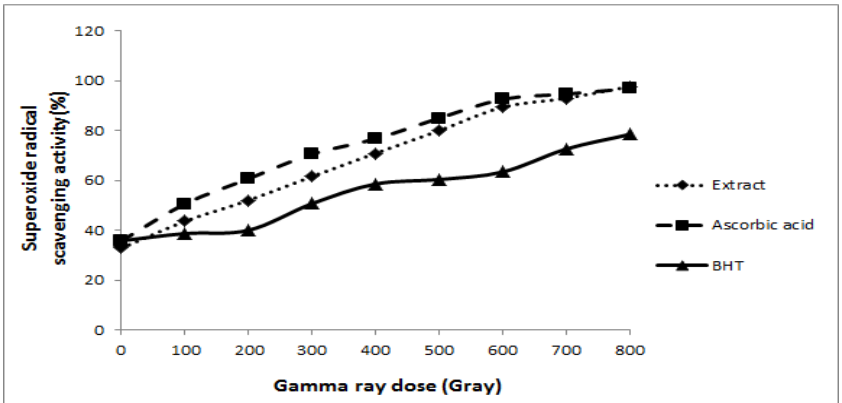

Figure (2): Superoxide radical scavenging activity of methanol extract of fenugreek

The scavenging capability was increased with increasing gamma radiation dose.

The appreciable level of phenolic compounds reported for fenugreek (El-Shora et al., 2015a) is consistent with the determined $O_2^{\cdot-}$ radical scavenging potential because phenolic compounds including flavonoids and possess high $O_2^{\cdot-}$ anion scavenging capability (Rajendran et al., 2004).

The antioxidant activity of phenolic compounds is due to their redox properties because they allow them to act as metal chelator, quencher of singlet oxygen, hydrogen donors and reducing agents (Lotito and Frei, 2006).

Hydroxyl radicals ($OH^{\cdot-}$) are the main active oxygen species causing tissue damage and lipid peroxidation (Catala, 2009). The influence of $OH^{\cdot-}$ radicals produced by Fe^{3+} ions was estimated by the degree of deoxyribose degradation which is an indication of thiobarbituric acid malondialdehyde (TBA–MDA) adduct formation. This complex forms a pink chromogen upon heating with TBA at low pH (Ruberto et al., 2000). Fenugreek extract exhibited potential scavenging activity for hydroxyl radical (Fig. 3). The antioxidant components in the plant extract competed with deoxyribose against the $OH^{\cdot-}$ radicals generated from the Fe^{3+} dependent system and prevented the reaction. The antioxidants in plant extract might act as chelators of Fe^{3+}

ions in system. This will prevent these ions from formation of a complex with deoxyribose or these antioxidants might donate hydrogen atoms and accelerating the conversion of H_2O_2 to H_2O (Wang et al., 2007).

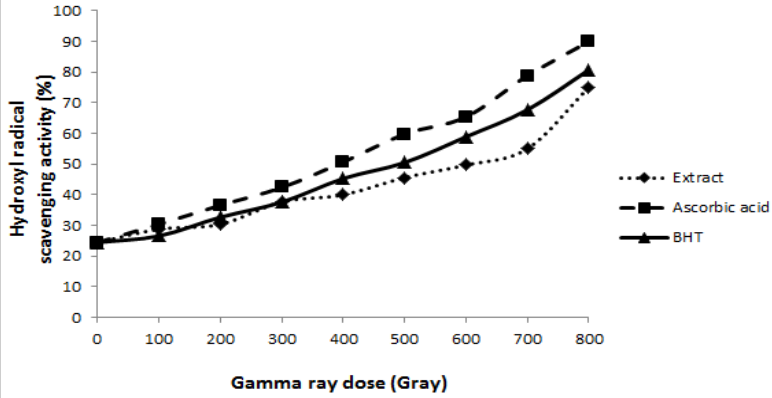

Figure (3): Hydroxyl radical scavenging activity of methanol extract of fenugreek

The capability of the fenugreek extract to inhibit or scavenge $OH^{\cdot}$ radicals reveals that the extract can inhibit remarkably the process of lipid peroxidation because $OH^{\cdot}$ radicals are involved highly in lipid peroxidation.

Lipid peroxidation means formation as well as propagation of lipid radicals which eventually causes destruction of membrane lipids (Catala, 2006). The inhibitory potential of fenugreek extract for lipid peroxidation was assessed by determination of produced MDA.

The inhibition of lipid peroxidation by fenugreek extract is dependent on the dose of gamma radiation (Fig. 4). Considering the damage effect of lipid peroxides in various disease conditions (Muller et al., 2007), the capability of fenugreek extract to inhibit lipid peroxidation might comprise part of the basis for the ethnopharmacolgical claims for its use.

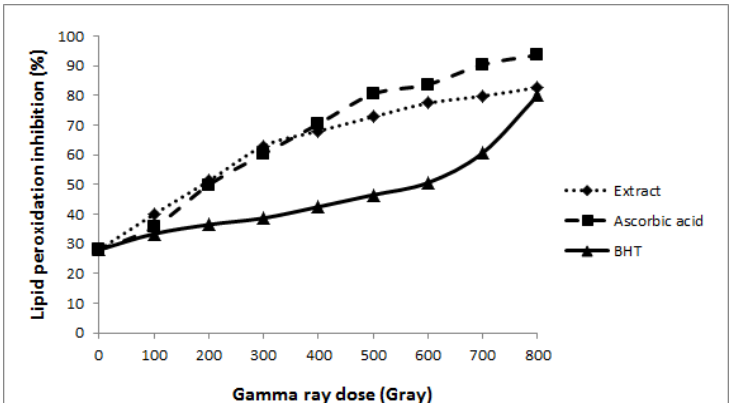

Figure (4): Inhibition of lipid peroxidation by methanol extract of fenugreek

The inhibition of ABTS radical cation by methanol extract of fenugreek expressed comparable scavenging activity with L-ascorbic acid and higher than the scavenging ability of butylated hydroxyl toluene (BHT) (Fig. 5). This indicates that the extract seems to have antioxidant capability due to the presence of polyphenols (El-Shora et al., 2015a) which may act on a similar fraction reduction by donating electrons (El-Shora et al., 2015b). This indicates that some extract components are electron donors which react with free radicals.

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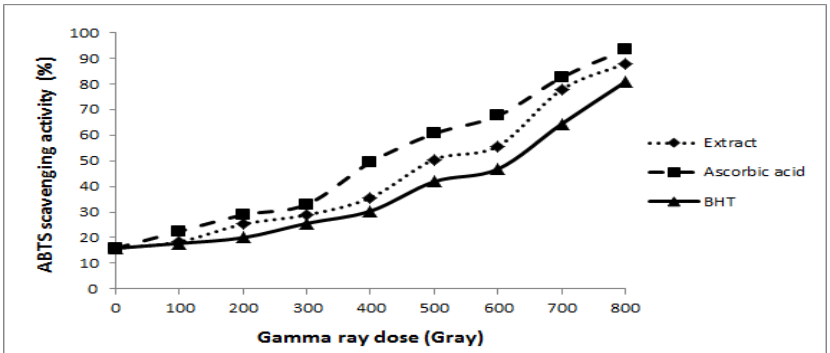

Figure (5): ABTS scavenging activity of methanol extract of fenugreek

Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects (Nabavi et al., 2009). As shown in (Fig. 6), fenugreek extract exhibited better hydrogen peroxide scavenging activity than BHT but lower than L-ascorbic acid. The scavenging activity was dependent on gamma radiation dose.

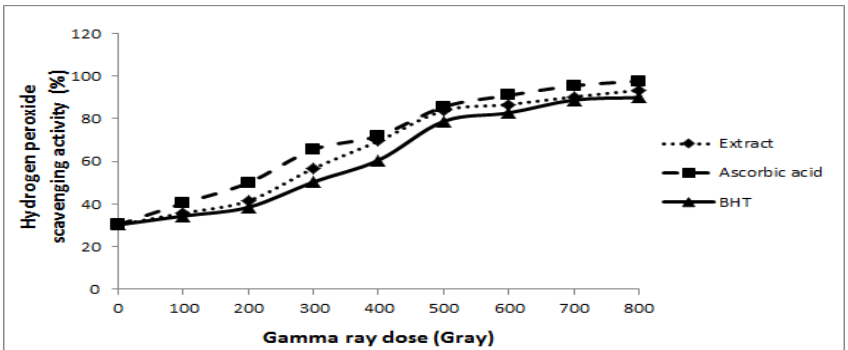

Figure (6): Hydrogen peroxide scavenging activity of methanol extract of fenugreek

Iron is essential for life as it is required for oxygen transport, respiration and for the activity of many enzymes. Chelating agents inhibit lipid peroxidation by stabilizing the transition metals (Angelo, 1992). Decrease in the red color ferrozine-Fe^{2+} complex indicates high scavenging activity of the compound. It was reported earlier that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of metal ions. The metal chelating ability of fenugreek extract is represented in (Fig. 7), and this result indicates that the plant extract exhibited strong ferrous ion chelating ability.

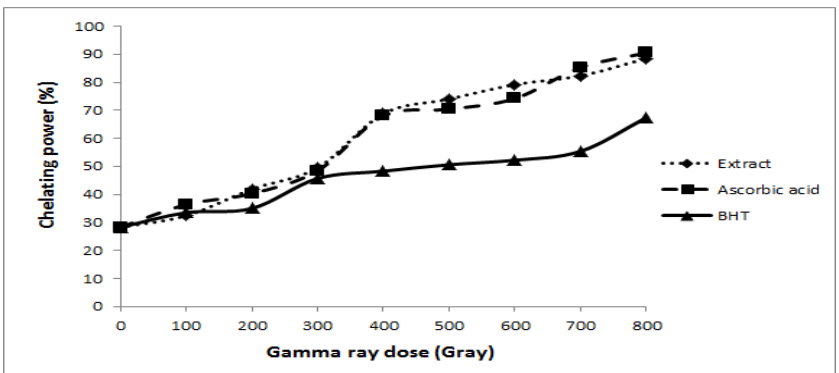

Figure (7): Chelating power assay of methanol extract of fenugreek

The results of FRAP scavenging activity of fenugreek extract (Fig. 8) showed that the values obtained are closed to the standard L-ascorbate. Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain through donating an H-atom.

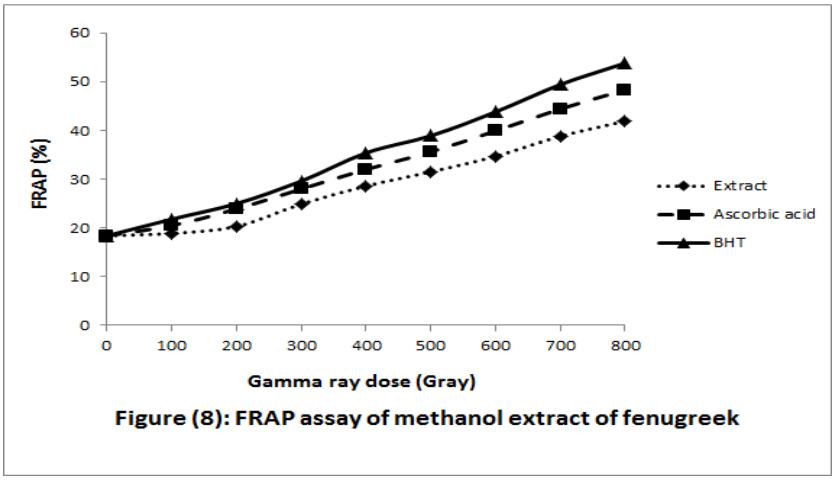

Figure (8): FRAP assay of methanol extract of fenugreek

CONCLUSION

The antioxidant plants have become important due to their possible uses as natural additives to replace synthetic ones. The results of the present work revealed that fenugreek extract exhibited potent antioxidant activity. Therefore, the present data suggest that methanolic extract can be used as a good source of natural antioxidants for health benefits and further isolation of bioactive compounds is required for identifying the unknown compounds to establish their pharmacological properties.

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