

## SCREENING OF LUTEIN IN FEW INDIAN PLANTS AND ITS HYDROXYL RADICAL SCAVENGING ACTIVITY

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### Summary

Plants are the best sources of biologically important lutein which can be used throughout the year. In the present study, Yellow flowers such as Sunflower (*Helianthus annuus*), Marigold (*Tageta erecta*), Shevanthi/Aster daisy (*Chrysanthemum*), Medicinal plants - Turmeric (*Curcuma longa*), Pome (*Punica grantum*), and Green leafy vegetables like Indian spinach (*Basella alba*), Agathi alba (*Sesbina grandiflora*), Pudina/Field mint (*Mentha arvensis*), Methi/Fenugreek (*Trigonella foecum graecum*) leaves were examined for lutein content and antioxidant activity. Biochemical characterization of these extracts indicates the presence of different phytochemicals such as  $\alpha$ -tocopherol, phenolics, ascorbic acid and flavonoids in minimum concentration compared to the lutein. The concentration of the lutein is higher in all the plant sources and its concentration varies from species to species. The hexane extracts of all the five plants were evaluated for their antioxidant activity by hydroxyl radical scavenging assay. The percentage of inhibition offered by all the plants extracts was more than 70% which is comparable to the standard antioxidants like BHA, Curcumin and  $\alpha$ -tocopherol.

**Key words:** Lutein, Green leafy vegetables, antioxidants, Phenolics, Curcumin.

**Abbreviations:** Age related macular degeneration (AMD), Cardiovascular diseases (CVD), Butylated hydroxy anisol (BHA), Thin layer chromatography (TLC), Bovine serum albumin (BSA), milliliter (mL), mg (milligram), Microgram ( $\mu$ g), Millimolar (mM).

### Introduction

Lutein is one of the important yellow carotenoid present in green leafy vegetables, yellow fruits, flowers, egg yolk etc. Since human beings are incapable of synthesizing lutein, has to depend on dietary sources for their requirements. Nearly 40-50 carotenoids are typically consumed in the human diet<sup>1</sup>.  $\beta$ -carotene, lycopene, lutein, violoxanthine, neoxanthine, cryptoxanthine and zeaxanthine are the important carotenoids available in green leafy vegetables<sup>2</sup>. Of all the carotenoids, lutein is an important hydroxy carotenoid required for the fine vision of the eyes, though it has no pro-vitamin A activity. Lutein is mainly accumulated in the macula of human eye<sup>3,4</sup> and hence it is called macular pigment. The deficiency diseases of the lutein and zeaxanthin are, Age related macular degeneration (AMD), cardiovascular diseases (CVD), various types of cancers such as prostate, colon, hepatic cancer, arthritis, alzheimer disease and cataract.

Among all the lutein deficiency diseases, Age related macular degeneration is one of the main leading cause of vision loss in the elderly population of western countries as well as in India for which effective treatment is lacking<sup>5</sup>. By consuming lutein rich diet, there is a chance of delay of the progression of AMD<sup>6</sup>. Green leafy vegetables and different types of fruits are rich in carotenoids<sup>7</sup>. Dietary carotenoids such as  $\beta$ -carotene,  $\alpha$ -carotene, lutein zeaxanthin, cryptoxanthin and lycopene are predominated in the blood<sup>8</sup> compared to the other carotenoids due to the consumption of carotenoid rich diets.

Consumption of different varieties of foods can overcome the nutritional deficiency diseases<sup>9,10</sup>. In this regard, plants are an abundant source of lutein and zeaxanthin, which have varieties of nutrients and non-nutrients<sup>11</sup>. Increased vegetable intake will lead to the increased concentration of carotenoids in the blood<sup>12,13,14</sup>. Earlier studies revealed that a significant correlation between habitual food intake and plasma lutein concentrations<sup>15,16</sup>. Some epidemiological studies suggested that high intake of green vegetables is associated with the reduced risk of free radical mediated degenerative diseases such as cancer, cardiovascular diseases<sup>17</sup> and age related eye diseases<sup>6</sup>.

Lutein and zeaxanthin act as efficient antioxidants in the macular region, give the protection to the eye from phototoxic damage by filtering high intensity blue light<sup>18,19</sup>. Number of publications have been reported the quantitative and qualitative ratio of carotenoids in different fruits and vegetables. So for marigold is considered as the richest source of lutein (0.57g/100g)<sup>20</sup>. Hence it has been used for the extraction of lutein commercially.

Scanty information is available on the screening of flowers and the green leafy vegetables as well as medicinal plants for their lutein content. For the first time attempt is made to isolate the lutein from all these plants by using simple but efficient extraction method. The antioxidant activity was also checked for these plants extracts which are rich in lutein. Apart from this, study also explores the presence of different phytochemicals along with the lutein.

### **Materials and methods**

#### **Sample preparation:**

Samples of Sunflower (*Helianthus annuus*), Marigold (*Tageta erecta*), Shevanthi/Aster daisy (*Chrysanthemum*), Turmeric (*Curcuma longa L*) leaves, Pome (*Punica grantum*) leaves, Indian spinach (*Basella alba*) leaves, Agathi alba (*Sesbina grandiflora*) leaves, Pudina/Field mint (*Mentha arvensis*) leaves, Methi/Fenugreek (*Trigonella foecum graecum*) leaves were freshly procured from reliable source. All the plant parts except leafy materials were removed washed with tap water and KMNO<sub>4</sub> 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,  $\beta$ -carotene were purchased from the Sigma Aldrich co. USA, Quercetin, A-tocopherol, Gallic acid, BSA were purchased from the Himedia Co. (Mumbai). All the reagents were of Analar 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 glass 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<sup>0</sup>C. The concentrated hexane extract of carotenoid was stored in dark and cold condition for further analysis. The same procedure was followed for the extraction of lutein from all the plant sources.

#### **Spectrophotometric analysis:**

Lutein was identified in the hexane extracts of all the samples by spectrophotometrically by using UV-Vis Spectrophotometer with PDA detector (Shimadzu UV-Vis, 601, Tokyo, Japan) using standard lutein for comparison and calculation. Purity of isolated lutein was checked by TLC method. In brief, the concentrated carotenoid sample was loaded to the silica plate of 0.5mm and developed in the solvent chamber containing 3:7 ratio of acetone and hexane (v/v). The separated bands were compared with the standard lutein. The band coinciding to standard lutein was eluted, dissolved in ethanol and spectrum was read between 200-600nm. Concentration was calculated on the basis of the molar absorption of standard lutein at 445nm. Further analysis was carried out for the partially purified lutein present in the hexane extract at room temperature in dim light.

**Proximate Analysis:**

Proximate analysis was carried out to identify the different plant pigments present in the partially purified hexane extract. The analysis involves the estimation of Protein, B-carotene, Total phenolics, A-tocopherol, Ascorbic acid and total sugars (reducing) etc.

***Protein estimation:***

The protein estimation was carried according to Bradford's method<sup>21</sup> using BSA as standard and hexane extracts of different plant materials into a series of test tubes. Volume was made up to 100 $\mu$ l with distilled water and 900 $\mu$ l of Bradford's reagent was added to each tube. Absorbance was read at 535nm. Concentration of protein was calculated accordingly using standard graph.

 ***$\alpha$ -tocopherol estimation***

$\alpha$ -tocopherol estimation was carried out according to Kivcak and merit et al.<sup>22</sup> 20-100 $\mu$ l of standard  $\alpha$ -tocopherol solution and 20 and 40  $\mu$ l of the hexane extracts was used for the estimation. Volume was made up to 3ml using chloroform, 1 ml of 2, 2-dipyridyl, and 1 ml of FeCl<sub>3</sub> solution, Incubated at 37<sup>0</sup>C for 15 minutes, and the absorbance of the reaction mixture was read at 520nm, concentration was calculated accordingly by using the standard graph.

***Total phenolics***

Total phenolics was determined according to the method of Folin Ciocalteu reaction<sup>23</sup> with minor modifications using gallic acid as a standard (0-100 $\mu$ g). Various concentrations of hexane extracts ranging from 0-100 $\mu$ g were taken in series of test tubes & the volume was made up to 500 $\mu$ l with distilled water. 500 $\mu$ l of the Folin-ciocalteu reagent was added to each tubes, the mixture was allowed to stand for 10 minutes followed by addition of 1.0ml of 20% Sodium carbonate, incubated at 10 minutes at 37<sup>0</sup>C. Absorbance was read at 750nm and the concentration was calculated using the standard graph accordingly.

***Ascorbic estimation***

Ascorbic estimation was carried out according to Sadasivam S., Manickam, A<sup>24</sup> Different concentrations (0-100 $\mu$ g) of hexane extracts were taken along with standard ascorbic acid. A drop of thiourea solution and 1ml of 2,4dinitrophenyl hydrazine reagent was added to each tube and the volume as made up to 100 $\mu$ l with 4% oxalic acid and incubated at 37<sup>0</sup>C for 3 hours. Then tubes cooled on ice water and 5ml of 85% sulphuric acid was added to each tube. Mix the reaction mixtures thoroughly. The orange color developed was read against a reagent blank at 540nm. The concentration was calculated on the basis of the standard curve.

***Total sugar estimation***

Sugar estimation was done according to Dubois method<sup>25</sup>. 10- 100  $\mu$ g of the working standard solution was pipetted into a series of test tubes 200 $\mu$ l of the extracted sample was pipetted into two separate test tubes. The volume in each tube was made up to 1000 $\mu$ l with double distilled water. 1ml of 5% phenol was added to each tube followed by 5ml of 96%

sulphuric acid, intensity of the colour was read at 520 nm. The amount of total sugar present in the given unknown sample solution was calculated using the standard calibration curve.

#### **Flavonoid estimation**

Flavonoid estimation was done according to Cheon et al<sup>26</sup> by using Quercetin as a standard. Various concentrations (0-100µg) of hexane extracts were taken in test tubes. Made up the volume to 1.5 ml with 95% ethanol. Then 100 µl of 10% of aluminium chloride, 100µl of 0.1M of potassium acetate was added to each tube. The the total volume was made up to 2.8ml of by using distilled water. O.D was measured at 415 nm and the concentration was calculated accordingly.

#### **Estimation of Total chlorophyll:**

Chlorophyll estimation was determined according to the method of Sadasivam and Manickam<sup>24</sup> with minor modifications. In brief, 0.5ml of the hexane extract was mixed with the 20ml of 80% acetone. Centrifuged at 5000 rpm for 5 minutes and supernatant was collected. The process was repeated for several times till the clear supernatant was obtained. All the supernatants were combined and volume was made up to 1 mL with 80% acetone. The absorbance of the solution was read at 645 and 663 nm. The amount of the total chlorophyll present in the extract, mg chlorophyll/gram extract was calculated accordingly using the calibration curve.

#### **Hydroxyl radical scavenging activity:**

Hydroxy-radical scavenging assay was used to assess the hydroxyl radical scavenging ability of hexane extracts of different plant materials<sup>27</sup>. The reaction mixture consists of FeCl<sub>3</sub> (100µM), EDTA (104µM), H<sub>2</sub>O<sub>2</sub> (1mM) and 2-deoxy ribose (2.8 mM) were mixed with or without hexane extracts at the concentration of 9uM, the volume was made up to 1ml with phosphate buffer (20 mM, pH 7.4). The reaction mixture was incubated at 37<sup>0</sup>C in a water bath for 1hour. Then the reaction mixture was heated at 98<sup>0</sup>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.

### **Results and Discussion:**

Ten different plants Sunflower (*Helianthus annus*) petals, Marigold (*Tageta erecta*) petals, Shevanthi/Aster daisy (*Chrysanthemum*) petals, Turmeric (*Curcuma longa*) leaves, Pome (*Punica grantum*) leaves, Indian spinach (*Basella alba*) leaves, Agathi alba (*Sesbina grandiflora*) leaves, Pudina/Field mint (*Mentha arvensis*) leaves, Methi/Fenugreek (*Trigonella foecum graecum*) leaves were used for the screening the lutein content. Among all, Marigold orange (*Tagetes Erecta*) petals, Indian Spinach (*Basella alba*) leaves, methy (*Trigonella foenum graecum*) leaves, sunflower (*Helianthus Annus*) petals, Shevanthi (*Chrysanthemum leucanthemum*) shows the high concentration of lutein (Table-1). The variation in the lutein

concentration may be due to the variations in the species. Apart from the species, there are other factors, which decide the lutein concentration, in each plant such as part of the plant, degree of maturity at the time of harvest, soil condition cultivation practices and post harvest handling practices.

**Table-1: Lutein composition in green leafy vegetables and yellow flowers**

Sl No.	Plant source	Concentration (mg/100g) dry weight
01	Marigold orange ( <i>Tagetes erecta</i> )	65.0±1.113
02	Pomegranate ( <i>Punica grantum</i> ) leaves	65.0±1.210
03	Indian Spinach ( <i>Basella alba</i> ) leaves	60.0±1.082
04	Methy ( <i>Trigonella foenum graecum</i> ) leaves	71.3±1.251
05	Pudina ( <i>Mentha arvensis</i> )	25.0±0.452
06	Agathi leaves ( <i>Sesbania grandiflora</i> )	20.0±1.762
07	Turmeric ( <i>Curcuma longa</i> ) leaves	17.2±0.359
08	Hibiscus ( <i>Hibiscus esculentus</i> ) leaves	21.0±0.382
09	Shevanthi ( <i>Crysanthemum</i> ) flower petals	61.0±1.022
10	Sunflower ( <i>Heliantus annus</i> ) petals	55.5±1.141

Lutein was estimated according to the described method by using different solvents and the concentration was calculated on the basis of the molar absorption of lutein as described in methods and materials.

Earlier, most of the carotenoids were mainly concerned on the isolation and purification of lutein from the marigold and green leafy vegetables. Marigold was the only source of lutein, the main draw-back of using marigold is that, contains lot of pesticides and insecticides which are very much harmful to the human health. Rajyalakshmi et al<sup>28</sup> and R. Lakshminarayana et al<sup>29</sup> have reported on the total carotenoids in different green leafy vegetables. In the present study, the lutein as well as other plant pigments such as a-tocopherol, flavonoids, chlorophyll, ascorbic acid etc by using green leafy vegetables, medicinal plants as well the yellow flowers are reported for the first time. More importantly, the concentration of lutein here in reported are different and higher compared to the previous reports. R. Lakshminarayana et al<sup>29</sup> reported that the concentration of lutein in spinacia oleracea, Sesbena grandiflora & Trigonell foecum graecum leaves was 77.58µg, 16.90µg and 59.60µg/100g. In the present study we report that the lutein concentration in these plants are 63.0µg, 20.0µg, 71.3µg/100g. The difference in the values of the lutein concentration may be due to the difference in the extraction procedure along with the species seasonal and geographical variations.

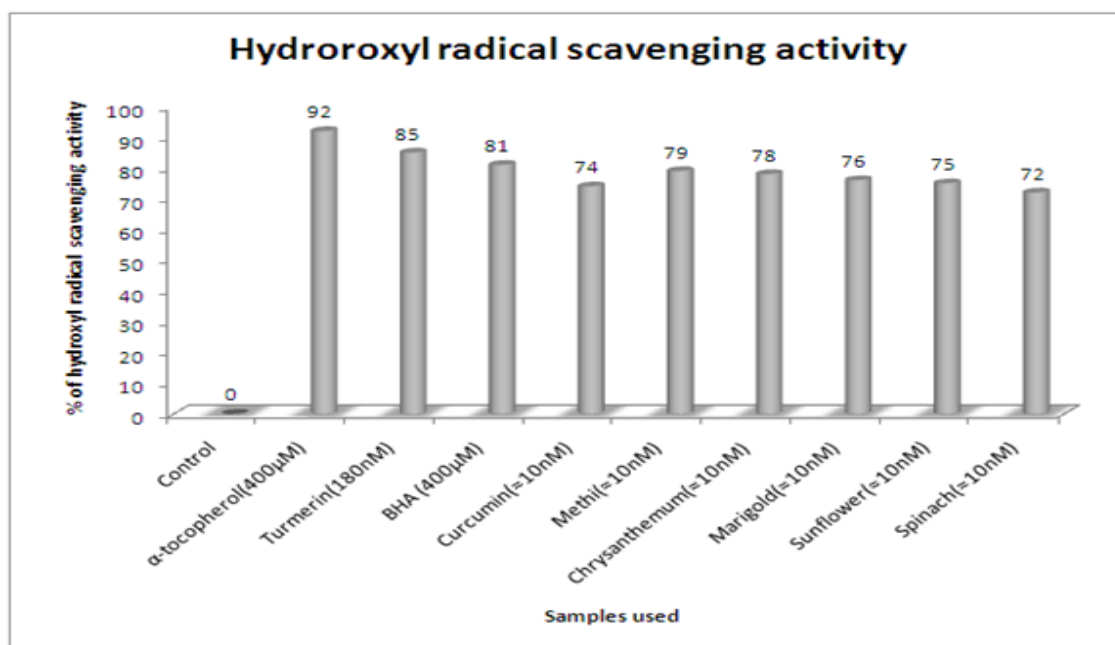
**Table-2: Proximate analysis of hexane extracts of plants of high lutein content**

Plant source	Total protein (mg/g)	Total phenols (mg/g)	Total flavonoids (mg/g)	Ascorbic acid (mg/g)	$\alpha$ -tocopherol (mg/g)	Total chlorophyll (mg/g)
Marigold orange ( <i>Tagetes erecta</i> )	N.D	N.D	0.531±0.003	N.D	1.06±0.004	0.003±0.001
Indian Spinach ( <i>Basella alba</i> ) leaves	N.D	0.45±0.004	0.73±0.002	N.D	1.08±0.003	1.005±0.002
Sunflower ( <i>Heliantus annus</i> ) petals	N.D	0.43±0.002	1.3±0.005	3.66±0.021	N.D	0.002±0.001
Shevanthi ( <i>Crysanthemum</i> ) petals	N.D	0.022±0.001	0.5±0.001	0.084±0.002	0.67±0.003	0.004±0.002
Methi( <i>Trigonella foenum graecum</i> ) leaves	N.D	0.067±0.002	0.36±0.002	N.D	1.2±0.005	0.013±0.002

Results are men of triplicates and methods used as in methods and materials. ND- Not detected

Biochemical characterization was carried out for five plant extracts; they have shown high concentration of lutein compared to the other plant pigments such as , flavonoids, total phenolics,  $\alpha$  -tocopherol and ascorbic acid. The results show that, Marigold orange contain 1.06g of  $\alpha$ -tocopherol, other pigments, such as phenolics, flavonoids, total sugars were not detected. Indian spinach has total phenolic (0.45mg/g) and flavinoids (0.73mg/g) in very low concentration compared to the  $\alpha$  -tocopherol(1.06mg/g). Whereas chrysanthemum has very low concentration of total phenolics (0.022mg/g) and ascorbic acid (0.084mg/g) when compared to the total flavinoids (0.5mg/g) and  $\alpha$ -Tocopherol (0.67mg/g). In case of Sunflower the concentration of ascorbic acid (3.66mg/g) is very high than that of flavinoids (1.3mg/g) and phenolics (0.43/mg/g). In methi leaves extract, very less concentration of phenolics (0.063mg/g) & average concentration of flavonoids(0.36mg/g), but the concentration of  $\alpha$  -tocopherol is more(1.2) compared to all the others. In all the sources, the total chlorophyll concentration is very negligible except in Indian spinach, which has 1.005mg100g (Table-2).

The hydroxyl radicals scavenging activity was checked for all the hexane extracts of all the five plants. The lutein present in the hexane extracts (6 $\mu$ g) scavenges the hydroxyl radicals up to the 79% which is approximately equal to the standard BHA (81%), Curcumin (74%) and less than that of standard  $\alpha$ -tocopherol (92%) (Fig-1). Hydroxyl radicals are the primary ROS capable of causing damage to the DNA. Lutein being a very good antioxidant, present in the hexane extracts of all the five plants, can prevent the DNA damage as effective as the standard antioxidants such as BHA, Curcumin and  $\alpha$ -tocopherol etc. Apart from this, our preliminary data shown that, the lutein isolated from the methi leaves can prevent the smoke induced cataract invitro (data not shown).



**Figure:-1 Hydroxyl radical scavenging activity of plant extracts** Hydroxyl radical scavenging activity of lutein in different plant extracts(10nM) was estimated by deoxyribose assay and control was without lutein or any scavenger and % hydroxyl radical scavenging activity of lutein in different plant extract was calculated as described in methods. The results are mean of five experiments.

The present study shows that dietary sources such as green leafy vegetables as well as yellow flowers are the good sources of the lutein with high concentration of Lutein compared to the other plant pigments such as a-tocopherol, flavonoids etc. By using these plants in diet as the source of lutein, It is possible to ameliorate the lutein deficiency and consequents it can prevented the free radical mediated diseases such as different varieties of cancer, AMD, cataract, CVD etc may be prevented.

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