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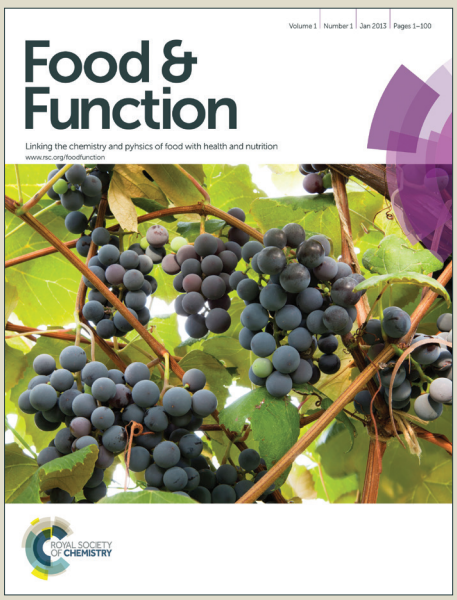

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Lutein derived fragments exhibit higher antioxidant and anti-inflammatory properties than lutein in lipopolysaccharides induced inflammation in rats.

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Running title: Anti-inflammation properties of oxidative derivatives of lutein

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Abbreviations:

AcN - Acetonitrile; CAT - Catalase; COX-2 - Cyclooxygenases-2; DCM - Dichloromethane; GSH - Glutathione; GPx - Glutathione peroxidase; GR - Glutathione reductase; GST - Glutathione-S-transferase; GNO - Groundnut oil; HPLC - High performance liquid chromatography; IL-6 -Interleukins-6; LC-MS - Liquid chromatography-mass spectroscopy; LPS – Lipopolysaccharides; MDA - Malondialdehyde; MeOH - Methanol; NO - Nitric oxide; PGE_2 - Prostaglandin E_2; SOD - Superoxide dismutase; TNF-α - Tumor necrosis factor-α; UV - Ultra violet.

Abstract

In the present study, we appraised the anti- inflammatory efficacy of the lutein oxidative degradation derivatives mediated through UV- irradiation over lutein in counteracting the inflammation induced by lipopolysaccharide (LPS) in rats (n=5/group). UV- irradiated lutein fragments were identified as anhydrolutein (B, $C_{40}H_{54}0$), 2,6,6-trimethylcyclohexa-1,4-dienylium (M1, C_9H_{13}), (2E,4E,6E,8E)-9-(4-hydroxy-2,6,6-trimethylcyclohex-1-1en-1-yl)-3,7-dimethylnona-2,4,6,8-tetraen-1-ylium (M2, C20H29O), 4-[(1E,3E,5E,7E)-3,7,-dimethyldeca-1,3,5,7-tetraen-1-yl]-3,5,5-methylcyclohex-3-en-1-ol (M3, $C_{21}H_{30}O$) and zeaxanthin (M4, $C_{40}H_{56}O$) and its isomers as 13′-Z zeaxanthin, 13′-Z lutein, all-trans zeaxanthin, 9-Z lutein. Induction of inflammation by LPS significantly increased the productions of nitrites (3.3 fold in serum and 2.6 fold in liver), prostaglandin E_2 (26 fold in serum), and pro-inflammatory cytokines like tumor necrosis factor-α (6.6 fold in serum), and interleukins-6 (4.8 fold in serum). Oxidative derivatives of lutein especially M1, M2 and M3, ameliorated acute inflammation in rats by inhibiting the production of nitrites, malondialdehyde (MDA), PGE_2, TNF-α, and IL-6 cytokines more efficiently than lutein in rats. The anti-inflammatory mechanism of derivatives might be related to the decrease of inflammatory cytokine and increase of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione S transferase, glutathione reductase), which would result in the reduction of iNOS, COX-2 and MDA and subsequently inflammatory responses.

Keywords: Inflammation, Lipopolysaccharide, Lutein, Oxidative derivatives, UV-irradiation.

Introduction

Lutein is an important xanthophyll present in many leafy vegetables and certain fruits and has been proven to be helpful in combating against several lifestyle complications including cancer, cardiovascular diseases and age related macular degeneration.[1–3] Lutein is oxidatively metabolized by cleavage enzymes (beta-carotene monooxygenase (BCO1)) in the body to produce physiologically active compounds that may exhibit their own antioxidant and anti-inflammatory properties. Reports have clearly shown the presence of fourteen different carotenoids in human plasma including, four oxidative metabolites of lutein.[4] Earlier studies focused on the metabolic fate of lutein and the subsequent metabolites formed.[5,6] Studies have also suggested that biological activities of carotenoids may be intervened by its ability to form metabolites and/or oxidation products.[7–9] Carotenoid oxidation/degradation products may possess either improved or diminished bioactivity than parent lutein molecule.

In vitro chemical oxidation (2,2' azobis dihydrochloride-mediated) of lutein leads to the formation of apocarotenoids which have been reported to inhibit cancer cell proliferation of HeLa cells.[10,11] Studies have also shown that carotenoids oxidative products have higher antioxidant potential when compared to their parent molecules. Catalano et al. [9] synthesized apo-14′-lycopenoic acid which showed potent antioxidant against H_2O_2 and cigarette smoke in human macrophages. Oxygenated cleavage product of lycopene, (*E*,*E*,*E*)-4-methy-8-oxo-2,4,6-nonatrienal induced apoptosis in HL-60 cells.[12] Diapocarotenoid oxidative product of lycopene stimulates the gap junction communication.[13] However, not much scientific evidence is available on the beneficial properties and oxidation/ degradation products of lutein.

Antioxidant lutein down regulate the production of inflammatory cytokines such as TNF-α, interleukins- 1β, and IL-6, and inflammatory eicosanoid like PGE_2.[14-17] Lutein is reported to inhibit LPS induced systemic inflammation in animals and RAW 264.7

macrophages[14,15] and also attenuate interleukins (IL) -1β. Studies have shown that Nrf2 and NF-κB DNA binding activity in the liver of turkey and guinea pig are significantly modulated by lutein.[16,17] However, the mechanism by which lutein oxidative metabolites modulate inflammatory responses has not been thoroughly investigated. We hypothesize that the lutein and its oxidative derivatives may show different effects than lutein on antioxidant defense mechanism; thus modulate the inflammatory cytokine and eicosanoid production. It is imperative to elucidate the structural characterization of lutein oxidation products before looking into its biological functions. Hence, the objectives of the present study are to characterize ultra-violet (UV) mediated lutein oxidative/photolyzed products, and further evaluate and compare its potential against LPS induced inflammation in rats.

Materials and Methods

Chemicals

Lipopolysaccharide (LPS, *Escherichia coli*-0111-B4, phenol extracted), standard lutein (99 %), bovine albumin (BSA), butylated hydroxyl toluene, glutathione reductase (GR), cytochrome C, xanthine oxidase, β-nicotinamide adenine dinucleotide phosphate monohydrate ($NADPH^+$), dinitro- 5-thiobenzoic acid, thiobarbituric acid, 1-chloro-dinitro-benezene (CDNB), 1,1,3,3-tetramethoxypropane (TMP) and glutathione (reduced and oxidized) were obtained from Sigma - Aldrich (St. Louis, USA). Analytical high-performance liquid chromatography (HPLC) - grade solvents like acetonitrile (AcN), methanol (MeOH), dichloromethane (DCM) and other chemicals mentioned elsewhere in this study were purchased from Sisco Research Laboratories (Mumbai, India). Rat feed pellets were procured from Sai Durga feeds (Bangalore, India). Refined groundnut (GNO) oil (peanut oil) was obtained from a local supermarket.

Preparation of lutein oxidation products/ derivatives from UV- irradiated reaction mixtures

Lutein oxidation products were prepared using liposome model proposed by Kim et al.[18], where phosphatidylcholine (PC, 20 μmol) and lutein (600 nmol) are dispersed in 5 ml of Tris-HCl buffer (pH 7.4). Briefly, lutein in DCM/MeOH was mixed with PC dissolved in chloroform, and the mixture was evaporated to dryness and re-suspended in Tris-HCl buffer with vigorous mixing (2 min) and sonication (5 min at 42kHz/80W). To obtain UV-oxidation products, lutein liposomes in quartz test tubes were exposed to UVC 254 nm TUV T5 lamp (Philips, India) for 48 hrs and then 0.1% BHT (w/v) in ethanol was added to terminate oxidation.

Separation and purification of lutein oxidized products

UV- irradiated lutein oxidized products were separated by preparative HPLC (LC-8A; Shimadzu, Kyoto, Japan) equipped with photodiode array detector (SPD-M20A, Shimadzu) and Varian Pursuit C-18 semi preparative column (250 x 21.2 mm; 10 μm) with a mobile phase containing AcN: DCM: MeOH (9:0.5:0.5; v/v/v) under isocratic condition with a flow rate of 5 ml/ min at 445 nm. The column temperature was maintained at 25 ± 2 ºC and the injection volume was 3 ml. Yellow coloured oxidized lutein rich fraction was further purified by reverse phase HPLC (LC-10 Avp, Shimadzu, Japan) on C-30 (4.6 mm×250 mm; 5μm, Princeton, USA) column with the column temperature maintained at 25 ± 2 ºC and the injection volume was 20 μl. The purification was achieved using a mixture of AcN: DCM: MeOH (6:2:2; v/v/v) containing 0.1% ammonium acetate as a mobile phase at a flow rate of 1 ml/ min. The fractionation and isolation procedures were carried out under dim yellow light to prevent isomerization and degradation by light irradiation. Lutein oxidized products were identified by UV–vis spectrum using an LC-10 Avp HPLC system (similar to analytical HPLC conditions mentioned above using C-30 column) equipped with a photodiode array detector (Shimadzu, Japan) and mass spectrum using an HPLC system (Alliance 2695, Waters, UK) connected to a Q-Tof Ultima mass spectrometer (Waters 2996 modular HPLC

system, UK) equipped with atmospheric pressure chemical ionization ($APCI^+$) module.[19] The APCI source was heated at 130 ºC, and the probe was kept at 500 ºC. The corona (5 kV), HV lens (0.5 kV), and cone (30 V) voltage were optimized. Nitrogen was used as sheath and drying gas at 100 and 300 l/h, respectively. The spectrometer was calibrated in the positive ion mode, and $(M+H)^+$ ion signals were recorded. Mass spectra of lutein and its oxidized products/ metabolites were acquired with a m/z 0-1000 scan range at 445 nm by a diode array detector. Purified lutein oxidation products (≥ 98%) were stored at -80 ºC for further experiments. Further, NMR studies of these compounds were not carried out because of the quantitative limitations, which is the potential limitation in the present investigation. Lutein and UV- oxidized lutein products were used to study the anti-inflammation properties against LPS induced inflammation.

Animals and treatments

The Institutional Animal Ethical Committee approved all the procedures for the use and care of animals. Male rats [OUTB-Wistar, IND-CFT (2c)] were bred at animal house facility of Central Food Technological Research Institute, Mysore, India and housed in steel cages at room temperature (28 ± 2 ºC) with 12-h dark-light cycles. The animals had free access to feed and water *ad libitum*. After 2 weeks of the adaptation period, the rats (200-250 g) were randomly assigned to seven groups (n = 5/group). The rats were gavaged with 200 mg of groundnut oil as a carrier of lutein or its oxidized/photolyzed derivatives. The concentration of lutein or its oxidized derivative administered to rats was 200 µM/day/rat and the control group received only GNO for 15 days. On the fifteenth day, inflammation was induced by intraperitoneal (i.p.) injection of LPS (10 mg/kg BW) in saline and control group received only saline. Rats were anaesthetized 24 h after LPS injection, and blood was collected by cardiac puncture. Serum was isolated by centrifuging the clotted blood at 3000 g for 20 min at 4 ºC. Serum and tissue samples were stored at -80 ºC until use. Liver samples (1

g) were homogenized at 4 ºC in buffered saline and centrifuged at 10000 g for 15 min at 4 ºC to remove nuclei and cell debris. The supernatant was used to assay antioxidant enzymes.

Measurement of nitrites and malondialdehyde levels

Nitrite levels in the serum and liver homogenate were measured by the Griess method.[20] Liver samples were homogenized and deproteinized by adding zinc sulfate, and the supernatant was obtained after centrifugation at 4000 g for 10 min at 4 ºC. Subsequently, 100 µl of serum or liver supernatant were applied into a microtiter plate and mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% napththyl ethylenediamine dihydrochloride and 5% phosphoric acid), incubated at room temperature for 10 min and absorbance was measured at 540 nm with Micro-plate reader (BIO-RAD Model 680). The concentration of nitrites was determined according to the regression equation of the standard curve of sodium nitrite. Malondialdehyde (MDA) levels in serum, liver and retina homogenates were evaluated by the thiobarbituric acid reacting substance (TBARS).[21] Briefly, the reaction mixture contains biological sample (200 µl), 1.5 ml acetic acid (pH 3.5, 20 %, v/v), 1.5 ml of thiobarbituric acid (0.8 %, v/v) and 200 µl of sodium dodecyl sulphate (8 %, v/v). The mixture was incubated for 1 h in a boiling water bath and extracted with 5 ml of n-butanol, and the upper n-butanol phase containing TBARS (pink complex) was read spectro-fluorometrically (Hitachi, F-2000, Japan) with an excitation at 515 nm and emission at 553 nm and quantified as malondialdehyde equivalents using 1,1,3,3-tetramethoxypropane (TMP) as a standard.

Measurement of serum cytokines and PGE_2

Serum levels of TNF-α and IL-6 were determined using a commercially available ELISA kits purchased from Abcam Inc. (Cambridge, UK), whereas PGE_2 EIA kit was procured from Cayman Chemical (Michigan, USA). The concentration of serum TNF-α, IL-6

and PGE_2 levels were presented as pg/ml and established according to the regression equation of the standard curve.

Measurement of antioxidant enzymes activity

Antioxidant enzymes were analyzed in serum and liver homogenates of rats. Total superoxide dismutase (SOD) activity was determined by the inhibition of cytochrome c reduction by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm.[22] One unit of SOD was defined as the amount of enzyme required to inhibit cytochrome c reduction by 50%. Solution A was prepared by mixing xanthine (5 μmol) in 0.001N NaOH and cytochrome C (20 μmol) in 100 ml 50 mM phosphate buffer (containing 0.1 mM disodium EDTA). Solution B containing xanthine oxidase in 0.01 M disodium EDTA was freshly made and kept on ice. For the assay, serum (50 μl) or liver homogenate (50 μl) and solution A (2.9 ml) are taken in a cuvette, and the reaction was started by the addition of solution B (50 μl) and absorbance was read for every 60 seconds for a period of 300 seconds at 550 nm. Total catalase (CAT) activity was measured with reduction of 10 mM hydrogen peroxide in phosphate buffer (pH 7.0) was monitored at 240 nm.[23] In brief, 50 μl of sample containing the enzyme were added to the reaction mixture containing 900 μl phosphate buffer (0.1 mM, pH 7.0), and 50 μl of hydrogen peroxide (8.8 mM) and decrease in absorbance was monitored for 5 min at 240 nm. One CAT unit is defined as the amount of enzyme required to decompose 1 μM of H_2O_2/min. Glutathione peroxidase (GPx) activities were determined in the reaction mixture consisted of 500 μl of 0.1 M phosphate buffer (pH 7.0), 50 μl of sample, 100 μl of glutathione reductase (0.24 U) and 100 μl of glutathione (GSH, 10mM) and pre-incubated for 10 min at 37 °C. Thereafter, 100 μl of NADPH(1.5 mM) and 100 μl of the pre-warmed t-butyl hydroperoxide solution (12 mM) were added, and decrease in absorbance was monitored at 340 nm for 5 min, and the activity was expressed as micromoles of oxidized NADPH/min/mg protein.[24] Glutathione

209 reductase (GR) activity was determined by measuring the decrease in absorbance in a
210 reaction mixture of 750 µl of potassium phosphate buffer (pH 7.2, 0.1 M) and 50 µl of the
211 enzyme source and incubated at 37 °C for 10 min and later 50 µl of 2 mM NADPH (in 1 %
212 $NaHCO_3$), and 50 µl of oxidized glutathione were added and the absorbance at 340 nm was
213 monitored using spectrophotometer.[25] Glutathione-S-transferase (GST) activity in liver
214 homogenates was determined following the formation of a conjugate of GSH and CDNB at
215 340 nm in a reaction mixture containing 50 µl GSH (20 mM), 50 µl CDNB (20 mM), sample
216 (50 µl) and 880 µl phosphate buffer (0.1 mM, pH 6.5) containing 1mM EDTA. The increase
217 in absorbance was monitored for 5 min using spectrophotometer.[26] Glutathione (GSH) level
218 was measured by monitoring the rate of 5-thio-2-nitrobenzoic acid formation at 412 nm.[27] In
219 brief, samples were deproteinized with trichloroacetic acid solution (5%) and mixed with
220 4.75 ml of sodium phosphate buffer (0.1M, pH 8.0) and 50 µl of 10mM dithionitrobenzoic
221 acid (DTNB) in buffer (pH 8.0) and allowed to stand for 5 min, and absorbance was read
222 spectrophotometrically at 412 nm and was quantified from the standard curve of reduced
223 glutathione.

224 **Statistical analysis**

225 The experimental data obtained for different parameters were subjected to
226 homogeneity of variances by the Bartlett test. When homogenous variances were confirmed,
227 the data were tested by analysis of variance and significant differences between the groups
228 were assessed by Tukey's test using SPSS Software (v. 10.0). The percent differences among
229 groups were calculated and considered significant at $p < 0.05$.

230 **Results**

231 **Purification and characterization of UV- irradiated lutein oxidation products**

232 Lutein, when exposed to UV- irradiation, leads to oxidation and slacking of its
233 chemical structure. This may result in the formation of lutein oxidation/ photolyzed products

234 and also other derivatives formed due to lutein cleavage. The tentative structures established
235 using LCMS results of the purified lutein metabolites were designated as A, B, M1, M2 and
236 M3, and scan range was 0-1000 m/z (Table 1 and Fig. 1). Lutein isomers (13′-Z zeaxanthin,
237 13′-Z lutein, all-trans zeaxanthin, 9-Z lutein), which were well separated and identified by
238 HPLC were not seen in MS profile of the same sample, that may be due to similar molecular
239 mass. Protonation of lutein/ zeaxanthin during MS analysis leads to the formation of
240 anhydrolutein (peak B) at 551.5 which corresponds to ($M+H^+-H_2O$) ion, formed due to the
241 loss of H_2O molecule from allylic hydroxyl group of lutein. The plausible pathway for the
242 formation of these oxidative/ photolyzed products from lutein has been demonstrated in Fig.
243 2. The UV- exposure of lutein (A or $M+H^+$) lead to the formation of fragments M1 (m/z
244 121.19, $M+H^+- C_{31}H_{46}O$); M2 (m/z 285.44, $M+H^+/2$); M3 (m/z 298.46, $M+H^+- C_{19}H_{27}O$) and
245 its isomer zeaxanthin/M4 (m/z 551.8, $M+H^+-H_2O$), which were further purified by
246 preparatory HPLC and reverse phase HPLC and used for anti-inflammatory studies. The
247 purity of concentrated oxidative/ photolyzed products (or metabolites) was ascertained by
248 HPLC and LC-MS ($APCI^+$) analysis and found to be ≥ 98 %, which is illustrated in Fig. 3
249 and Fig. 4 respectively.

250 **Nitrites and malondialdehyde levels as modulated by lutein and its metabolites**

251 In this study, we have determined the effect of lutein metabolites on the production of
252 nitrites and MDA in LPS challenged rats. The serum and liver accumulated nitrite ($p <$
253 0.001), as a stable oxidized product of NO when stimulated with LPS and pretreatment with
254 lutein and lutein metabolites significantly ($p < 0.05$) inhibited nitrite production (Fig. 5).
255 Among the experimental groups, significant reduction of nitrites levels were observed in
256 serum and liver of M1 + LPS (55 % and 69 %), M2 + LPS (44 % and 58 %), M3 + LPS (37
257 % and 46 %), M4+ LPS (30 % and 50 %) and lutein + LPS (35 % and 40 %) fed groups,
258 respectively, compared with the LPS group. Treatment of lutein caused a reduction in LPS

induced nitrites production, which was remarkably ($p < 0.05$) less effective than M1 (30 % and 48 %) and M2 (13 % and 28 %) in serum and liver, respectively. On the contrary, there was no significant difference between M3, M4 and lutein in reducing levels of nitrites. Similarly, LPS induced inflammation resulted in elevated levels of MDA in serum, liver and retina compared to control (Fig. 6). However, sharp decline ($p < 0.05$) in the MDA levels in serum, liver and retina was observed in M1+ LPS (56 %, 60 % and 69 %), M2 + LPS (51 %, 57 % and 47 %), M3 + LPS (34 %, 51 % and 38 %), M4 + LPS (34 %, 44 % and 41 %), lutein + LPS (44 %, 34 % and 33 %), respectively, when compared to LPS group. Effectiveness ($p < 0.05$) in reducing MDA levels in serum, liver and retina over lutein was found in M1 (22 %, 43 % and 53 %) and M2 (13 %, 34 % and 20 %), respectively, unlike M3 and M4. These results of nitrites and MDA levels in serum and tissues clearly show that the lutein metabolites, particularly M1and M2 are more potent antioxidant, than lutein and other oxidative derivates (M3 and M4) in inhibiting the production of nitrites and MDA levels under LPS induced inflammation.

Serum cytokines and PGE_2 levels as modulated by lutein and its metabolites

TNF-α, IL-6, PGE_2 are critical mediators of the inflammatory process and organ injury in endotoxemia and sepsis. In Fig. 7A, the TNF-α levels increased significantly ($p < 0.001$) in the serum after LPS injection, which was markedly ($p < 0.05$) reversed by pretreatment of lutein (43 %), M1 (70 %), M2 (66 %), M3 (69 %) and M4 (36 %) when compared to LPS group. However, efficient reduction in comparison with lutein was found in M1 (47 %), M2 (39 %) and M3 (44 %). Likewise, both IL-6 (Fig. 7B) and PGE_2 (Fig. 7C) levels were increased significantly ($p < 0.001$) in the serum after LPS injection, and this effect decreased significantly by the pretreatment with lutein (33 %, 28 %), M1 (60 %, 52 %), M2 (54 %, 49 %), M3 (57 %, 54 %) and M4 (39 %, 17 %), respectively. But, when treatment groups were compared to lutein, oxidation products M1 (41 % and 33 %), M2 (31 % and 29

284 %) and M3 (36 % and 35 %) were more potent in reducing IL-6 and PGE_2. Results clearly
285 showed that lutein oxidative products (M1, M2 and M3) inhibited the production of
286 inflammatory mediators more efficiently than lutein.

287 **Effect on antioxidant enzymes by lutein and its metabolites**

288 The activities of radical scavenger enzymes like SOD, CAT, GPx, GST and GR were
289 investigated after challenging rats with LPS and are illustrated in Table 2. In serum and liver,
290 LPS treatment resulted in decreased ($p < 0.05$) activity of SOD (73 % and 64 %), CAT (67 %
291 and 78 %), GPx (56 % and 59 %), and GR (25 % and 55 %), respectively, when compared to
292 control. Similarly, activity of GST was also lowered significantly in liver (46 %) of the LPS
293 group over the control. In addition to antioxidant enzymes, GSH levels in serum (55 %) and
294 liver (59 %) was found to reduce ($p < 0.05$) in LPS group, when compared to control.
295 However, pretreatment of lutein and lutein metabolites increased ($p < 0.05$) the activity of
296 antioxidant enzymes and GSH levels in both serum and liver. Moreover, lutein metabolite
297 groups (M1, M2 and M3) significantly increased the activities of SOD (35 % to 90 % and 19
298 % to 28 %), CAT (24 % to 82 % and 13 % to 38 %), GPx (45 % to 109 % and 12 % to 29 %),
299 GR (10 % to 25 % and 50 % to 79 %) and GSH (47 % to 59 % and 37 % to 64 %) in serum
300 and liver, respectively, more efficiently than lutein. Similarly, the percentage increase in GST
301 activity in liver of lutein metabolite groups (M1, M2 and M3) ranged from 28 % to 57 % over
302 lutein. In contrast, M4 was less effective than other lutein metabolites and showed no
303 significant difference in comparison with lutein group. These results clearly show that the
304 lutein metabolites (M1, M2 and M3) are more superior to lutein in upregulating the
305 antioxidant enzymes in inflammation conditions caused by LPS.

306 **Discussion**

307 UV irradiation of lutein results in the formation of fragments with different chemical
308 identity. These fragments may exhibit potential of anti-oxidant or anti-inflammatory activity

as they are derived from a physiologically potent lutein molecule. Tissues like retina gets exposed to light and the lutein present in the retina may undergo photo-oxidation to provide lutein derivatives. In the present investigation, the lutein products derived upon UV- exposure were characterized as 2,6,6-trimethylcyclohexa-1,4-dienylium (M1, m/z 121), (2*E*,4*E*,6*E*,8*E*)-9-(4-hydroxy-2,6,6 trimethylcyclohex-1-en-1-yl)-3,7-dimethylnona-2,4,6,8-tetraen-1-ylium (M2, m/z 285), 4-[(1*E*,3*E*,5*E*,7*E*)-3,7-dimethyldeca-1,3,5,7-tetraen-1-yl]-3,5,5-methylcyclohex-3-en-1-ol (M3, m/z 298) and its isomer zeaxanthin (M4, m/z 551.8). These oxidative/ photolyzed derivatives were studied for their antioxidant and anti-inflammatory properties (Table 1 and Fig. 1). Formation of shorter polyene chain cleavage products from lutein possibly because of light exposure through a number of the reaction mechanism are given in Fig. 2. Systematic LC-MS ($APCI^+$) studies have been done, and the fragmentation pattern with molecular ion peak was evaluated and characterized. Samples were handled carefully, taking all precautions to avoid the possibility of further oxidation. Based on the results (Table 1), we speculate that formation of lutein oxidation/ photolyzed products *in vitro* may possibly be due to oxidative degradation of lutein, as in the case of M1 or by central cleavage of the lutein to form M2, which indicates the action of high excitation energies generated by UV- light, which on oxidation forms M3, which is also reported earlier by Nagao[7], where β-carotene cleaves on excitation to retinol and retinoid. Even though the molecular weights of M2 and M3 resembles that of retinol and 4-oxo retinoic acid, they do not have any spectral similarity.

Increasing evidence suggests that many of the biological actions of lutein may be ascribed to its ability to form metabolites and/ or oxidation products. It has been reported that lutein may be metabolized by eccentric cleavage, catalyzed by carotene-9′,10′-dioxygenase[28], *in vitro*[10,11] and *in vivo*[5,6] resulting in the generation of lutein oxidation products. Sharoni et al.[29] reported that these oxidation products involved in various physiological functions

334 including signal transduction, modulation of various transcriptional factors (Nrf2 and NF-
335 kB), or as photosensitizer in vision. Thus, it is worth evaluating the biological effects of
336 lutein oxidation/ photolyzed products in comparison to parent molecule lutein. The results of
337 the present study showed that UV- oxidative metabolites of lutein (M1, M2, and M3) are
338 more potent than lutein in attenuating the inflammation induced by LPS, thus indicating the
339 superior anti-inflammatory properties, which may be due to the presence of the hydroxyl
340 group at C-3 position and/or higher absorption of these oxidation products *in vivo*. UV-
341 degradation products of β-carotene and lycopene suppressed the immune function of human
342 peripheral blood mononuclear cells.[30] Catalano et al.[9] reported that the apo-14′-lycopenoic
343 acid is more potent than apo-10′-lycopenoic acid and lycopene in reducing oxidative stress
344 caused by H_2O_2 and cigarette smoke in human macrophages. β-carotene metabolite, β-apo-
345 13-carotenone, activated the retinoid receptor (RXRα) by interfering with nuclear receptor
346 signaling.[31] Higher antioxidant and cytotoxic effect of oxidized lutein over lutein were
347 reported in HeLa cells.[10,11] These results strongly suggest that oxidation products have
348 potential biological effects compared to intact carotenoids.

349 Results of the present study demonstrated that UV- oxidative metabolites of lutein are
350 more potent than lutein in inhibiting the levels of nitrites, PGE_2, TNF-α, IL-6 in LPS
351 challenged rats, which is in agreement with Shanmugasundaram and Selvaraj[16] and Kim et
352 al.[17], who demonstrated that lutein exerts both antioxidant and anti-inflammatory effects by
353 attenuating NF-κB DNA binding activity. Nitric oxide is an important inflammatory mediator
354 produced by iNOS and plays an important role in the inflammatory response.[32,33] Results of
355 the present study confirmed that LPS injection resulted in overproduction of nitrites, and
356 levels of nitrites in the serum and tissues were reduced significantly by the pre-treatment of
357 lutein oxidative metabolites. Further, results showed that oxidative metabolites more

efficiently suppressed PGE_2 levels in the LPS stimulated rats than parent molecule lutein. Kim et al.[17] demonstrated that the lutein down regulates the inducible COX-2 expression, which is responsible for PGE_2 production in arachidonic acid metabolism. Chang et al.[34] reported that the induction of COX-2 activity and subsequent generation of PGE_2 are closely related to NO (nitrites) production. The COX-2, as well as the iNOS gene, possess binding sites for several transcriptional factors, including nuclear factor-κB (NF-κB) and one possible mechanism of the inhibition of COX-2 expression could be through the suppression of NF-κB showed by fucoxanthin against LPS induced inflammation.[35] In our experiment, we observed that lutein and lutein oxidative metabolites, suppresses nitrites and PGE_2 production in the rats, possibly through the inhibition of the iNOS and COX-2 enzymes respectively.

It has been reported that activation of TNF receptor 1 (TNF-R1) triggered signal transduction and lead to the activation of NF-κB and JNK/AP-1, which in turn activates many pro-inflammatory and immunomodulatory genes including cytokines (IL-6, IL-8, TNF-α etc.), endothelial cell adhesion molecules, and surface adhesion ligands on neutrophils and monocytes.[36] Results from the present study indicated that lutein oxidative metabolites may reduce the TNF-α concentration more competently than lutein. Lutein/oxidative metabolites of lutein influence the monocytes and macrophages that produce TNF-α, thereby revealing its anti-inflammatory action. The results of TNF-α inhibition by lutein or lutein metabolites correlate with the decreased nitrites production in treated groups. However, the mechanism of nitrites induced suppression of TNF-α synthesis is not known. A potential link could be PGE_2 as it has been reported that NO activates COX enzymes and thereby leads to a marked increase in PGE_2 production.[37] Lutein/ lutein oxidative metabolites suppressed the levels of LPS induced PGE_2 and TNF-α, further supported the argument for a regulatory role of NO (nitrites) on TNF-α production. IL- 6 is a well known pro-inflammatory cytokine and

382 regarded as an endogenous mediator of inflammation.[15] Similar, results were seen in the case
383 of IL-6 that was reduced by lutein oxidative metabolites more profoundly than lutein.

384 It has been shown that several natural antioxidant compounds directly inhibit the
385 expression of cytokines, PGE_2, iNOS and COX-2 and thus reduced the inflammation. The
386 LPS induced inflammatory response has been linked to the production of neutrophils derived
387 free radicals and release of other neutrophils derived mediators.[15]. He et al.[38] demonstrated
388 that LPS increases the levels of oxygen derived species, which might attack plasma
389 membrane, resulting in the accumulation of MDA. Present study also exhibited similar results
390 and further revealed that oxidative metabolites of lutein considerably reduced the
391 accumulation of MDA in serum, retina and liver after LPS administration. In addition, GSH
392 levels were significantly up-regulated by lutein or oxidative metabolites of lutein. In the
393 present study, activities of CAT, SOD, GPx, and GR in serum and liver were found to be
394 lower in the LPS group, which subsequently increased towards normalcy in lutein or
395 oxidative metabolites of lutein treated groups (Table 2). Thereby, it is assumed that the
396 suppression of MDA levels in serum, retina and liver is probably due to the increased
397 activities of CAT, SOD, GPx, and GR. These results were in agreement with He et al.[38], who
398 reported that mRNA expression, as well as activities of SOD and GPx, increased after lutein
399 treatment in LPS challenged mice. Further, results also revealed that oxidative/ photolyzed
400 metabolites of lutein upregulated the antioxidant enzymes more profoundly than lutein, which
401 may be due to stronger oxygen quenching capability, considering their molecular structure.

402 In summary, lutein oxidative/ photolyzed metabolites were found to have higher anti-
403 inflammatory effect than the parent molecule lutein. A possible mechanism for the anti-
404 inflammatory effect of oxidative metabolites is the suppression of production of nitrites,
405 MDA, PGE_2, IL-6 and TNF-α by direct blocking NOS enzyme activity and upregulation of
406 antioxidant defense enzymes. These results suggest that along with lutein, oxidative

metabolites of lutein also possess potential anti-inflammatory activity and might have a beneficial effect on the treatment for inflammatory diseases.

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Figure Legends:

Fig. 1: A typical HPLC and LC-MS profile of standard lutein (A, B) and lutein oxidation products resulted after UV- exposure to lutein liposomes for 48 h (C, D).
Refer to Table 1 for possible structures and respective molecular mass. HPLC and LC-MS conditions adopted were outlined under materials and method section.

Fig. 2: Proposed pathway of lutein fragmentation *in vitro* by UV- irradiation.
Refer to Table 1 for possible structures and respective molecular mass.

Fig. 3: HPLC profile of purified lutein oxidation products/ metabolites after purification by preparatory and reverse phase HPLC.
HPLC conditions adopted were outlined under materials and method section.

Fig. 4: Typical LC-MS profile of four different lutein oxidation products/ metabolites after purification by preparatory and reverse phase HPLC.
HPLC and LC-MS conditions adopted were outlined under materials and method section.

Fig. 5: Effect of lutein or lutein oxidation products/ metabolites (200 μM/day for 15 days) on the nitrites in the serum (A) and liver (B) of rats treated with LPS.
Values are mean ± SD (n = 5). Groups not sharing a similar letter are significantly different ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's test.

Fig. 6: Effect of lutein or lutein oxidation products/ metabolites (200 μM/day for 15 days) on the malondialdehyde concentration in the serum (A), liver (B) and retina (C) of rats treated with LPS.

512 Values are mean ± SD (n = 5). Groups not sharing a similar letter are significantly different
513 ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's test.
514
515 Fig. 7: Effect of lutein or lutein oxidation products/ metabolites (200 μM/day for 15 days) on
516 the serum TNF-α (A), IL-6 (B) and PGE_2 (C) levels in rats treated with LPS.
517 Values are mean ± SD (n = 5). Groups not sharing a similar letter are significantly different
518 ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's test.
519

520 Table 1: Fragmented ions derived after the exposure of lutein with UV- irradiation for 48 h,
521 elucidated by LC-MS, APCI$^+$ ion mode showing their molecular structure, molecular mass
522 and molecular formula.

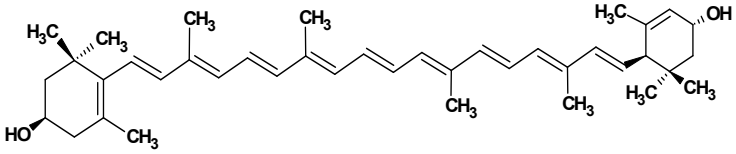
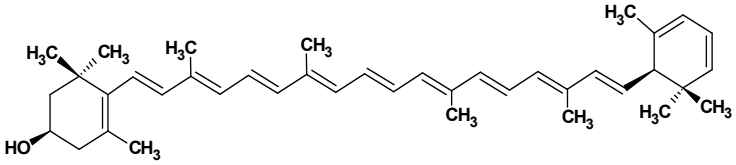
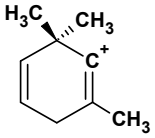
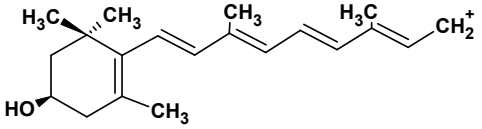
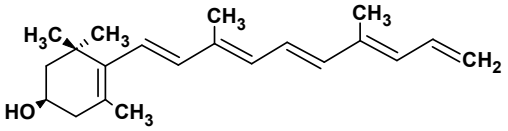
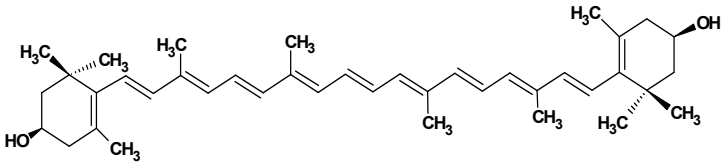
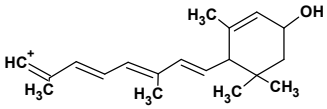
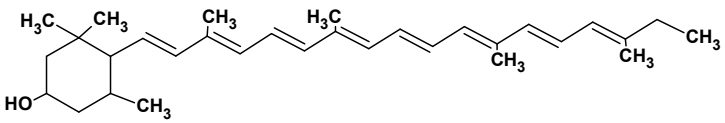
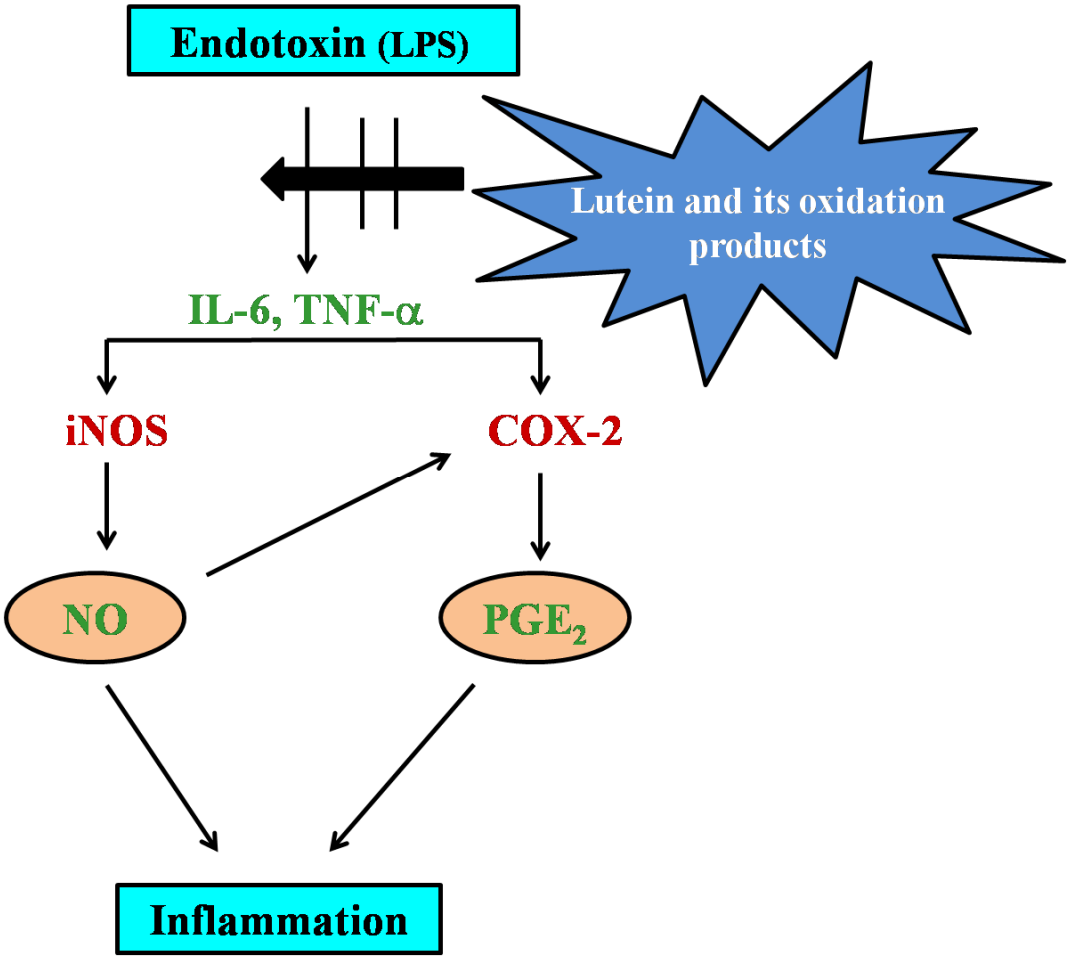
Fragments	Structure of the compound	Approx. Molecular mass	Molecular Formula
A	 Lutein	568.8	$C_{40}H_{56}O_2$
B	 Anhydrolutein	551. 5	$C_{40}H_{54}O$
M1	 2,6,6-trimethylcyclohexa-1,4-dienylium	121.1	C_9H_{13}
M2	 (2*E*,4*E*,6*E*,8*E*)-9-(4-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl)-3,7-dimethylnona-2,4,6,8-tetraen-1-ylium	285.4	$C_{20}H_{29}O$
M3	 4-[(1*E*,3*E*,5*E*,7*E*)-3,7-dimethyldeca-1,3,5,7-tetraen-1-yl]-3,5,5-methylcyclohex-3-en-1-ol	298.4	$C_{21}H_{30}O$
M4	 Zeaxanthin	551.8	$C_{40}H_{56}O_2$
y		271.5	$C_{19}H_{27}O$
z		434.6	$C_{31}H_{46}O$

Table 2: Effect of feeding lutein or lutein oxidation products (200 μM/d/rat) for 14 days on the antioxidant enzymes in the serum and liver of the rats treated with LPS.

Parameters	Control	LPS	Lutein + LPS	M1 + LPS	M2 + LPS	M3 + LPS	M4 + LPS
Serum							
SOD (U/mg protein)	1.9 ± 0.3^{a}	0.5 ± 0.2^{b}	2.0 ± 0.3^{a}	3.8 ± 0.3^{c}	2.7 ± 0.4^{a}	2.9 ± 0.4^{ac}	2.2 ± 0.5^{a}
CAT (μmol/min/mg protein)	0.9 ± 0.2^{a}	0.3 ± 0.1^{b}	1.7 ± 0.6^{c}	3.1 ± 0.8^{d}	2.1 ± 0.8^{cd}	2.1 ± 0.7^{cd}	1.5 ± 0.4^{c}
GPx (mmol/min/mg prot)	1.6 ± 0.1^{a}	0.7 ± 0.1^{b}	1.1 ± 0.3^{a}	2.3 ± 0.3^{ac}	2.2 ± 0.2^{ac}	1.6 ± 0.2^{a}	1.0 ± 0.2^{a}
GR (μmol/min/mg protein)	40.5 ± 3.5^{a}	30.3 ± 4^{b}	60.3 ± 1.9^{c}	75.4 ± 2.8^{d}	$64.8 \pm .4^{c}$	78.4 ± 1.4^{d}	61.3 ± 9.8^{c}
GSH (μg/ml)	7.8 ± 1.7^{a}	3.5 ± 0.2^{b}	10.8 ± 3.3^{a}	17.2 ± 2.3^{c}	15.9 ± 1.6^{c}	14.8 ± 2.0^{c}	11.5 ± 3.4^{a}
Liver							
SOD (U/mg protein)	3.7 ± 0.5^{a}	1.3 ± 0.6^{b}	5.7 ± 0.8^{ac}	6.8 ± 0.5^{ac}	7.1 ± 0.6^{c}	7.3 ± 0.6^{c}	5.4 ± 1.6^{ac}
CAT (μmol/min/mg protein)	5.5 ± 0.8^{a}	1.4 ± 0.3^{b}	6.8 ± 1.5^{a}	8.7 ± 0.9^{c}	9.4 ± 0.9^{c}	7.7 ± 0.7^{ac}	6.4 ± 0.8^{a}
GPx (mmol/min/mg protein)	11.6 ± 3.0^{a}	5.1 ± 1.6^{b}	16.2 ± 2.7^{ac}	20.9 ± 3.5^{c}	18.1 ± 3.2^{c}	18.9 ± 3.6^{c}	17.2 ± 1.4^{ac}
GR (mmol/min/mg protein)	1.8 ± 0.2^{a}	0.8 ± 0.3^{b}	1.4 ± 0.6^{a}	2.1 ± 0.6^{a}	2.5 ± 0.3^{a}	2.4 ± 0.5^{a}	1.3 ± 0.6^{a}
GST (μmol/min/mg protein)	68.9 ± 3.5^{a}	36.6 ± 2^{b}	56.3 ± 5.4^{a}	72.3 ± 3.1^{a}	88.3 ± 6.7^{c}	80.8 ± 2.7^{c}	$61.2 \pm 7a.5^{a}$
GSH (μg/g)	23.5 ± 1.4^{a}	9.6 ± 0.9^{b}	22.1 ± 3.1^{a}	36.3 ± 4.3^{c}	34.8 ± 2.8^{c}	30.4 ± 5.1^{c}	26.4 ± 2.8^{a}

549 Values are mean ± SD (n = 5). Values not sharing a similar superscript within the same row in a group are significantly different ($p < 0.05$) as
550 determined by one-way ANOVA followed by Tukey's test. CAT, catalase; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione
551 reductase; GST, glutathione-S-transferase; LPS, lipopolysaccharides; SOD, superoxide dismutase.

552 Graphical Abstract



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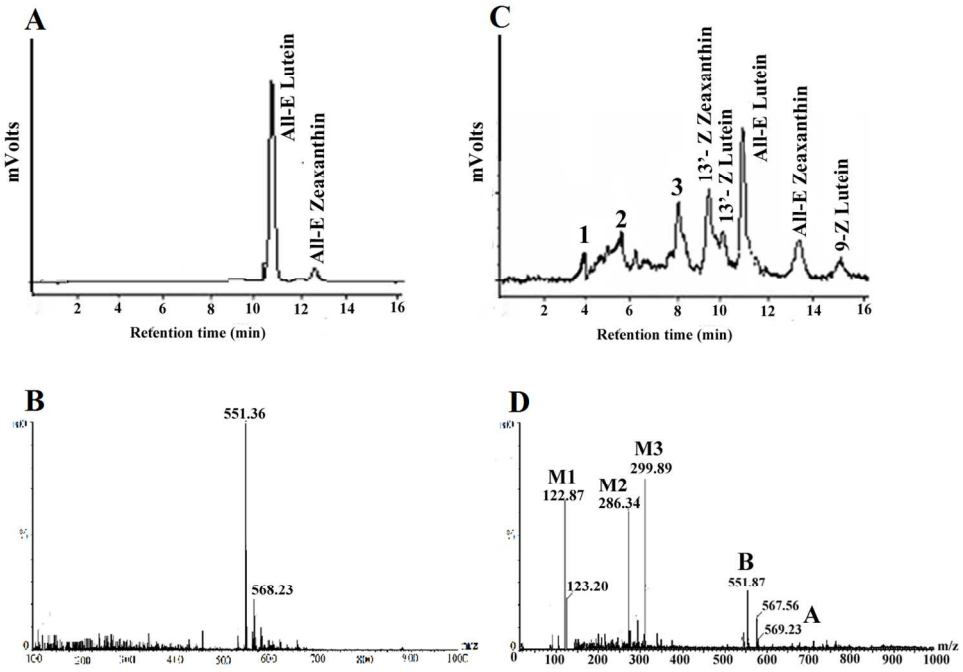

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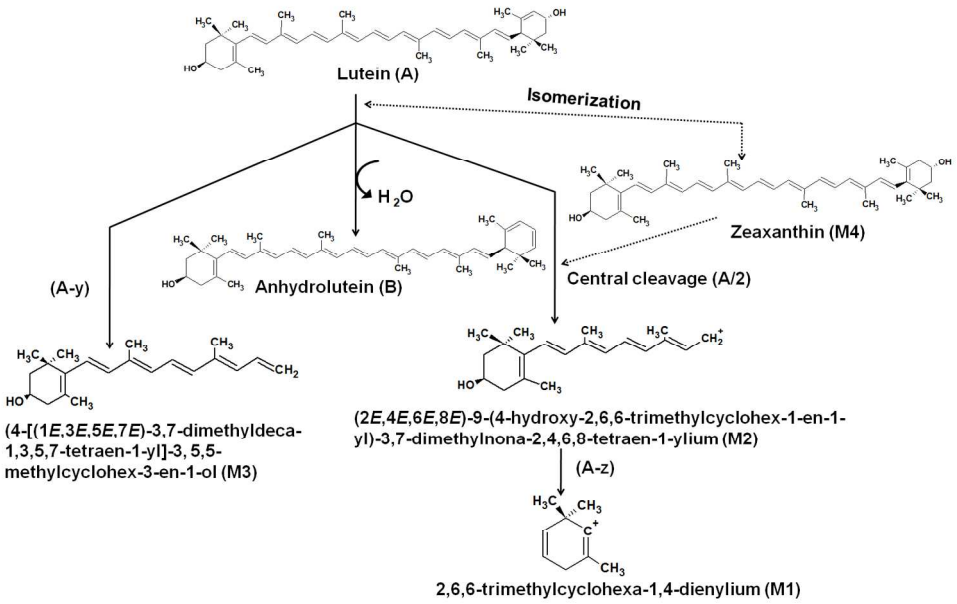

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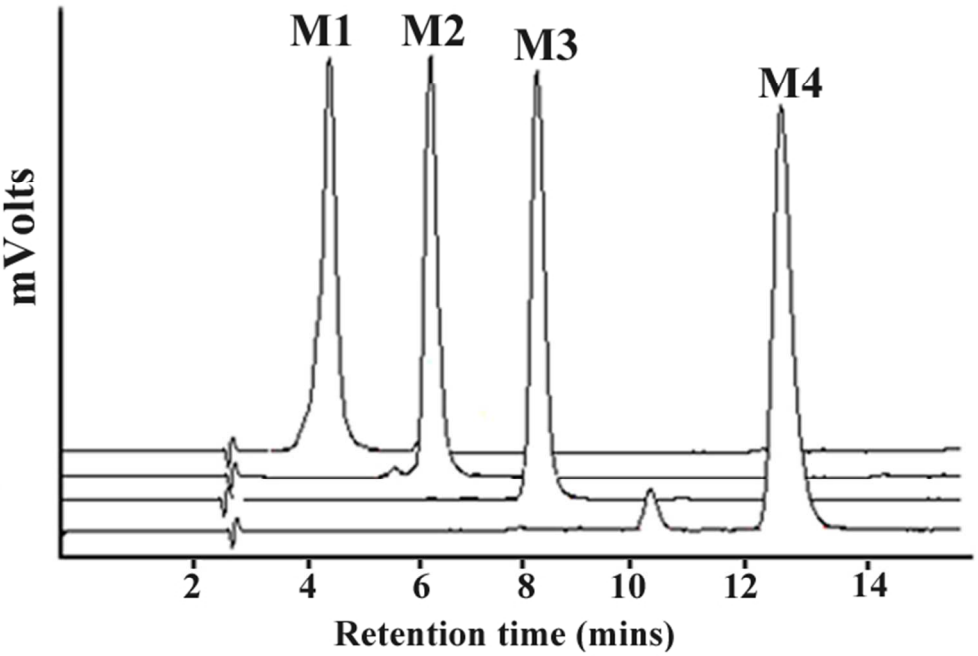

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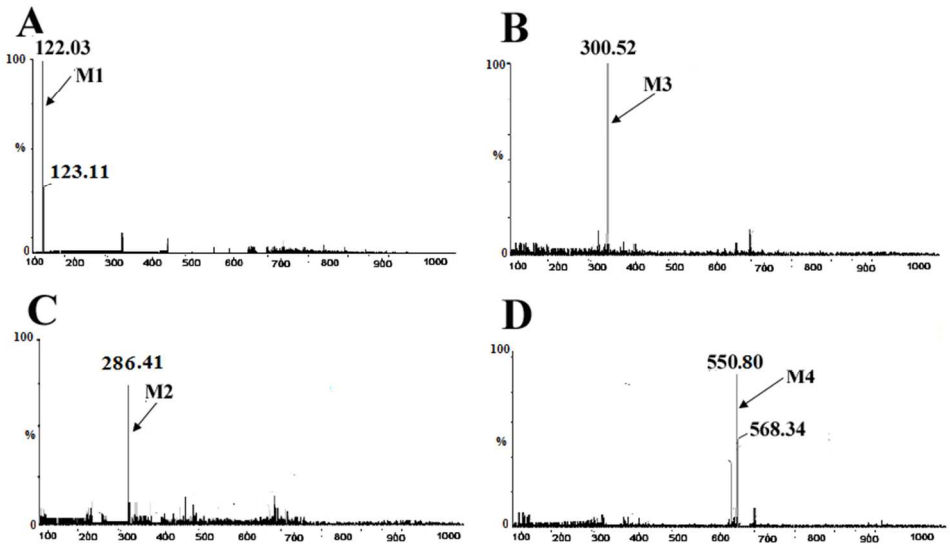

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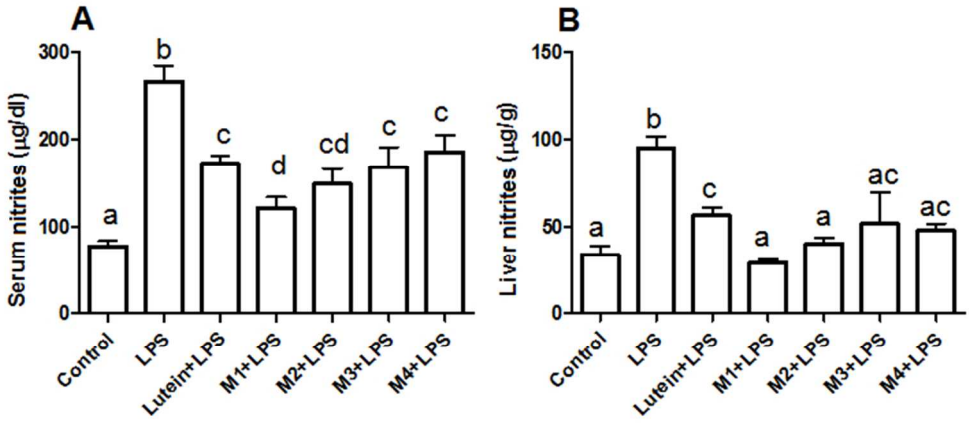

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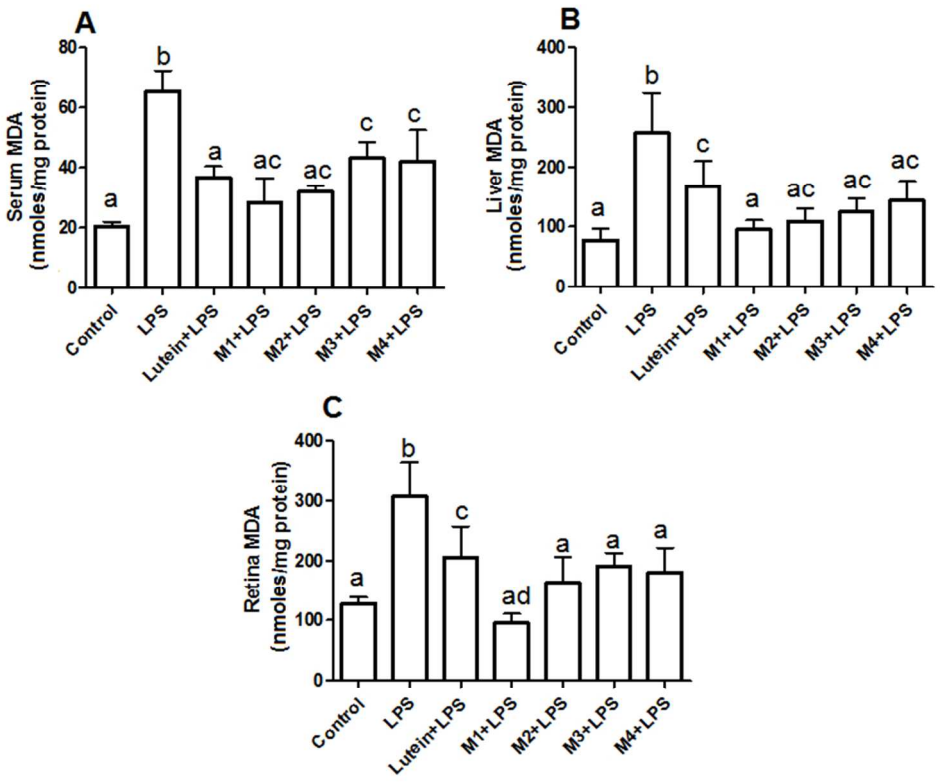

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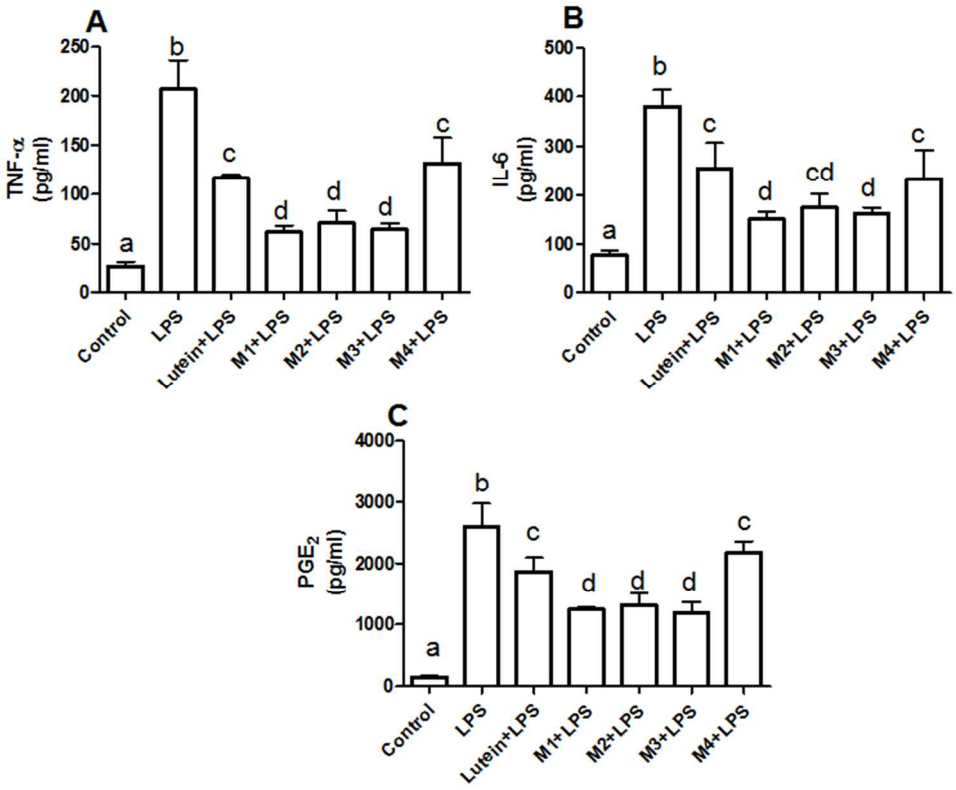

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