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EXTRACTION, QUANTIFICATION AND MULTIPLE FACETS OF LUTEIN

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ABSTRACT

Tagetes erecta, also called Aztec marigold belongs to species of genus *Tagetes*. *Tagetes erecta* is reputed for its high therapeutic values. These are rich in alkaloids, terpenes, flavonoids, phenolic compounds etc. Hexane was added to the dried and cleaned form of marigold flower petals to prepare oleoresin. The oleoresin was saponified using potassium hydroxide and ethyl alcohol further the mixture was homogenized and purified to obtain pure lutein and then it was subjected to analytical procedure like HPLC. The therapeutic activities like Antioxidation, Anti-Inflammation and Neuroprotective properties along with its action on Eye, Heart and Brain were also mentioned.

INTRODUCTION

Lutein belongs to the carotenoid family, namely to xanthophyll.[1] It is a yellow plant pigment with structural formula depicted in following figure.[1,2] Lutein is found to be present in many kinds of vegetable and fruits, especially in leafy vegetables, but also in marigold flower.[3] Lutein effectively acts as an antioxidant, particularly to protect the eyes, because it has the ability to neutralize free radicals formed by the action of ultraviolet radiation on eye retina.[4] Humans cannot synthesize lutein, so they acquire solely by consuming vegetables, fruits or dietary supplements.[5] Plant material has all-trans-isomer of lutein but cis-isomer of lutein is also generated along with the other agents by the action of temperature and light, and other factors are also taken in consideration during extraction and analysis of sample.[6,7] In plants, lutein exists either in the form of free lutein in leafy vegetables like cabbage, broccoli and spinach or in the form of esters with fatty acids in following vegetable and fruits: papaya, orange, mango, green or red pepper, yellow corn etc.[8] The lutein content in the natural

source depends on their kind, level of maturity, variety, the way of processing by heat, part of fruit, storage and also preservation.[9]

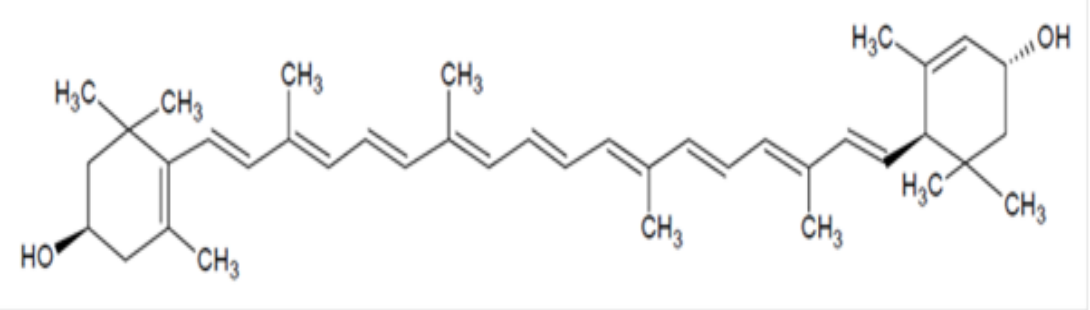

Figure 1: Structural formula of lutein.

Countries like Mexico, Peru, Spain, China and India grow Marigold flower for business purpose as it is rich source of lutein. Dried marigold flowers contain 0.1-0.2% dry matter (DM) of carotenoids, in which 80% are lutein diesters.[10] A non-polar oleoresin extract is obtained by extracting dried and ground flowers.[11] Conversion of trans-lutein into cis-lutein is catalysed by heat, light, air, oxygen and acids (in the process of technological treatment).[12] Lutein ester is obtained by purifying non-polar extract; its subsequent saponification leads to the isolation of free lutein.[13] Both lutein ester and free lutein in the form of powder, oil, or as bead lets are used for manufacturing dietary supplements or for enhancing drinks and foods with carotenoids.[14] Mainly the studies that deal with examining lutein content in fresh or dried marigold flowers is in non-polar concentrates.[15] It is very essential to carry out the quantitative analysis before implementing qualitative analysis (in this case HPLC analysis of lutein). Extraction of lutein with organic polar or non-polar solvents and their combinations is most common procedure yet, the least demanding instrumental method employed. Based on the nature of the sample, lutein extraction can be done by sample saponification.[16] The ability of lutein to stabilize and solubilize is based on the extraction agent which either may cause a positive effect or it can cause a negative effect on HPLC analysis along with this the effects of organic solvents on the environment and their safe use were also monitored.[17] Extraction of lutein using supercritical fluid extraction with carbon dioxide do not give competent results.[18,19] However it can be approved for separating stereoisomers of Lutein i.e. (3R,3'S,6'S)-lutein(2), (3R,3'S,6'R)-lutein(3), (3R,3'R,6'S)-lutein(4).[20] Lutein i.e. (3R,3'R,6'R)-lutein(1) can be determined using HPLC analysis technique, chromatographic columns with C18 or C30 sorbents are commonly applied, the

latter packing is also suitable for the separation of individual trans- and cis-isomers of lutein.[21]

Chemical constituents

The oleoresin extract obtained from dried marigold flower petals by treating with hexane consists of lutein, lutein esters, other carotenoids and waxes. Purified lutein is further obtained from the oleoresin by saponification and crystallisation methods.

Chemical formula: $C_{40}H_{56}O_2$

Molecular weight: 568.88

Lutein is a free-flowing orange red powder and insoluble in water, soluble in hexane. Lutein is generally used as a nutrient supplement and also as colouring agent. Lutein is xanthophyll or an oxy-carotenoid, which contains basic C-40 isoprenoid structure and two cyclic end groups common to all carotenoids. Lutein is known to be one of the major components and the main pigment of Tagetes erecta.[22]

Extraction of lutein

Isolation of Lutein from marigold extract

The oleoresin (obtained when dried petals were treated with hexane) was mixed with ethyl alcohol to which potassium hydroxide was added, which further dissolves in the solution to give alcoholic-alkali solution.[23]

Saponification reaction mixture consist of three ingredients in the ratio of about 1:3:0.25 part of oleoresin: alcohol: potassium hydroxide respectively by w/v/w.[24]

Potassium hydroxide (6 g) dissolved in ethyl alcohol (75ml) in a round bottom flask (250ml) into which oleoresin (25g) was added and the flask is shaken vigorously and subjected to water bath at 70^{o}C.[25] This process refers to saponification of oleoresin which results into formation of free xanthophyll along with alkali salts of fatty acids.[26]

The progress in the saponification reaction can be monitored by HPLC analysis to inspect the complete saponification which is confirmed by absolute disappearance of the lutein ester peak.[27]

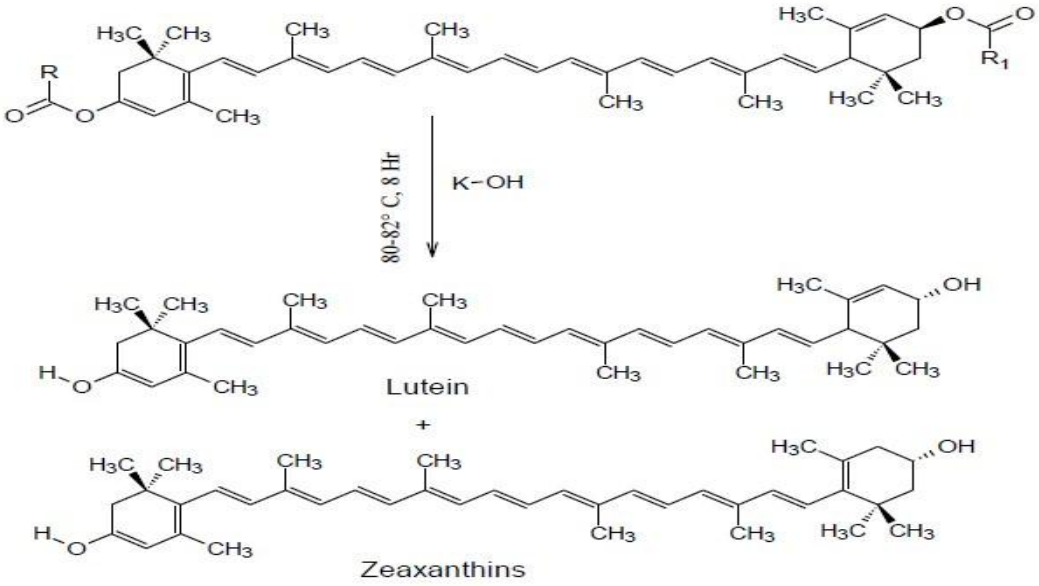

Figure 2: Scheme for conversion of Lutein and Zeaxanthin esters to free form.

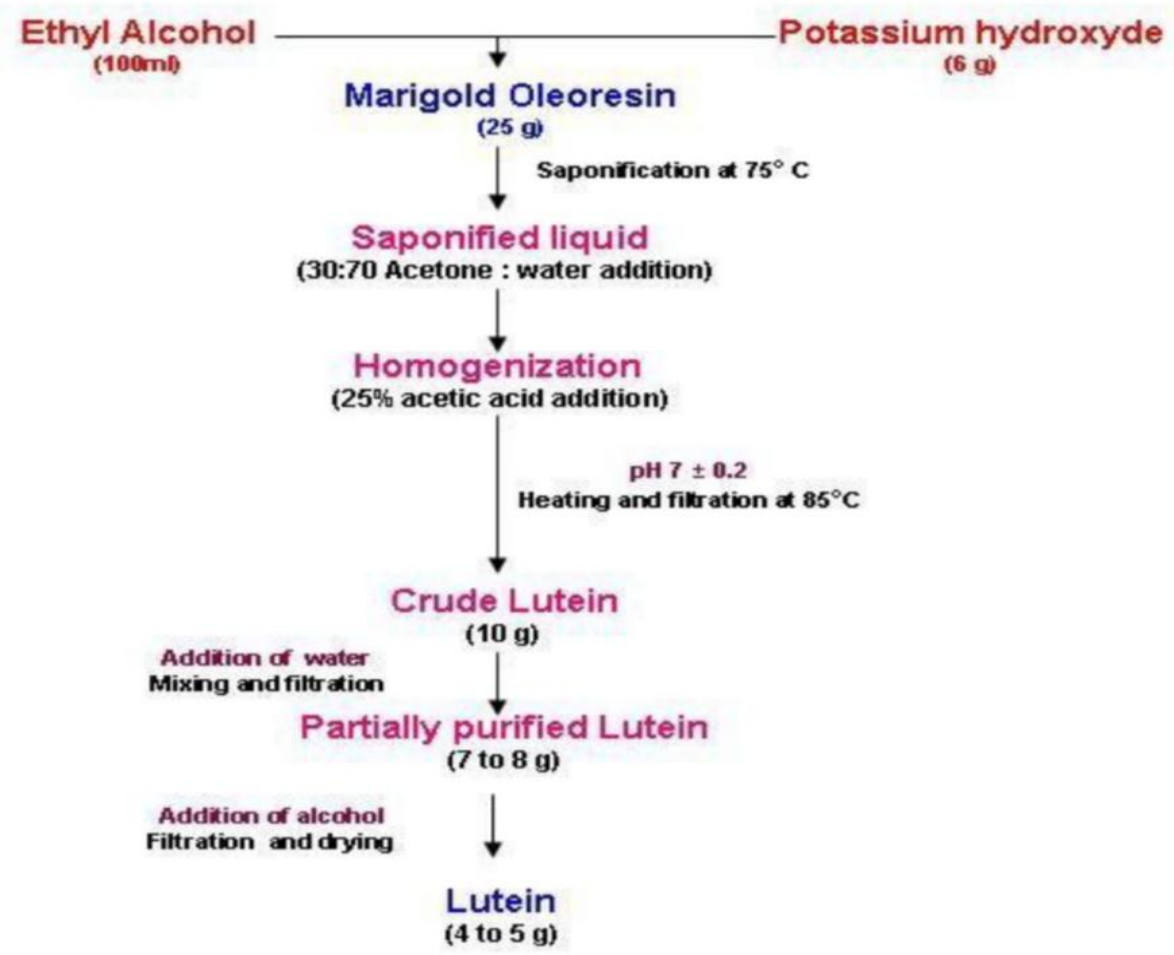

Figure 3: Saponification, isolation and purification of Lutein from marigold

HPLC Analysis

The analysis was carried out by taking an aliquot of 1 ml of saponification mixture and 30 ml of extractant i.e. hexane: acetone: toluene: absolute alcohol 10:7:7:6 v/v in an 100 ml amber coloured standard flask to which 30ml of hexane is added and shaken for 60 seconds.[28] Further, the solution mixture is diluted with 10% Sodium sulphate and kept rendered to sunlight (in a dark environment) for 30 minutes. The upper phase was analysed by HPLC. Silica column of 254mm x 4.5mm, 5µm dimensions was used. Mobile phase for the separation was Hexane: Ethyl acetate 75:25 respectively. An isocratic environment should be maintained at flow rate 2 ml /min.

The volume of sample injected was 20 µl. Detection wavelength was set at 474 nm. The alcohol was removed from the reaction mixture by vacuum distillation.[29]

The final saponified product was then homogenized at room temperature with water for 30 minutes resulting in a brownish yellow oily liquid which has the lutein + fatty acid soap along with other impurities present in the free form. The solution was neutralized by adding 25% solution of acetic acid followed by the addition of acetone.[30] The temperature was increased to 80-85^0 C and the mixture was stirred continuously for an interval of 15 minutes. The resultant mixture was filtered using Buckner funnel and the filtrate was disposed of. The Xanthophyll gets distinct in crude crystal form and the impurities can be dissolved using acetone/ water which were further removed by filtration.[31] This obtained lutein crude crystals should be thoroughly washed with distilled water until pH becomes neutral and filtrate nearly becomes colourless. The resulting lutein that is obtained consists around 50-60% of pure xanthophyll by spectrophotometric analysis.[32] Final purification was performed by crystallization in ethyl alcohol by dissolving 60% pure crystals in ethyl alcohol. The crystals were further filtered off and dried in vacuum at 50^o-60^{o}C. The purity of this product was noted greater than 85% monitored by the spectrophotometer and by AUC % by HPLC method Lutein content was over 90%.[33]

Effects of lutein on human body

- **Antioxidant, Anti-Inflammatory and Neuroprotective Properties**

Studies show that, a mouse receiving three months lutein supplementation had an anti-inflammatory, neuroprotective and antiangiogenic effect and the outer nuclear layer thickness examined was greater than the non-supplemented mouse. In the same unit it was observed that the retinal expression of the pro inflammatory mediators like inducible nitric oxide

synthase, THF- α, cyclooxygenase-2, IL-1β, and vascular endothelial growth factor was lower in the supplemented mouse as compared to the non-supplemented mouse.[34] The consumption of lutein facilitate neuroprotective action against transient cerebral ischemic injury in mice as it has been possible to increase reduced/oxidized GSH ratio along with the activities of antioxidant enzymes such as superoxide dismutase, GSH peroxidase, and catalase.[35] Lutein represses STAT3 actuation by extracellular signal-regulated kinase activation and inflammatory cytokines, retaining a-wave electroretinogram amplitude and slowing DNA damage in mouse models.[36] Lutein has a neuroprotective role in retinal ganglion cells against N methyl-D-aspartate-induced retinal damage in rats. Lastly, lutein treatment significantly reduces the oxidative stress in rat model of skeletal ischemia/ reperfusion injury by down regulating oxidative stress and inflammatory mechanism.[37]

- **Lutein and Cognitive Function**

Recent research papers suggest the predominance of lutein accumulated in the brain, is positively associated with improved cognitive function in the elders.[38] Macular pigment density is a stable measure of zeaxanthin and lutein in the retina and is consistent with verbal learning and fluency, better global cognition, and processing and perceptual speed in old people.[39] Moreover, lutein improves cognitive functioning in old women after 4-month supplementation[40] visual motor behaviour and ameliorates visual processing speed in young population[41] Because of the motivating findings of positive influence of lutein on brain function, growing interest targets on identifying possible beneficial effect of lutein in neurodegenerative diseases such as Alzheimer disease (AD) and Parkinson disease (PD). It has been proposed that lutein may prevent neuronal damage occurring in AD patients by virtue of its antiapoptotic, mitochondrial protective and antioxidant properties. A randomized, double-blind clinical trial reported improvements in visual function and increase of macular pigment density in patients with AD after lutein supplementation while cognitive function was not influenced when AD patients were daily supplemented for six months with macular carotenoids (10mg meso-zeaxanthin, 10mg lutein, and 2 mg zeaxanthin)[42] PD-mice model showed lutein to protect nigral dopaminergic neurons by diminishing mitochondrial dysfunction, amplifying antioxidant defence mechanisms and apoptotic death.[43] Lutein enhanced antiapoptotic marker (Bcl-2) expressions and inhibited the activation of proapoptotic markers, with significant reduction in motor abnormalities and thus reversed the loss of nigral dopaminergic neurons. These findings prove that beneficial and useful

employment of lutein for neurodegenerative therapy even if its potential protective function against these diseases remains to be explored.[44]

- **Lutein and Cardio metabolic Health**

Lutein exerts positive influence in decreasing the risk of Coronary Artery Disease (CAD) and promoting cardiovascular health as it possesses antioxidant and anti-inflammatory capacity. Prevention of atherosclerosis development by oxidizing low density lipoprotein levels, decreasing malondialdehyde and reducing inflammatory cytokines such as interleukin- (IL-) 10 is possibly due to lutein as proved in animal studies.[45] Also, when ApoE-deficient mice was given lutein for 24 weeks it came to know that NADPH oxidase was inhibited and peroxisome proliferator-activated receptor expression was increased by lutein, which in turn protects against high fat diet-induced atherosclerosis.[46] Lutein rich diet in humans gives beneficial cardiovascular effect, especially in preventing formation of arterial plaque, is reported in the Carotid Ultrasound Disease Assessment (CUDAS) studies and the Atherosclerosis Risk in Communities (ARIC).[46] While the effect of lutein on hypertension is doubtful.[47] lutein counteracts oxidative stress created after myocardial ischemia/reperfusion damage.[48] An inflammatory response with increased generation of highly reactive oxygen species upon reperfusion occurs due to accumulation of neutrophils, which are responsible for myocytes apoptosis.[49] Hence, Contractile dysfunction, reducing morbidity and mortality associated with CAD can be stopped by limiting myocardial injury. Recent meta-analysis also indicated a reduced risk of coronary heart disease, stroke, and metabolic syndrome in lutein-supplemented subjects or high lutein blood concentration subjects.[50]

- **Lutein and the eye**

Human eye consists of macular pigment which is composed of three carotenoids including lutein in equal concentrations to meso-zeaxanthin and zeaxanthin.[51] The macula lutea is a circular yellow area 5-6 mm in diameter which is located in the central and posterior portion of the primate retina. The macula has large part of photoreceptors and it is in charge of high-resolution visual acuity and central vision. Retina has neuronal lipid bilayer membranes and it is vulnerable to oxidative damage due to exposure to high oxygen concentration. Lutein is soluble in polyunsaturated phospholipid membrane domains, functions against oxidative stress in retinal tissues. Retinal endangerment to hypoxia-ischemia is obvious especially as a result of photochemical damage, primarily situated on central region of the retina in the outer layers, with respect to both photoreceptors and retinal pigment epithelium.[52] Laboratory

studies recommended that oxidative events trigger photochemical damage leading to apoptosis of retinal cells. In specific, ocular exposure to UV, sunlight and short blue light-emitting lamps may cause retinal degeneration and cataract through a photooxidation reaction. During a photo-oxidative reaction, phototoxic chromophore present in the eye have ability to absorb light but they turn to an unstable state (singlet and then a triplet state) that produces FR.[53] Phototoxic reactions damage can be prevented by lutein as Antioxidant quenchers. In fact, being a chemical structure with substantial conjugated bonds, lutein is capable to absorb light of the blue range wavelength (400–500 nm) that prevents light-induced retinal damage.[54,55] Moreover, during oxidative stress conditions, lutein functions as an effective quencher of singlet molecular oxygen in the retina and prevents lipid peroxidation and the accumulation of FR accountable for photoreceptor apoptosis.[56] Oxidative stress is the main result of retinal ischemia which was found to underlie retinopathy of prematurity (ROP) and diabetic retinopathy (DR).[57] In both ROP and DR early ischemia due to abnormal retinal blood supply causes abnormality in neovascularization and subsequent haemorrhages and blindness. In preterm infants, hypoxic injury is caused due to delayed retinal vascular development because of the suppression of growth factor in a hyperoxia environment and imbalance between an increased metabolic demand.[58] Decrease in blood flow and DR hyperglycaemia cause retinal ischemia.[59] Hyperglycaemia induces changes in the human system such as leukostasis, vasoconstriction and proinflammatory state that also induces hypoxia in the retina. Retinal hypoxia can be caused due to the early proinflammatory changes. Also, lutein protects against senile cataract. Protection is done by influencing changes in glutathione oxidation, which is responsible for the increased susceptibility of the nucleus to cause oxidative damage in older lenses.[60] Protective effects of lutein have been also described in age-related macular degeneration (AMD). AMD is a major cause of blindness and visual impairment among people 65 years or older which is due to the decreased capability in naturally protective antioxidant systems and the increase visible and UV light-absorbing endogenous phototoxic chromophores that generates reactive oxygen species.[61] In the retinal pigment epithelium, lutein counteracts stress induced changes encouraging suppresses inflammation and tight junction repair both by induction of endogenous antioxidant enzymes and by direct scavenging. Sustained supplementation of lutein along with meso-zeaxanthin and zeaxanthin was described to be effective in increasing contrast sensitivity, macular pigment and visual function in early AMD.[62] The three carotenoids also showed positive effects on visual performance in various retinal diseases.[63] In a double-masked clinical trial namely 'Age-Related Eye Disease Study 2 (AREDS2)',

volunteers were assigned to receive four different treatments: (1) 10 mg lutein + 2 mg zeaxanthin; (2) lutein + zeaxanthin + DHA + EPA; (3) fish oil containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); and (4) placebo. Lutein and zeaxanthin combination remarkably decrease the progression to advanced AMD.[64] Lutein, zeaxanthin and meso-zeaxanthin consumption is effective in improving contrast sensitivity in population who does not have retinal abnormality (healthy population) by enhancing retinal concentrations of given carotenoids.[65] In a meta-analysis report it was given that lutein, zeaxanthin, and meso-zeaxanthin supplementation improve optical density, macular pigment in both healthy population and AMD with a dose-response relationship.[66]

CONCLUSION

The present experiment was performed with the objective of extracting a suitable economical system for extraction of lutein from dried marigold petal powder. Different solvents have been employed among which ethyl alcohol, potassium hydroxide, acetone, acetic acid was found to be suitable for extraction which was further confirmed by HPLC and later importance of lutein in human diet was discussed. As lutein holds anti-inflammatory properties, along with assurance of safety, it can be considered as a promising molecule in various fields of application. Neonatal age is a sensitive stage relating to the dangerous effects of oxidative stress on the developing tissues. Neonates, do not have defence mechanism against the oxidative cellular injury because of the several prooxidant events like deficient antioxidant systems and the exposition to a relatively hyperoxia environment with enhanced generation of FR. Oxidative stress damage can be worsened by other neonatal conditions (inflammation, hypoxia, ischemia, and free iron release. As a result, a great subject of interest has been focusing on antioxidant treatments. Tests performed on human adult diseases like AMD, atherosclerosis and senile cataract showed the efficacy of lutein in counteracting oxidative damage. This evidence calls for a more investigation in infants. AS humans cannot synthesize lutein, lutein supplementation should be consumed in general diet, especially during pregnancy and to the neonates.

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