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Research Article

FREE RADICAL SCAVENGING ACTIVITY OF LUTEIN – ISOLATED FROM METHI LEAVES (*TRIGONELLA FOENUM GRAECUM*)

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ABSTRACT

Plants are the rich source of antioxidants, which plays a very important role in maintaining human health. Their antioxidant property protects cells of different organs of human beings against free radicals and free radical mediated diseases. Even though, there is lack of knowledge on the antioxidant effect of lutein present in plants. In the present study, lutein was isolated from the methi leaves (*Trigonella foenum graecum*) which is used as a dietary source. The procedure adopted for the isolation and purification of lutein is simple and less time consuming. Free radicals scavenging activity of isolated lutein was assessed by Hydroxyl radical scavenging assay, DPPH radical scavenging assay, Ferric ion reducing assay, Ferrous ion chelating assay and Superoxide scavenging assay. The isolated lutein scavenged 89% of hydroxyl radicals at 20nM/ml and DPPH radical at 3 to 4 fold lower concentration compared to the standard antioxidants. It exhibited Ferrous ion chelation, Ferric ion reducing activity and Superoxide radical scavenging activity very effectively. The results proven that lutein isolated from methi leaves has an efficient antioxidant ability. Hence Methi leaves could be used as the best source of lutein to prevent the free radical mediated diseases.

Key words: Antioxidants, Free radicals, Lutein, Deoxyribose, Methi leaves, dietary source, DPPH.

INTRODUCTION

Cells of all living organisms from plants to human beings are continuously exposed to oxidative stress which leads to the formation of Reactive Oxygen Species (ROS) and other free radicals, which strongly implicated in the pathophysiology of diseases[1] such as cancer, cardiovascular disease, arteriosclerosis, cataract etc. Reactive oxygen species (ROS) and free transition metal ions cause extensive oxidative damage to cellular biomolecules such as DNA, proteins and lipids. Subsequently, oxidative stress contributes to the pathogenesis of oxidative stress-related diseases[2]. Although synthetic antioxidants appear to be promising, their toxicity and surplus side effects rules out their extensive use. Obviously, there is great interest in the use of naturally occurring antioxidants for treatment or prophylaxis of various oxidative stress-related diseases[3]. The administration of an antioxidant source comprising of multi administration of an antioxidant source comprising of multiple constituents could offer protection against cancer[4] and combat oxidative stress induced physiological malfunctions

Many natural antioxidants derived from plants play very important role because of their safe use with no side effects. Lutein is one such multidimensional lipid soluble antioxidant present in plants especially in green vegetables. Even though more than 700 carotenoids have been isolated from natural sources, lutein is an important hydroxyl carotenoid antioxidant which is more efficient free radical quencher [5].

Epidemiological studies shown that, there is inverse relationship between vegetable intake and plasma lutein concentration[6]. Human plasma lutein has been inversely associeated with cytochrome activity and human cancer[7]. In animal models it prevents colon[8, 9] and breast cancer[10,11]. Lutein can also enhanced the recovery of cells oxidative change by stimulating DNA strand break repair[12]. Lutein has been shown to enhance antibody production in response to T-dependent antigens in spleen cells in vitro, as well as in mice in vivo[13]. Hence plants sources such as green vegetables, fruits can be used to derive the maximum health benefits because of their lutein contents. The present study showed that the Methi (*Trigonella foenum graecum*) is one of the best dietary sources of lutein. It also showed that the lutein scavenges the free radicals very effectively than the standard available antioxidants at lower doses.

MATERIALS AND METHODS

Preparation of sample

Methi/Fenugreek (*Trigonella foecum graecum*) leaves were freshly procured from market. Plant material was identified by Prof. Shivamurthy, Taxanomist, Department of Botany, University of Mysore, Karnataka, India. Leafy materials were removed washed with tap water and $KMNO_4$ to make them free from pesticides and insecticides. The extraction and analysis were carried out immediately in the dim light at room temperature.

Chemicals

Standard Lutein, Curcumin, BHA, β-carotene were purchased from the Sigma Aldrich co. USA, Neutral alumina was purchased from Merck, Pvt. Ltd., Germany, Methanol, Dichloromethane & Acetonitrile were of HPLC grade, purchased from Merck, India, mubai. Quercetin, α-tocopherol, Gallic acid, BSA were purchased from the Himedia Co. (Mumbai). All other reagents were of Analytical grade were purchased from the Merck Co., (India); and S.D. fine chem., Mumbai, India.

Extraction of carotenoid

Extraction of carotenoid was carried out by taking 10 g of the each fresh samples with 100 ml of the 5% ethanolic KOH. The mixture ground well for 5 minutes using pestle and mortar at room temperature in dim light. The extraction was repeated till the resultant extract was colourless. Total volume of the ethanol extract was 500ml and which was concentrated to 50ml. Hexane was added to the ethanol extract in the ratio of 1:2 in a separating funnel, shaken well for 5 minutes and kept in the dark for 15 minutes. Two phases were separated and the solvent partitioning was repeated till the hexane extract was colourless. All the hexane phases were pooled and flash evaporated at 30^0C and redissolved in known volume of hexane. It was applied to open column chromatography in order to purify the individual carotenoids. The purity of the eluted lutein was confirmed by HPLC.

Open column chromatography

Carotenoids such as β-carotene and lutein were separated by open column chromatography[14] (25x 2.0 cm). Neutral alumina was used as an adsorbent (70-230 mesh). A known amount of an aliquot of

hexane extract was applied to the column. The β-carotene was eluted with hexane and the lutein was eluted with Methanol: dichloromethane (1:1 v/v), and the violaxanthin and neoxanthin were eluted with ethyl acetate: hexane (5:5 v/v) and ethyl acetate: hexane 1:9 (v/v) respectively. The eluent of the lutein was concentrated by using flash evaporator and liquid nitrogen and redissolved in known volume of Hexane. The purity of the lutein was analyzed by TLC and HPLC and compared with that of reference standard.

Thin layer chromatography (TLC) and HPLC analysis

The purity of the column eluted lutein of methi was analyzed by thin layer chromatography by using the silica gel-G as the adsorbent[15]. Development of the TLC plate was carried out using Hexane and Acetone (70: 30, v/v) as the mobile phase (Results not shown)

For HPLC analysis, an aliquot of hexane extract was redissolved in 1ml of HPLC solvent Methanol: dichloromethane: acetonitrile (67:23:10, v/v)). 20 µl of the sample was loaded to the injector (Rheodyne, Cotati, CA). The ultasphere C18 column (250 x 4.6 mm i.d., 5µm, Beckman, Fullerton, CA) was used and an isocratic condition was maintained at flow rate of 1 ml/min. The absorbance of the eluent was monitored at 450 nm with waters 996 photodiode array detector. The purity of the eluted lutein was compared against reference standard (Fig-1).

Free radical scavenging activity

The purified lutein of methi leaves was used for the assessment of its free radical scavenging activity by Hydroxyl radical, DPPH, Ferric ion reducing, Ferric ion chelating activity and SOD activity.

Hydroxyl radical scavenging activity

Hydroxyl-radical scavenging assay[16] was used to assess the hydroxyl radical scavenging ability of hexane extracts of different plant materials. The reaction mixture consists of $FeCl_3$ (100µM), EDTA (104µM), H_2O_2 (1mM) and 2-deoxy ribose (2.8mM) were mixed with or without hexane extracts at the concentration of 9µM, the volume was made up to 1ml with phosphate buffer (20mM, pH 7.4). The reaction mixture was incubated at 37^{0}C in a water bath for 1hour. Then the reaction mixture was heated at 98^{0}C for 20 minutes after the addition of 1% TBA. The tubes were cooled which was followed by the addition of 1 ml of acetone to stabilize the colour. The absorbance was read at 535 nm. All the readings were corrected by including the appropriate controls. The control without any antioxidant or hexane extract was considered as 100% deoxyribose oxidation. The percentage of hydroxyl radical scavenging activity of the hexane extracts was determined accordingly in comparison with control.

Diphenyl-2-picrylhyrazyl (DPPH) Scavenging assay

DPPH radical scavenging activity was assessed according the method described previously[17]. 10µM concentration of lutein in hexane extract was mixed with 1 ml of freshly prepared 0.5mM DPPH ethanolic solution and 2ml of 0.1M acetate buffer. α-Tocopherol (400µM) was used as a positive control under the same assay conditions. The resulting reaction mixtures were incubated at 37^{0}C for 30 mints, and the absorbance was measured at 517 nm.

Ferric ion reducing power assay

Assay was carried according to the method described previously[18] with minor modifications. In brief, 100µl of potassium ferricyanide solution (4mM) was mixed with 200µl of 0.2 M phosphate buffer, pH 6.5 and 200 µl of 1% potassium ferricyanide then incubated at 50^{0}C for 20 mints. 10% trichloroacetic acid (250 µl) was added to the mixture and centrifuged at 3000 x g for 10 mins at room temperature. The resulting supernatant was taken and mixed with 500 µl of distilled water and 100 µl of 0.1% ferric chloride then incubated at 37^{0}C for 10 mins. The absorbance at 700 nm was measured. This assay was done in triplicate. Increased absorbance indicated increased reducing power.

Superoxide scavenging activity by alkaline DMSO method

Superoxide radical scavenging activity of the lutein isolated from methi leaves was teted[19]. 5-50 µg of lutein isolated from the methi leaves was mixed with the reaction mixture containing 0.1 ml of NBT(1mg/ml in DMSO) and 0.3ml of the extract and standard in DMSO, 1ml of alkaline DMSO (1ml DMSO in 0.1 ml of 5mM NaOH) was added to five a final volume of 1.4 ml and the absorbance was measured at 560nM. Control was without lutein. The percentage of super oxide radical scavenging by the Lutein of methi extracts and standard was calculated.

Ferrous ion Chelating assay

Ferrous ion chelating activity was measured according to the method of Suter and Richter[20] with minor modifications. The reaction solution containing ferrous chloride (200 uM) and potassium ferricyanide (400 µM), with or without Lutein at various concentrations ranging from 20 to 100 µg,, was made to 1 ml with distilled water and mixed thoroughly. The reaction mixture was incubated at 200C for 10 min. Formation of the potassium hexacyanoferrate complex was measured at 700nm using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan). The assay was carried out at 20^{0}C to prevent Fe^{2+} oxidation. Lower absorbance indicated a higher iron chelating capacity. The negative control was without any chelating compound or test sample. The percent ferrous ion chelating activity was calculated accordingly by comparing the absorbance of the test samples with that of the negative control.

RESULTS AND DISCUSSION

The method adopted for the isolation of lutein from methi leaves is simple and less time consuming. The extraction with saponification was performed, which does not allowed the chlorophyll and other water soluble contaminants except lutein with negligible amount of β-carotene. Carotenoids isolated from methi leaves was measured spectrophotometrically, the absorbance spectra of the Methi leaves extract indicated the presence of Lutein and β-carotene. The concentration of lutein was calculated on the basis of the molar absorption of lutein. The column eluted lutein was 98 ± 1 % pure, which is almost similar to the purity obtained by Hua-Bin Li (2002) from microalga. Its purity was tested by Thin layer chromatography, which showed the single band and the Rf value was similar to that of standard lutein.

HPLC analysis of the purified lutein showed the single peak (Fig.1) and its retention time was 4.764 min, it was comparable and exact to that of standard lutein peak. The solvents used for the elution of lutein are more suitable and reproducibility it was indicated by single peak of the isolated lutein except very negligible amount of β-carotene.

Hydroxyl radicals are known to be the most reactive of all the reduce forms of dioxygen and are thought to initiate cell damage in vivo[21]. The lutein of the methi leaves scavenges the hydroxyl radicals up to the 79% at 10µM concentration, whereas standard BHA, α-tocopherol at 400µM scavenged 82-85% (Fig.2). Since, there is an inverse association between the intake of lutein and DNA damage.

Figure.3 shows that, Lutein more effectively scavenges DPPH radicals up to 90% at 20µg concentration compared to the α-tocopherol which achieved the same activity at double the concentration of 40 µg. The radical scavenging activity of lutein is thought to be due to its hydrophobic nature and the presence of alternative conjugated double bonds.

The reducing power of the lutein might be due to its hydrogen donating ability to support this, lutein has shown maximum absorbance 2.5 at 20µg concentration which is five fold less concentration than that of the BHA. BHA showed the maximum absorbance of 2.5 at 100µg concentration. It is shown in fig.4. It implies that, lutein acts as an electron donar and could react with free radicals and convert them into highly stable products and terminates the radicals chain reactions. It has been proved that the

antioxidant efficiency increases with the increasing reducing power[22].

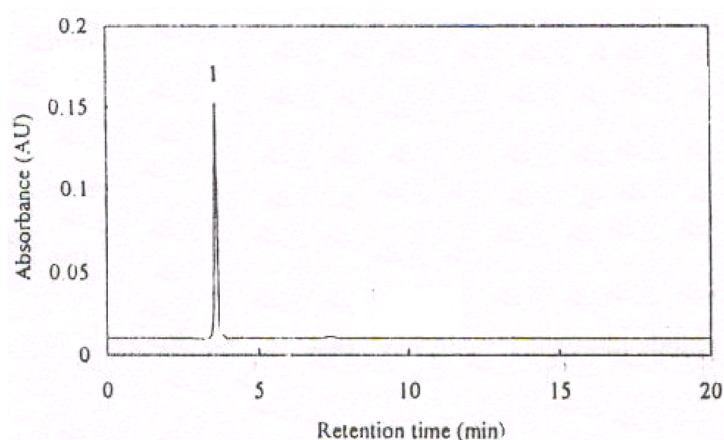
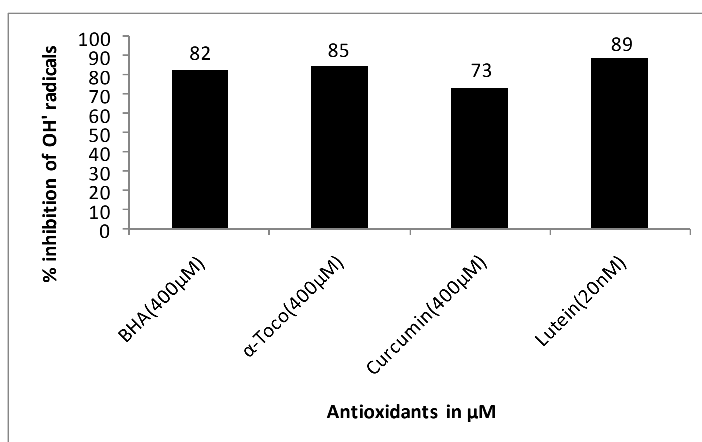
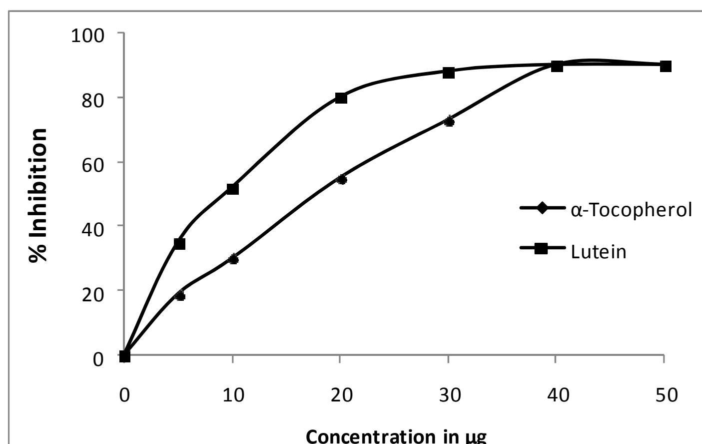


Fig. 1: HPLC profile of isolated lutein



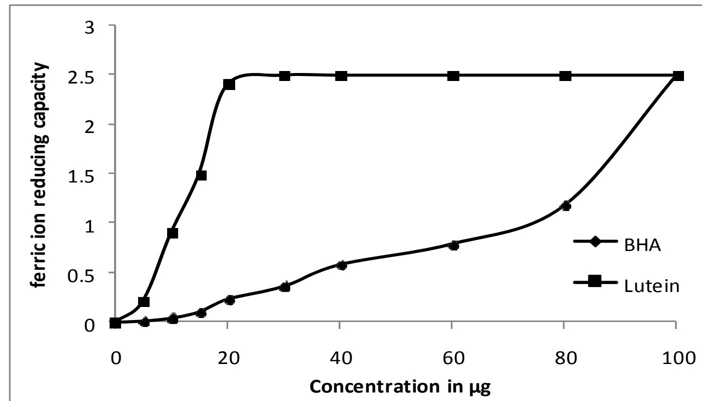
Hydroxyl radical scavenging activity of Lutein isolated from methi leaves(0.02μM) BHA-Butylated hydroxyl anisole (400μM), A-tocopherol (400μM) & Curcumin (400μM) were used as positive controls. The negative control was without any antioxidant or lutein. Results are shown as mean ± SD (n=3).

Fig. 2: Hydroxyl radicals scavenging activity of Lutein from methi leaves



Free radical scavenging activity of Lutein from methi leaves was measured at different concentration ranged from 0-50μg by DPPH assay. The control was without lutein or A-tocopherol. The absorbance was measured at 517nm. The resuts are mean ± SD (n=5)

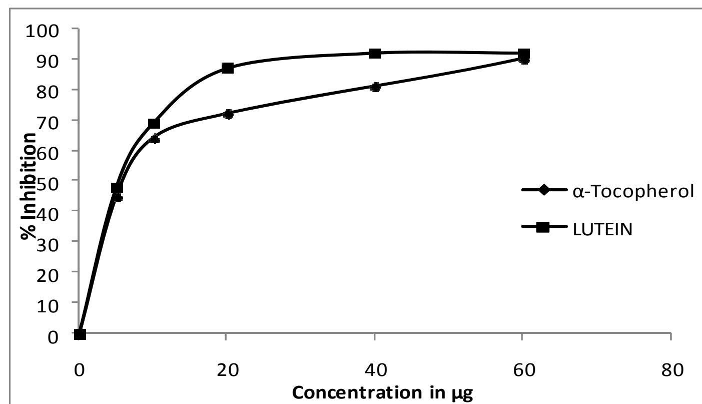
Fig. 3: DPPH Radical Scavenging activity of Lutein from methi leaves



Dose- dependent Ferric ion-reducing capacity of the Lutein isolated from methi leaves. BHA was used as positive control. The control was without any reducing agent or lutein. The absorbance was measured at 700nm. Results are shown are mean of ± SD (n=5).

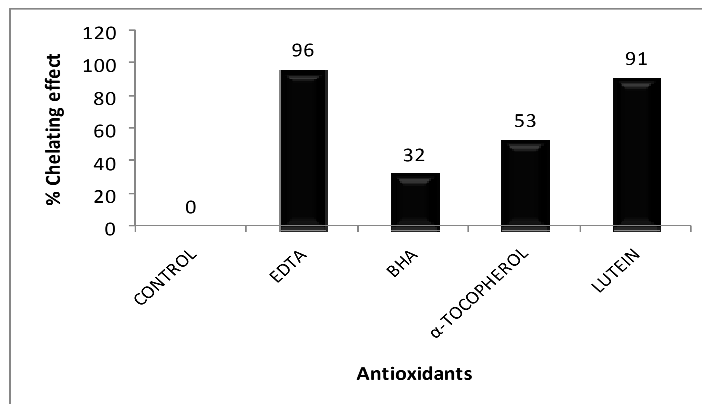
Fig. 4: Ferric ion reducing activity of Lutein from Methi leaves.

Fig. 5 indicated that lutein scavenged the superoxide radicals very effectively up to 85% at 20µg concentration whereas α-tocopherol achieved the 85% at 60 µg concentration, which is 3 fold higher concentration than the lutein. So, lutein could be used to prevent the formation of Superoxide and its adverse effect on cells. The ferrous ion chelating effect of lutein isolated from methi leaves is shown in fig. 6. It is evident that the strong reductive capacity of antioxidants may affect ion, especially Fe^{2+} and Cu^{2+}. Ferrous is a vital substance for normal physiology, but an excess of it may result in cellular damage. If iron undergoes the Fenton reaction, these reduced metals may form highly reactive hydroxyl radicals and thereby contributing to oxidative stress. Lutein isolated from the methi leaves was tested for ferrous ion chelation activity. It exhibited 91% chelation at 20 ug, it was similar to that of standard iron chelator- EDTA.



The inhibitory effect of lutein from methi leaves on Superoxide radical production was estimated by NBT reduction. The concentration of lutein was ranged from 0-80ug. α-Tocopherol was used as the positive control. The control was without any inhibitor. Results are shown are mean ± SD (n=3).

Fig. 5: Superoxide radical scavenging activity of Lutein from Methi leaves.



Ferrous ion chelating activity of lutein from Methi leaves (0.02µM). The positive control was α-tocopherol(400µM). The negative control was without any antioxidant or lutein. Results are shown are mean ± SD (n=5)

Fig. 6: Ferrous ion chelating activity of Lutein from methi leaves

CONCLUSION

In conclusion, Methi leaves showed high content of lutein and prevents free radical formation more effectively than the standard, available synthetic antioxidants. Hence Methi leaves can be used as a good dietary source of the much needed lutein to protect and ameliorate AMD & cataract.

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