ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Sunflower *(Helianthus annuus* L.) petals: A new biological source of Lutein

Chethan Kumar M* **and T Veerabasappa Gowda**

Post Graduate Department of Bio-technology, JSS College of Arts, Commerce and Science (Autonomous under University of Mysore; Re-Accredited by NAAC with **A** grade), Identified by UGC as College with Potential for Excellence, Ooty Road, Mysore – 570 025, Karnataka, India.

ABSTRACT

Lutein (L) is a carotenoid, which, along with its stereo isomer Zeaxanthin (Z), are but two of the more than 600 plant pigments in the carotenoid class which are potential as preventive medicine against diuresis, diarrhea, and several inflammatory diseases. The present investigation is aimed at isolation of Lutein from sunflower *(Helianthus annuus* L.*)* petals and its ability to quench reactive oxygen species. The hexane extract of *Helianthus annuus* L. had negligible amounts of polyphenols, flavonoids and microgram quantities of α–tocopherol, proteins, sugars and ascorbic acid. The spectral analysis showed the presence of Lutein rich fraction when compared with signature wavelength at 421nm, 446nm and 472nm of standard Lutein. Lutein at 20µg/ml effectively inhibited peroxidation of lipids, hydroxyl radical formation and DPPH radical formation to the tune of 86%, 92% and 90% respectively, whereas, α-tocopherol, curcumin and butylated hydroxy anisole, when used at dose ~12 times more (400µM) than Lutein showed 75–95% inhibition of lipid peroxidation and scavenging of hydroxyl and DPPH radicals

Keywords: *Helianthus annuus* L; Antioxidant; Lutein; DPPH radical; *2*-Deoxy-D-ribose.

Corresponding author
Email: chethankumar.m@gmail.com

ISSN: 0975-8585

INTRODUCTION

Lutein (L) is a carotenoid, which, along with its stereo isomer Zeaxanthin (Z), is concentrated in the macula lutea. Lutein and Zeaxanthin are but two of the more than 600 plant pigments in the carotenoid class with the molecular formula $C_{40}H_{56}O_2$. Lutein is structurally related to α-carotene and Zeaxanthin to β-carotene. Therefore, they have different preferences for interaction with membrane structures with differences in local functions. Humans and primates do not synthesize Lutein and Zeaxanthin *de novo*, and therefore depend entirely on dietary sources of these compounds. The highest concentration of Lutein is found in food sources with yellow colour, the intense coloration being due to the extensive conjugation in the polyene chain [1].

Naturally occurring macular carotenoids exist as protein complexes called carotenoproteins. Heating food denatures the carotenoproteins and facilitates the absorption of Lutein and Zeaxanthin, thus improving their bioavailability. However, excess heating results in the isomerization of all trans double bonds to cis configurations, destroying up to 65% of xanthophylls. Interestingly, Lutein appears to be the most heat stable xanthophylls. Lutein and Zeaxanthin are absorbed from the duodenum via chylomicrons, and thus process is dependent on the presence of bile and pancreatic juices. Following absorption, these carotenoids reach the liver via the portal circulation where they are repackaged as plasma lipoproteins for subsequent release into the systemic circulation. In the plasma, it has been suggested that they are carried predominantly by the Apo E containing high-density lipoprotein. Several factors influence the bioavailability of macular carotenoids from the gastrointestinal tract [2].

The processes governing the capture and stabilization of Lutein and Zeaxanthin at the macula remain elusive. The differences in capture of these compounds suggest that tissue-specific xanthophyll binding proteins may mediate Lutein and Zeaxanthin capture in each tissue. Our understanding of the macular yellow spot has accelerated in the past few decades. Advances in measuring xanthophyll density *in vivo*, combined with an expanded understanding of the biochemical and molecular nature of Lutein and Zeaxanthin, are unveiling several potential roles for these pigments in the human eye. Their antioxidative properties provide a solid basis for investigating their potential in slowing the development of cataract and age-related macular degeneration (AMD), in which oxidative damage is believe to play a role in the pathophysiology.

Helianthus annuus L. (Sunflower, Asteraceae) has been an important resource of natural oil and lipid-rich nutrients for centuries. It is widely used in human foods because of its highest protein concentrate in the seed flour. Additionally, it is used as a preventive medicine against diuresis, diarrhea, and several inflammatory diseases [3]. Recently, it was shown that aqueous extract of sunflower seed inhibits cell damage induced by hydrogen peroxide or amyloid ß-peptide in SH-SY5Y neuroblastoma cells [4]. The plant is well known for its allelopathic compounds, several phenol and terpene compounds [5]. Leaves of *Helianthus annuus* L. possess antimicrobial activity against many bacteria [6]. The plant extracts is shown to heal inflammation during experimental wound. In this context, the present investigations are aimed at isolating Lutein from sunflower *(Helianthus annuus* L.*)* petals, which are usually dumped as waste after harvesting the seeds. These petals are rich

source of colored compounds. Hence an attempt is also made to study its ability to quench reactive oxygen species.

MATERIALS AND METHODS

Plant materials

Petals of *Helianthus annuus* L. were collected from authentic vendors in the local market of Mysore, Karnataka. The petals were washed in double distilled water to remove any extraneous material and air dried in an open space under aseptic condition for about 3-5 days, and ground to fine powder and sieved through 100mesh. The powder was stored in air tight polypropylene bottle at room temperature (RT). Standard Lutein was obtained as kind gift from Dr. ML Shankar Narayan, Cancor India Ltd. Cochin. Kerala

Isolation of Lutein from *Helianthus annuus* L. petal extract

Lutein was isolated by suspending 5g of petal powder in 100ml of hexane. The resultant suspension was homogenized for 15min, filtered through Whatmann No.1 filter paper and the volume was measured. The residue (pellet) of extract was weighed after drying to constant weight to calculate the solubility [7] of the petal powder. The solubility was found to be 30%. The filtrate was evaporated at 55^{O}C and concentrated to half of its volume obtained originally. The above prepared hexane extract, 10ml of was added to 10% KOH in 20ml ethanol. The solution was evaporated at 68^{O}C for 15min. The resultant solution was washed with 30ml of 1:1 mixture of water:ethanol, centrifuged the mixture at 8000rpm for 10min at 10^{O}C. The resultant residue was re-constituted in small volume of 1:1 mixture of water:ethanol.

Spectral analysis of Lutein isolated from *Helianthus annuus* L.

Spectral analysis of Lutein standard (10µg/20µl) and Lutein fraction isolated from *Helianthus annuus* L. petal hexane extract (10µl) was scanned between the wavelength region of 400–500nm. The appearance of signature wavelength for Lutein at 421, 446 and 472nm was recorded in Hitachi 2900UV double beam spectrophotometer.

Proximate composition

The proximate composition of the *Helianthus annuus* L. petal was done carried out using methods described in literature such as polyphenols [8], flavonoids [9], sugars [10], protein [11], α–tocopherol [12] and ascorbic acid [13].

Antioxidant activity

Preparation of human erythrocyte ghosts

Fresh blood was obtained from the peripheral blood of normal healthy male donors aged 26±2 years. The ghost membranes were prepared from the erythrocytes according to

the method of Dodge et al [14]. The ghost membranes were suspended in 0.9% saline in aliquots and stored at –20^{O}C for further use. The protein content of the ghosts was determined by Lowry's method [11].

Determination of antioxidant activity by Thiobarbituric Acid Reactive Substances (TBARS) assay

Lipid peroxidation was induced in erythrocyte ghost and linoleic acid micelles by ferrous sulphate-ascorbate system, known inducer of lipid peroxidation [15] [16] according to the procedure of Shimazaki [17] and an assessment of oxidation was achieved by measurement of TBARS according to the method of Dahle et al [18]. 6µM of linoleic acid (solubilized in hexane) or 100µl of ghost suspension (300µg membrane protein) were subjected to peroxidation by ferrous sulphate: ascorbic acid (10:100µmole) in a final volume of 0.5ml Tris buffered saline (10mM Tris, pH7.4, 0.15M NaCl) with or without out Lutein (20µg/ml). A similar assay was done with other known antioxidants such as butylated hydroxy anisole (BHA)/curcumin/α-tocopherol each at 400µM (30µl) concentration serving as positive controls. The contents were incubated for 60min at 37^{O}C. The reaction was terminated by adding 1ml of 1% thiobarbituric acid, the contents were kept in boiling water bath for 15min, cooled, centrifuged at 3000rpm for 10min at room temperature. The absorbance of the supernatants obtained was measured at 535nm. The assay was carried out with appropriate blanks and controls. Antioxidant activity was expressed as percent inhibition of lipid peroxidation.

Determination of hydroxyl radical scavenging activity by *2*-Deoxy D-ribose assay

2-Deoxy D-ribose assay is done to determine the hydroxyl radical scavenging activity of Lutein [19]. The reaction mixture containing $FeCl_3$ (100µM), ascorbate (100µM), EDTA (104µM), H_2O_2 (1mM), 2-deoxy-D-ribose (2.8mM) were mixed with 20µg of Lutein in 1ml of 20mM potassium phosphate buffer, pH7.4 and incubated for 1hr at 37^{O}C. A similar assay was done with other known antioxidants such as butylated hydroxy anisole/curcumin/α-tocopherol each at 400µM (30µl) concentration serving as positive controls. The reaction mixture was heated at 95^{O}C in boiling water bath for 15min following the addition of 1ml of TBA (0.5%). Finally the reaction mixture was cooled on ice and optical density was measured at 535nm. The assay was carried out with appropriate blanks and controls. Antioxidant activity was expressed as percent inhibition of hydroxyl radical formation.

***1, 1*– diphenyl-*2*-picrylhydrazyl (DPPH) radical scavenging activity**

1, 1– diphenyl-*2*-picrylhydrazyl (DPPH) radical scavenging activity was assessed according to the method of Schimada et al [20] and Wang et al [21] with minor modifications. Lutein at 20µg/ml was mixed in 1ml of freshly prepared 0.5mM ethanolic DPPH solution and 2.0ml of 0.1M acetate buffer, pH5.5. Similar assay was carried out with positive controls such as butylated hydroxy anisole/curcumin/α-tocopherol each at 400µM (30µl) concentration. The resulting solutions were incubated at 37^{O}C for 30min prior being spectrophotometrically measured at 517nm. Lower absorbance at 517nm represents higher DPPH scavenging activity. Blank test or control received the appropriate quantity of the solvent without any inhibitor or test sample. The % inhibition was calculated by comparing

with that of control. Antioxidant activity was expressed as percent inhibition of DPPH radical formation.

Statistical analysis

Statistical analysis was done using students *t*-test. All the values represent mean of triplicates and are expressed as Mean±SEM. $p<0.01$, $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Phytochemicals, such as antioxidants and anti-inflammatory agents, help prevent or delay the progression of many pathological conditions. Observational and clinical trials conducted to support the safety of higher intakes of the phytochemicals such as Lutein and Zeaxanthin and their association in reducing the risks of cataracts and improving clinical features of AMD. Additional phytochemicals of emerging interest, like green tea catechins, anthocyanins, resveratrol, and Ginkgo biloba, shown to ameliorate ocular oxidative stress, hence deserve greater attention in clinical trials [22].

Proximate composition

The hexane extract of *Helianthus annuus* L. had negligible amounts of polyphenols and flavonoids. The protein, sugars, ascorbic acid and α–tocopherol content was also analyzed and are present in microgram quantities per gram (Table No 1). Polyphenols and flavonoids constitute one of the most numerous and ubiquitous groups of plant metabolites and are an integral part of both human and animal diet. However, interest on polyphenols and flavonoids has increased greatly because of the antioxidant and free radical scavenging abilities associated with their potential effects on human health. Their concentration in the hexane extract was negligible but their contribution to antioxidant activity by the extract was significantly less [23].

Sugars, ascorbic acid and α–tocopherol are well known antioxidants. Nagai and others [24] have reported that sugar cane extracts possess significant antioxidant activity; such extracts also exhibit interesting physiological functions, enhancement of resistance to infections as well as vaccine adjuvant and anti-inflammatory features. Many carbohydrates are excellent scavengers for metal ions. Glucose, fructose and the sugar alcohols (sorbitol and mannitol), have the ability to block the reactive sites of ions such as copper, iron and, to a lesser extent, cobalt. Furthermore, Maillard reaction products are known to have antioxidant properties [25]. One of the vital roles of ascorbic acid (vitamin C) is to act as an antioxidant to protect cellular components from free radical damage. Ascorbic acid has been shown to scavenge free radicals directly in the aqueous phases of cells and the circulatory system [26].

Vitamin E is a fat-soluble antioxidant that stops the production of ROS formed when fat undergoes oxidation, by limiting free-radical production and possibly through other mechanisms, vitamin E might help prevent or delay the chronic diseases associated with free radicals [27]. High Concentrations of vitamin E in sunflower petals makes it unfit for human consumption as high vitamin E when consumed increase the risk of hemorrhagic

stroke and premature death. In addition, high doses of vitamin E may inhibit the absorption of vitamin A. The microgram concentrations of these components in hexane extract could be contributing for antioxidant activity along with Lutein in the hexane extract without exhibiting any toxicity. There was no significant synergistic effect between Lutein and other compounds in the hexane extract towards antioxidant activity. The Lutein standard also showed similar percentage of scavenging of free radicals in comparison to Lutein rich fraction.

Spectral analysis of Lutein isolated from *Helianthus annuus* L. petal hexane extract

The spectral analysis of hexane extract of sunflower *(Helianthus annuus* L.) showed the presence of Lutein when compared with Lutein standard with a signature wavelength at 421nm, 446nm and 472nm (Figure No 1 and 2). The presence of sugars, α-tocopherol and ascorbic acid did not interfere for absorption at the designated wavelengths. A slight depression in the spectrum observed at UV region in comparison to Lutein standard could be due to small amounts of polyphenols present in the hexane extract.

Lutein is a yellow colored pigment. Although Lutein is not categorized as a vitamin, dietary Lutein is believed to be an essential nutrient for normal vision. Lutein is fat soluble, a deficiency may occur if fat digestion is impaired. Lutein is found in egg yolk and in many plants and vegetables, including red peppers, mustard, broccoli, zucchini, corn, garden peas, spinach, leek, collard greens and kale. Lutein is responsible for the coloring of many fruits and vegetables. Lutein is an antioxidant which is believed to be an essential nutrient for normal vision [28]. Studies have also indicated that Lutein improves heart health, protects our skin against UV damage, reduces diabetes induced oxidative stress, and possesses anti-inflammatory and anti-cancer properties [29]. The central part of the retina, called the macula, contains macular pigments in which Lutein is concentrated. The yellow colored pigments protect the retina from damage of the photo-oxidative affect of high-energy radiations. Lutein offers eye protection by lowering the risk of age related vision loss, which causes gradual loss of central vision [30]. Age related vision loss or age related macular degeneration is caused by steady damage of the retina.

There are reports which determine the effect of food supplements or eating foods rich in antioxidants that can protect against age-related macular degeneration, a disease in which the central portion of the retina deteriorates so that only peripheral vision remains. A team of Australian researchers who followed 3,654 subjects of age 49 or older found no statistically significant association between AMD and dietary intake of carotene, zinc, or vitamins A or C, either from diet, supplements, or both. Other published studies have had conflicting results, with some finding correlations and others finding none [31, 32].

Free radicals can be defined as species with an unpaired electron. The reactivity of free radicals varies from relatively low, as in the case of oxygen molecule itself, to a very high, as in the case of the short lived and highly reactive hydroxyl radical ($^{\bullet}OH$). Fatty acids are susceptible to attack by Reactive Oxygen Species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), peroxy radicals ($R\text{-}OO^{\bullet}$), singlet oxygen ($^1O_2$) and in particular hydroxyl radical ($^{\bullet}OH$), hence any reaction which forms ROS would surely stimulate lipid

peroxidation. Hydrogen abstraction is easier in unsaturated fatty acids than their saturated fatty acids [33].

Superoxide radical is generated by four electron reduction of molecular oxygen into water. In living organisms, $O_2^{\bullet-}$ is removed by the enzymes called Super Oxide Dismutase (SOD). The H_2O_2 formed due to four electron reduction of O_2 into H_2O and dismutation of $O_2^{\bullet-}$ is not a free radical, but an oxidizing agent. H_2O_2 can generate $^{\bullet}OH$ via the superoxide driven Fenton reaction [34]. The $^{\bullet}OH$ formed by this reaction and four electron reaction of O_2, is highly reactive and cause damage to deoxyribonucleic acid (DNA) and initiates lipid peroxidation.

Oxidative stress arises from an imbalance between generation and elimination of the ROS often leading to cell damage and eventually death. The molecules which counteract this process and help to protect the body against damages caused by ROS are termed as Antioxidants. Antioxidants neutralize free radicals by accepting or donating an electron to eliminate the unpaired electron, which in the process becomes a free radical with very low potential. Organisms possess natural defenses against free radicals in the form of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidases, reductases and transferases and thioredoxin reductases. Organisms also synthesize non enzymatic antioxidants such as glutathione, Coenzyme Q, sulphydryl and non protein sulphydryl groups. Organisms can also obtain antioxidants through diet. Therefore, molecules which quench these detrimental radicals can act as potent antioxidants, which in turn reduce the local tissue damage caused by inflammatory mediators generated due to the action of PLA_2s.

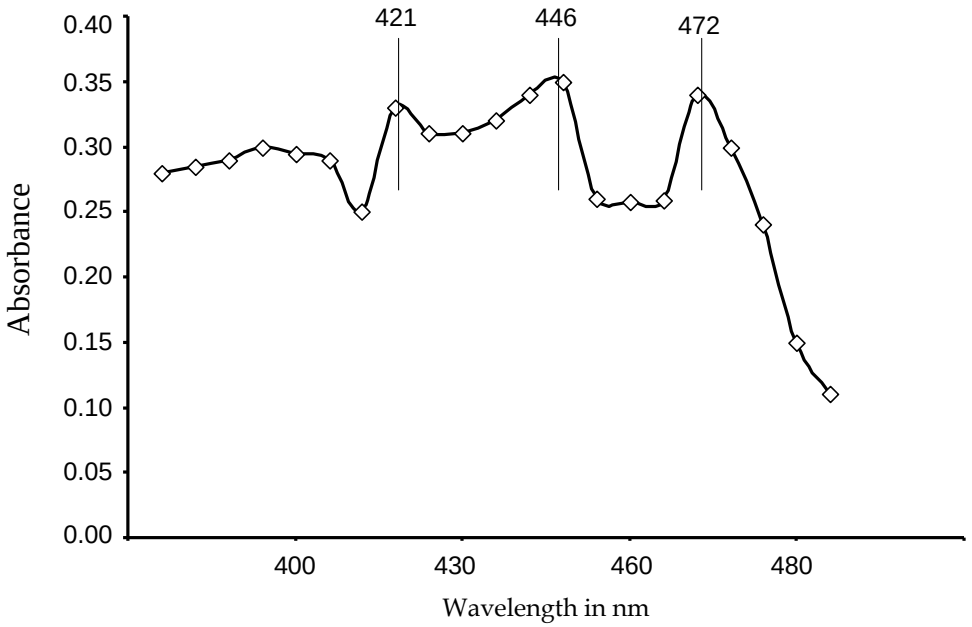
Several molecules either synthetically derived or plant and or plant based extracts have shown to act as antioxidant and antiinflammant [35]. Among the Aloesin derivatives obtained from Aloe vera, Isorabaichromone showed potent antioxidative and antiinflammatory activity [36]. Among the many plant derived molecules, polyphenols such as flavonoids, coumaric acids act as potent antioxidants [37]. Flavonoids act by forming a stable radical that can react with another flavonoid radical to produce two non radicals [38].

Antioxidant activity

In the present investigation, Lutein isolated from Sunflower *(Helianthus annuus* L.*)* was investigated for its ability to quench free radicals generated by $FeSO_4$: Ascorbate. The antioxidant activity of Lutein was tested in three different systems such as inhibition of lipid peroxidation, hydroxyl radical formation and DPPH radical formation. Lutein at 20µg effectively inhibited peroxidation of lipids, hydroxyl radical formation and DPPH radical formation to the tune of 86%, 92% and 90% respectively, whereas, Butylated Hydroxy Anisole (BHA) quenched hydroxyl and DPPH radicals by 94% and 93% respectively and inhibited lipid peroxidation by 91%. But the dose used was 20 times more than Lutein.

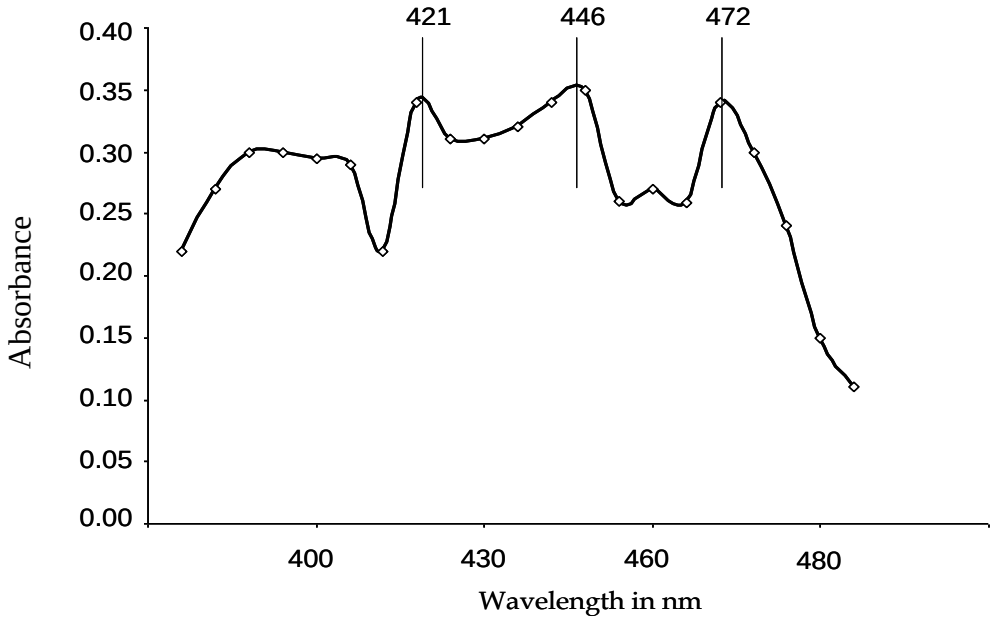
ISSN: 0975-8585

Figure 1: Spectrum of Lutein standard



Spectral analysis of Lutein standard (10µg/20µl) scanned between the wavelength region of 400–500nm. The appearance of signature wavelength for Lutein at 421, 446 and 472nm was recorded in Hitachi 2900UV double beam spectrophotometer.

Figure 2: Spectrum of Lutein isolated from hexane extract of *Helianthus annuus* L.



Spectral analysis of Lutein rich fraction isolated from *Helianthus annuus* L. petal hexane extract (10µl) was scanned between the wavelength region of 400–500nm. The appearance of signature wavelength for Lutein at 421, 446 and 472nm was recorded in Hitachi 2900UV double beam spectrophotometer.

ISSN: 0975-8585

Table 1: Proximate composition of hexane extract of *Helianthus annuus* L. petal

Analysis	Hexane extract of *Helianthus annuus* L.
	µg/g
Proteins	21 ± 8
Sugars	98 ± 2
Polyphenols	10 ± 1
Flavonoids	30 ± 4
Ascorbic acid	27 ± 3
α-tocopherol	74 ± 2

The proximate composition was done for hexane extract of *Helianthus annuus* L. petals according to standard methods as described in methods section. The results are Mean±SD of triplicates.

However, use of BHA as an antioxidant has limitations because of its ability to induce toxicity [39]. Therefore, Lutein even at lower dose acts as potent antioxidant. In comparison to BHA, Curcumin and α-Tocopherol which were used at dose 12 times more (400µM) than Lutein showed 75–85% inhibition of lipid peroxidation and formation of hydroxyl and DPPH radicals (Table No 2).

Table 2: Antioxidant activity of Lutein in three model systems

Treatment	Effective Dose (µM)	% inhibition of lipid peroxidation	% inhibition of hydroxyl radical formation	% inhibition of DPPH radical formation
Lutein rich fraction from hexane extract of *Helianthus annuus* L.	20 µg/ml	86 ± 3.25	92 ± 4.25	90 ± 4.12
Lutein standard	35 µM	84 ± 3.01	94 ± 3.20	88 ± 3.19
BHA (Butylated Hydroxy Anisole)	400 µM (30µl)	91 ± 3.96	94 ± 4.76	93 ± 5.04
Curcumin	400 µM (30µl)	77 ± 3.19	88 ± 3.72	89 ± 4.11
α-Tocopherol	400 µM (30µl)	75 ± 3.16	89 ± 3.22	90 ± 3.43

Table showing effective concentration at which Lutein acts as potent antioxidant. The three assays were carried out as mentioned in methods**.** The results are mean of three experiments and the values are expressed as Mean±SEM.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the facilities provided by JSS Mahavidyapeetha, Ramanuja Road, Mysore and Prof. BV Sambashivaiah, Principal, JSS College of Arts, Commerce and Science (Autonomous), Ooty Road, Mysore. The authors thankfully

acknowledge the financial support given by University Grants Commission (UGC) under UGC-Minor Research Project – MRP-283/08-09/KAMY013/UGC-SWRO dated 30-03-2009.

REFERENCES

[1] Canovas R, Cypel M, Farah ME, Belfort R Jr. Arq Bras Oftalmol 2009; 72(6): 839-844.
[2] Carpentier S, Knaus M, Suh M. Crit Rev Food Sci Nutr 2009; 49(4): 313-326.
[3] Lewi DM, Hopp HE, Escandon AS. Methods Mol Biol 2006; 343: 291-297.
[4] Park JY, Heo JC, Woo SU, Lee SH. Korean J Food Preserv 2007; 14: 213-219.
[5] Macias FA, Varela RM, Torres A, Molinillo JMG, Heliespirone A. Tetrahedr lett 1998; 39: 427.
[6] Mitscher LA, Rao GSR, Veysoglus T, Drake S, Haas T. J Nat Prod 1983; 46(5): 745.
[7] Sujatha R, Srinivas L. Toxicol In vitro 1995; 9: 231–236.
[8] Kujala TS, Loponen JM, Klika KD, Pihlaja K. J Agric Food Chem 2000; 50: 6490–6496.
[9] Woisky R, Salatino A. J Apic Res 1998; 37: 99-105.
[10] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Anal Chem 1956; 28: 350–356.
[11] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. J Biol Chem 1951; 193(1): 265–275.
[12] Kivcak B, Mert T. Fitoterapia 2001; 72: 656–661.
[13] Sadasivam S, Manickam A. In: Biochemical Methods. 2nd ed. India: New Age Int. Pub. 1997, pp. 184–186.
[14] Dodge JT, Mitchell C, Hanahan DJ. Arch Biochem Biophys 1963; 100: 119–128.
[15] Shalini VK, Luthra M, Srinivas L, Harinarayana Rao S, Basti S, Reddy M, Balasubramanian D. Ind J Biochem Biophys 1994; 41: 261–266.
[16] Fenton HJH. J Chem Soc 1984; 106: 899–910.
[17] Shimazaki H, Ueta N, Mowri HO, Inoue K. Biochim Biophys Acta 1984; 792: 123–128.
[18] Dahle LK, Hill EG, Holman RT. Arch Biochem Biophys 1962; 98: 253–261.
[19] Halliwell B, Gutteridge JMC, Aruoma OI. Anal Biochem 1987; 165: 215–219.
[20] Schimada K, Fujikawa K, Yahara K, Nakamura T. J Agric Food Chem 1992; 40: 945–948.
[21] Wang L, Yen JH, Ling HL, Wu MJ. J Food Drug Anal 2003; 11: 60–66.
[22] Rhone M, Basu A. Nutr Rev 2008; 66(8): 465-472.
[23] Nagulendran KR, Velavan S, Mahesh R, Hazeena Begum V. 2007; 4(3): 440-449.
[24] Nagai Y, Mizutani T, Iwabe H, Araki S, Suzuki M. In: Proceedings of the Intl. Meeting of Sugar Industry Technologists; New York 2001, pp 97–104.
[25] Davis EA. Am J Clin Nutr 1995; 62: 170S-7S.
[26] Robert E Beyer. J Bioenerg Biomem 1994; 26(4): 349-358.
[27] Blumberg JB, Frei B. Free Radic Biol Med 2007; 43:1374-1376.
[28] Hossein B, Michele M, Gislin D. BMC Opthalmolo 2006; 6: 23
[29] Choudhary R, Tandon RV. Pak J Physiol 2009; 5(1): 76-83.
[30] Amresh C, Usha C, Dinesh V. Br Med J 2003; 326(7387): 485–488.
[31] Smith W, Mitchell P, Webb K, Leeder SR. Ophthalmol 1999; 106(4):761-767.
[32] Age-Related Eye Disease Study Research Group (AREDS). AREDS report no. 8. Arch Ophthalmol 2001; 119:1417-1436.
[33] Packer L, Glazer AN. Methods in Enzymology. Vol. 186 Part B San Diego, CA, Academic Press. 1990, pp. 855.
[34] Halliwell B, Gutteridge JMC. 2nd ed. Oxford, UK, Clarendron Press. 1985, pp. 279-315.

[35] de las Heras B, Slowing K, Benedi J, Carretero E, Ortega T, Toledo C, Bermajo P, Iglesias I, Abad MJ, Gomes-Serranilios P, Liso PA, Villar A, Chiriboga X. J. Ethanopharmacol 1998; 61: 161-166.
[36] Yagi A, Kabash A, Okamura N, Haraguchi H, Moustafa SM, Khalifa TI. Planta Med 2002; 68: 957-960.
[37] Prabhu S, Jainu M, Sabitha CS, Devi S. J. Ethanopharmacol 2006; 107: 126-133.
[38] Bergman M, Varshavsky L, Gottlieb HE, Grossman S. Phytochem 2001; 58: 143-152.
[39] Labrador V, Fernandez FP, Perez MJM, Hazen MJ. Cell Biol Toxicol 2007; 23: 189-199.