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The Enhanced Oral Bioavailability Of Total Lutein Oxidized Products (Lops) Extracted From *Tagetes Erecta* Flower Petals In C57BL/6 Mice

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ABSTRACT

The lutein extracted from shade-dried *Tagetes erecta* flower petal powder by simultaneous solvent extraction and saponification was exposed to sunlight (31±2ºC) for 10 days. Bioavailability and absorption kinetics of total lutein oxidised products in comparison with lutein (parent molecule) were analysed on C57BL/6 mice. Time course plasma kinetics was studied by collecting blood, liver, intestine and eyes after the 1st, 2nd, 3rd, 6th and 9th hour of intubation. The oral bioavailability of total lutein oxidized products was enhanced in contrast to lutein in plasma by 25% (2nd hr), liver by 11% and eyes by 55%. The plasma kinetic properties like area under concentration and area under moment concentration of total lutein oxidized products were 1139.418 pg/h/ml and 17750.69pg/h^2/ml respectively with a half-life of 11.353h. The mean residence time of total lutein oxidized products was 15.573h with a volume of distribution of 2.874 and clearance of 0.175. The concentration of total lutein oxidized products in eyes at the 9th hour was 59.80pg/ml. Whereas, the mean lutein concentration in plasma, liver, and eye was significantly less in lutein in comparison with total lutein oxidized products. Thus, the above data suggest that total lutein oxidized products reach target tissue unaltered and enhance the absorption rate in comparison to the parental compound lutein.

Keywords: Lutein, *Tagetes erecta* flower, total Lutein oxidised products (LOPs), C57BL/6 mice, Bioavailability.

INTRODUCTION

Lutein is a dietary fat-soluble pigment which is accumulated in various tissues /organs of the human body like blood, skin, liver, intestine, breast (lactating women), brain and the majority in the macular region of the eye.[1] Various pre-clinical and clinical studies suggest health beneficial aspects of lutein. Some of them include neuroprotective effects[2], ophthalmological effects[3], antibacterial effects[4], photoprotection[5], wound healing[6], anti-inflammatory[7] and anticancerous effects[8]. Having known the mechanism of lutein in curing life-threatening diseases, yet finding it difficult to formulate into a drug. One of the reasons may be the poor bioavailability and bioaccessibility of lutein.[9]

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The uptake and absorption of lutein depend on the release of lutein from the food matrix, transfer of lutein into lipid micelles, uptake of lutein into the intestinal mucosal cells and transfer of lutein /metabolites to the lymph system. In every step, there is a huge loss of lutein due to varying temperatures, pH, and oxidative enzymes involved in the digestion process which accounts for poor bioavailability. There are various factors involved in altering the bioavailability of a compound. Some of them include physico-chemical properties, food matrix and processing, variety of dietary components, nutritional status, gut health and genotype.[10]

Choosing a suitable vehicle /formulation is an important criterion to enhance lutein bioavailability. Lutein solubilized in mixed micelles containing lysophosphatidyl choline increased bioavailability in comparison with phosphatidyl choline in male Wistar albino rats.[11] Another study suggests that olive oil improves lutein absorption in comparison with other vegetable oils.[12]

An European patented study reports that lutein in ester forms has higher bioavailability than free lutein as esterified lutein is more stable.[13] In our previous study, we reported the anti-obese potential of total Lutein oxidized products (LOPs) exhibiting better stability, lipase inhibitory activity, and thermodynamic and pharmacokinetic properties in comparison with the parental compound lutein.[14] In this study, we are trying to understand the bioavailability of total LOPs in contrast with lutein.

EXPERIMENTAL SECTION

Chemicals

Porcine pancreatic lipase, 4-Nitrophenyl palmitate and standard lutein were obtained by Sigma Aldrich Co. USA and all other chemicals including hexane, sodium chloride, Triton X100, methanol, diethyl ether, ethanol, chloroform, dichloromethane, tocopherol, potassium hydroxide, and isopropanol were of analytical grade.

Extraction of Lutein and synthesis of total LOPs

Marigold flowers were purchased from the local market of Mysore District, Karnataka. Petals were separated from the flower and rinsed thoroughly using double distilled water. Cleaned petals were shade dried, powdered and sieved using the mesh size of 260 microns to obtain a fine powder. The powder was then stored in an air-tight container in dark at room temperature until further use. The extraction of lutein from marigold flower petals was done according to the method described by Frederick Khachik[15,16]. The synthesis of total LOPs was done according to Nagashree et al. Briefly, extracted lutein was exposed to sunlight with an average solar intensity 5.89KWh/m^2/day for 10 days for a fixed time interval (from 10 am to 2 pm). The obtained product after 10 days of exposure to sunlight was named as total LOPs which was characterised by rp-HPLC and LC-MS. total LOPs is a combination of eight major compounds namely, LOP1: Lutein; LOP2: 4-((1E,3E,5E,7E,9E)-3,7-dimethyldodeca-1,3,5,7,9,11-hexaen-1-yl)-3,5,5-trimethylcyclohex-3-enol; LOP3: 3,5,5-trimethyl-4-((1E,3E,5E,7E,9E)-3,7,12-trimethyltrideca-1,3,5,7,9,11-hexaen-1-yl)cyclohex-3-enol; LOP4: (2E,4E)-3-Methyl-5-(2,6,6-trimethyl cyclohexa-2,4-dien-1-yl)penta-2,4-dien-1-ylium, LOP5: 4-((1E,3E,5E,7E)-3,7-dimethyldeca-1,3,5,7,9-pentaen-1-yl)-3,5,5-trimethylcyclohex-3-enol; LOP6: 4-((1E,3E,5E)-3,7-dimethylocta-1,3,5,7-tetraen-1-yl)-3,5,5-trimethylcyclohex-3-enol; LOP7:3,5,5-trimethyl-4-((1E,3E,5E,7E,9E,11E,13E,15E)

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3,7,12,16tetramethyloctadeca1,3,5,7,9,11,13,15,17-nonaen-1-yl)cyclohex-3-enone; and LOP8: 3,5,5-trimethyl-4-((1E,3E,5E,7E,9E,11E)-3,7,12-trimethyltetradeca-1,3,5,7,9,11,13-heptaen-1-yl)cyclohex-3-enol which was elucidated by LC-MS in previous article[14].

Preparation of mixed micelles

The preparation of lutein/ total LOPs mixed micelles was done according to lakshminarayana et al [11]. Briefly, 2.5mM of mono-oleyl glycerol, 7.5mM of oleic acid, 12mM of sodium taurochlorate, 0.5mM of cholesterol and 200µM of lutein/ total LOPs were dissolved in methanol and the solvent was dried under nitrogen. The dried mixture was resuspended using 0.5ml of phosphate buffered saline (p^H-7) giving a vigorous shaking using a vortex mixer until an optically clear solution was obtained.

Animals and Diet

Animal experiments were conducted after due clearance from the Institutional Animal Ethical Committee (IAEC approval copy attached). Male C57BL/6 mice (n=28) weighing 22±2g were housed in individual cages at room temperature with a 12hr light /dark cycle (28±2^{0}C). The animals received daily fresh pellets (AIN 93G) with water adlibitium. The left-over diets were weighed and discarded. After 7 days of acclimatization, mice were deprived of food for 12 hours before administration of total LOPs.

Single dosage intubation and sample collection

Total LOPs in mixed micelles were dispersed in 200µl of olive oil and administered orally for each mouse [n=24], divided into 5 groups (n = 4/group) to study time course plasma kinetics and its tissue levels after 1, 2, 3, 6 and 9 h of intubation. The remaining four mice are treated as control (administration of olive oil without lutein/total LOPs). In the case of lutein, (lutein in mixed micelles were dispersed in 200µl of olive oil) as there are many works of literature on bioavailability study, usage of animals was reduced to 4 mice. At 1, 2, 3and 6th hour, 100 µl of blood was drawn from the caudal vein and at 9th-hour mice were sacrificed. All mice after their time course were anaesthetized with diethyl ether and sacrificed by exsanguinations. Blood was collected directly from the heart into heparinised test tubes, and centrifuged at 1000×g for 15 at 4℃ to obtain plasma. The liver, intestine, and eyes were excised and washed with ice-cold isotonic saline, and stored at−70◦C until further analysis.

Extraction of total LOPs and Lutein from plasma and tissues

Total LOPs and Lutein were extracted from plasma, liver, intestine and eyes according to Lakshminarayana et al with slight modifications [17]. For plasma, 0.8ml of plasma was made up to 1ml by adding saline. 2ml of dichloromethane and 1 ml of methanol were added and vortexed thoroughly. To this mixture, 1.5ml of hexane was added and centrifuged at 1000g for 5 minutes. For lutein extraction, an additional compound of 2mM α-tocopherol was used to prevent oxidation. Centrifugation resulted in the formation of three layers, in which total LOPs/Lutein was found in the upper dichloromethane/ hexane layer which was collected into a separate tube. The addition of 1ml of dichloromethane and 1.5 ml of hexane and centrifugation were repeated twice to ensure the complete extraction of total LOPs/Lutein from plasma. The collected hexane layer was pooled and analysed for Lutein/ total LOPs using HPLC.

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In the case of tissues, liver (whole), intestine (mucosal layer from the duodenum to jejunum) and eye (pooled) samples were homogenised separately using the ice-cold isotonic saline solution in a hand homogeniser. Tissue homogenates were saponified with 2ml of 10M KOH at 60ºC for 45 minutes. During the process, for every 15 minutes 2ml of ice-cold water was added and vortexed. After saponification extraction of total LOPs/Lutein was carried out in the same manner as in plasma.

rp-HPLC of plasma and tissue extracts

Reverse-phase high-performance liquid chromatography was performed to estimate the amount of LOPS/Lutein present in the plasma and tissue extracts using Agilent 1260 Infinity Quaternary equipped with G1311B/C Quaternary Pump, G1329B Autosampler, G1330 Quaternary Pump, G1329B Autosampler, G1330B Thermostat, and G4212B VWD. The HPLC system was equipped with an Eclipse Plus C18 column (4.6X150mm I.D., 5µm particle). The analysis of the chromatographic data was carried out on Open lab CDS ChemStation software (A.01.05). The fractions were eluted in an isocratic fashion at the rate of 1ml/min using Acetonitrile: Dichloromethane: Methanol in the ratio 9:0.5:0.5.

Absorption kinetics of total LOPs/Lutein from plasma response

Kinetics of total LOPs and Lutein absorption from plasma response was calculated using the software pk solver version 2.0 non-compartmental analysis of plasma data after extravascular input using a trapezoidal linear method developed by Zhang Yong, China pharmaceutical University. Pharmacokinetic properties like observed peak concentration (C_{max}), time at a maximum concentration (T_{max}), half-life ($T_{1/2}$), area under concentration (AUC), the area under moment concentration (AUMC), mean residence time (MRT_{last}), the volume of distribution (V_d), Clearance rate (CL) were computed.

Statistics

All experiments were conducted in triplicate and the data were expressed as mean ± standard deviation (SD). Two-way ANOVA was used for the comparison between each group. Turkeys' multiple comparison post-hoc test was done using GraphPad Prism 9.4.1 software

RESULTS

Total LOPs and lutein obtained from plasma and tissue homogenates were detected and quantified using rp-HPLC. 40µl of plasma/tissue homogenates containing total LOPs/lutein were dissolved in the mobile phase and made up to 1ml. 10µl of each sample was injected and programmed with a runtime of 10 minutes. Lutein was detected at a retention time of 1.687 and total LOPs were detected at a retention time of 3.208 min which was confirmed by injecting standard lutein.

Absorption kinetics (plasma response) of total LOPs and Lutein.

Time-parameterised Post prandial total LOPs and lutein levels in plasma are shown in table 1. Various absorption kinetics parameters i.e., Area under concentration (AUC), an area under moment concentration (AUMC), Mean residence time (MRT), half-life ($T_{1/2}$) volume of distribution (Vd) and clearance rate (CL) were analysed. Both total LOPs and lutein in plasma reached maximum concentration at the 2nd hour. The mean peak concentration was increased to 25% in the case of total LOPs when compared with lutein. AUC and AUMC of

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total LOPs were 1139.418 pg/h/ml and 17750.69pg/h^2/ml with a half-life of 11.353h and 5.586h respectively. Whereas, lutein had a reduced AUC of 979pg/h/ml and AUMC of 8597.27 pg/h^2/ml with a half-life of 5.586 h. MRT of total LOPs and lutein was 15.573 h and 8.776 h with a Vd of 2.874 and 1.645 respectively. The rate of clearance is least in case of total LOPs (0.175pg/min) and lutein CL was 0.204pg/min [Table 1; Fig. 1].

Tissue response of total LOPs and Lutein

Postprandial total LOPs accumulation in the liver was maximum at the 6th hour (127.83pg/ml). total LOPs were not detected in 1st hour after intubation and the highest concentration was recorded in the 9th hour (160.73pg/ml). In the case of eyes, the total LOPs concentration was very less, hence all the eyes (n=8) were pooled and injected into HPLC. No significant amount of total LOPs was detected from 1st hour to 3rd hour. At the 6th hour, 37.60pg/ml of total LOPs were seen which later increased to 59.80pg/ml during the 9th hour [Table 2].

Concerning Lutein, animals were sacrificed at the 9th hour only. The concentration of lutein obtained in liver, intestine and eyes were 64.40pg/ml, 59.52pg/ml and 38.09pg/ml respectively. The concentration of total LOPs in the liver was elevated by 11% and in the eyes by 55% in comparison with Lutein [Table 2]. total LOPs exhibited good plasma and immune response and enhancement in bioavailability in contrast with lutein.

DISCUSSION

In this study oral gavaging of total LOPs to C57BL/6 mice reached the different target sites like the bloodstream, liver, intestine and eyes at different time intervals. In plasma and liver, total LOPs were detected at 1st hr, whereas in intestine and eyes (pooled) total LOPs were found at the 6th hour and 9th hour respectively

Lakshminarayana et al in 2008 showed that photo-oxidised lutein products when oral-gavaged with mixed micelles in male albino rats, lutein metabolites like Anhydrolutein, Lutein di epoxide, (2E, 4E)-3-methyl-5-(2,6,6-trimethylcyclohex-2,4-dien-1-yl) penta-2,4-dien-1-ylium, 2,6,6-trimethylcyclohexa-2,4-dienylium, and 5,6-Epoxy-3-hydroxy-12'-β, ε-carotene-12'-al were detected in plasma and liver. Anhydrolutein was also detected in the intestine.[18] Frederick Khachik in 1997 identified lutein and zeaxanthin oxidised products in human and monkey retinas. Oxidised products like 3-hydroxy-beta and epsilon -carotene-3'-one were found in the human retina. Monkey's retina consisted of lutein oxidised products like 9-cis-lutein, 9'-cis-lutein, 13-cis-lutein and 13'-cis -lutein through HPLC analysis.[19]

Postprandial plasma response concerning total LOPs was 25% more bioavailable with higher AUC, AUMC, half-life, the volume of distribution and clearance rate. total LOPs was 11% and 55% more bioavailable in the liver and eyes respectively. Similarly, Bowen et al showed that esterified lutein is 61.6% more bioavailable with higher AUC and maximum serum concentrations than the free form of lutein with human subjects.[20]

Our previous in-vitro study depicts that total LOPs are more stable, more lipophilic, less vulnerable to altered temperature and possess better ADME (absorption, digestion, metabolism and excretion) properties in comparison with lutein[14]. These criteria may help the total LOPs to decrease the loss of compounds involved during the process of absorption to the target organ and hence may increase the bioavailability.

DECLARATIONS

Ethics approval and consent to participate

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All animal studies were conducted under a protocol approved by the Institutional Animal Ethical Committee (IAEC approval No. KCC/IAEC/014/2020)

Competing interests

The authors declare no competing interests

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Table 1: Absorption kinetics of total LOPs and Lutein in plasma

Kinetic Parameters	TOTAL LOPS (Plasma response)	LUTEIN (Plasma response)
T_{max} (h)	2	2
$T_{1/2}$ (h)	11.353±0.5	5.586±0.009
C_{max} (pg/ml)	152.899±9.1****	122.149±0.8
AUC (pg/h/ml)	1139.418±13.7*	979.505±21.8
AUMC (pgh^2/ml)	17750.69±1261.5****	8597.27±230.09
MRT_{last} (h)	15.573±0.9	8.776±0.03
V_d	2.874±0.09	1.645±0.03
CL	0.175±0.002	0.204±0.004

Data are represented as mean± standard deviation with n=4/group. *P<0.01, ****P<0.0001. Time at maximum concentration (T_{max}), Maximum concentration (C_{max}), Area under concentration (AUC), an area under moment concentration (AUMC), Mean residence time (MRT), half-life ($T_{1/2}$) volume of distribution (Vd) and clearance rate (CL). Kinetics of total LOPs and Lutein absorption from plasma response was calculated using the software pk solver

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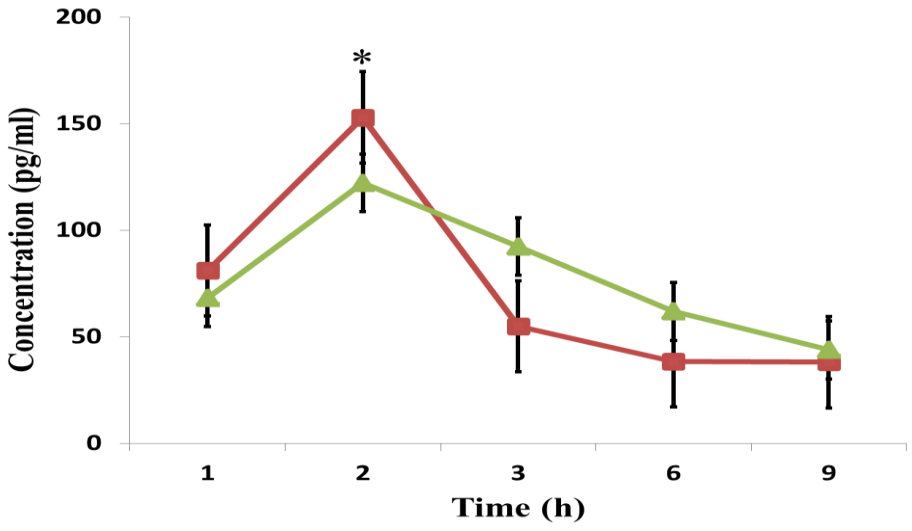
Table 2: Postprandial total LOPs and Lutein levels in liver, intestine and eyes.

Groups	Organs	Concentration in pg/ml at the different time duration				
		1st hour	2nd hour	3rd hour	6th hour	9th hour
total LOPs	Liver	42.19±0.9	46.06±0.1	83.50±1.7	127.83±7.18	71.56±2.05*
	Intestine	ND	ND	ND	ND	60.86±9.3
	Eyes	ND	ND	ND	37.60±0.76	59.80±1.23*
LUTEIN	Liver		-	-	-	64.40±1.17
	Intestine	-	-	-	-	59.52±1.63
	Eyes	-	-	-	-	38.09±1.06

Data are represented as mean± standard deviation with n=4/group. *P<0.01. Liver (whole), intestine (mucosal layer from duodenum to jejunum) and eye (pooled) samples were homogenised separately using an ice-cold isotonic saline solution in hand homogeniser. Tissue homogenates were saponified with 2ml of 10M KOH at 60ºC for 45 minutes. The analysis of the chromatographic data was carried out on Open lab CDS ChemStation software (A.01.05). The fractions were eluted in an isocratic fashion at the rate of 1ml/min using Acetonitrile: Dichloromethane: Methanol in the ratio 9:0.5:0.5.

Fig. 1 Postprandial plasma response in total LOPs and Lutein



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Data are represented as mean± standard deviation with n=4/group. $^{*}P<0.01$.

0.8ml of plasma was made up to 1ml by adding saline. 2ml of dichloromethane and 1 ml of methanol were added and vortexed thoroughly. To this mixture, 1.5ml of hexane was added and centrifuged at 1000g for 5 minutes. Centrifugation resulted in the formation of three layers, in which total LOPs/Lutein was found in the upper dichloromethane/ hexane layer which was collected into a separate tube.