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ORIGINAL CONTRIBUTION

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Fucoxanthin restrains oxidative stress induced by retinol deficiency through modulation of Na^+Ka^+-ATPase and antioxidant enzyme activities in rats

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Abstract *Background* Retinol deficiency is a major public health problem world wide, affecting children and women, in particular. It causes a variety of disorders in the body affecting various cellular functions. *Aim of the study* To study the effect of fucoxanthin (FUCO), a non-provitamin-A carotenoid in comparison with retinol (ROH) on changes in antioxidant molecules, lipid peroxidation and membrane bound enzymes in tissue and microsomes, induced by ROH deficiency in rats. *Methods* After induction of ROH deficiency by feeding a diet devoid of ROH for 8 weeks, rats were divided into two groups (n = 20/group) and administered orally a dose of either FUCO (0.83 μmol) or ROH (0.87 μmol). A group of ROH deficient rats (n = 5) and rats (n = 5) fed with ROH sufficient diet was considered as baseline and control groups respectively. Over a period of 8 h, activity of catalase (CAT), glutathione transferase (GST), level of lipid peroxidation (LPx), fatty acids in plasma, liver and liver microsomes and activity of Na^+K^+-ATPase in liver microsomes were evaluated. *Results* ROH restriction increased LPx ($P < 0.05$) in liver (~19%) and plasma (~34%) while the activities of CAT (90 ± 1%) and GST (17 ± 4%) decreased compared to control. Significant elevation (91%) was observed for Na^+K^+-ATPase activity in liver microsomes of ROH deficient when compared to control group and levels were lowered on administration of ROH (37–69%) and FUCO (51–57%), towards control over a period of 8 h. ROH and FUCO suppressed ($P < 0.05$) the LPx level (%) in plasma (34–62, 7–85), liver homogenate (9–71, 24–72) and liver microsomes (83–92, 61–87), while the activities of CAT in plasma (89–97%, 91–95%) and liver microsomes (84–93%, 85–93%) and GST in liver homogenate (43–53%, 44–51%) and liver microsomes (36–52%, 22–51%) were increased ($P < 0.05$) compared to ROH deficient group. *Conclusions* Results show that FUCO, a non-provitamin-A carotenoid protects cell membrane by modulating Na^+K^+-ATPase (51–57% lowering) and the activities of CAT and GST at the tissue and microsomal level which are affected by ROH deficiency. This may be due to its antioxidant nature. These in turn reduce LPx caused by ROH deficiency.

Key words antioxidant enzyme – fucoxanthin – lipid peroxidation – retinol – retinol deficiency

Introduction

Free radicals are produced continuously in all cells as part of normal cellular function. However, excess free radical production originating from endogenous sources due to vitamin A deficiency might play a role in many diseases. Use of antioxidant vitamins and phytochemicals are reported to avert free radicals induced tissue damage by scavenging or decomposing them. In-vitro and in vivo radical scavenging properties of retinol (ROH) have been well established [9, 25]. ROH is known to act effectively as an antioxidant and radical scavenger [27]. Barber et al. [6] reported oxidative damage to liver mitochondria in rats due to vitamin A deficiency of tissue membrane and related the peroxidation-induced damage to the membrane and its function.

Growing evidences advocate that retinoids and carotenoids protect cells from oxidative stress caused either by pro-oxidants or nutrient deficiency [6]. Pro-oxidative nature of ROH deficiency in rat's heart tissue has been well documented [24]. Increased lipid peroxidation (LPx) due to vitamin A deficiency is reported to be indicative of serious damage to cell membrane structure-function, in turn contributing to pathological abnormalities in tissues as well [6, 24]. Gatica et al. [11] reported vitamin A deficiency induced pro-oxidant environment and inflammation in rat aorta. ROH is reported to activate membrane bound ATPase and antioxidant enzymes and participate in the regulation of Na^+, K^+, Ca^+ and Mg^+ ions across the membrane [18]. Kaul and Krishnakantha [18] studied the effect of ROH deficiency on membrane function in rats and reported an elevated activity of microsomal enzyme Na^+K^+-ATPase and altered membrane fluidity in tissue. ROH deficiency related changes at cellular level could be modulated by feeding either ROH itself or antioxidant molecules like carotenoids.

Carotenoids, in general, afford various physiological effects. Nara et al. [19] has studied the antioxidant property of canthaxanthin, β-carotene and 8′-apo-β-carotenal under in vitro conditions. They have also studied the antioxidant effect of β-carotene against pro-oxidant induced LPx. Fucoxanthin (FUCO) which is the major non-provitamin A carotenoid found in brown seaweed, reported to show a strong antioxidant property against certain types of cancer in vitro [19]. Previously, Sugawara et al. [31] and Asai et al. [5] have reported that FUCO hydrolyzed to fucoxanthinol (FUOH) and amarouciaxanthin (AAx) in mice and the metabolites exhibit higher antioxidant property than FUCO in vitro [28]. FUCO along with its metabolites has been shown to establish anti-obesity and anti-carcinogenic properties [28]. Although modulation of LPx by FUCO has been established by in vitro [8], in vivo experimental evidence on the role of FUCO in altering LPx arising from oxidative stress due to vitamin A deficiency is not investigated in detail. Hence, the present study aimed at evaluating the effect of FUCO on oxidative stress indicators (catalase, glutathione transferase, Na^+K^+-ATPase) and its possible role in suppressing LPx resulting from ROH deficiency. The effect of FUCO was compared with ROH in rats.

Materials and methods

Materials

Chemicals

All-trans ROH (95%), retinyl palmitate, DL-α-tocopherol, fatty acid standards, thiobarbituric acid (TBA), adenosine tri-phosphate, di sodium (ATP), ouabain, 1,1,3,3-Tetramethoxypropane (TMP) and boron trifluoride-methanol solution were purchased from Sigma Chem. Co. (USA). HPLC grade acetonitrile, hexane, methanol and dichloromethane were purchased from Sisco Research Laboratories (Mumbai, India). Vitamin A-free casein, vitamins, minerals, cellulose, dextrin and methionine were purchased from Hi-Media (Mumbai, India) and were of high purity food grade. Choline chloride was purchased from Loba Chem. Lab (Mumbai, India). FUCO (>97%) was extracted and purified from brown seaweed *Padina tetrastromatica*. Cornstarch and peanut oil were obtained from local super market. All other chemicals used were of analytical grade.

Animals and diets

Animal experiments were conducted after due clearance from the Institutional animal Ethics Committee. Weanling male albino rats (OUTB-Wistar, IND-CFT 2c) weighing 35 ± 2 g were housed in individual stainless steel cages in the institute animal house facility at room temperature (28 ± 2°C). A 12-h light and dark cycle was maintained and the rats had free access to food and water ad libitum.

Methods

Extraction and purification of FUCO

FUCO was extracted and purified from *P. tetrastromatica* by the procedure previously described

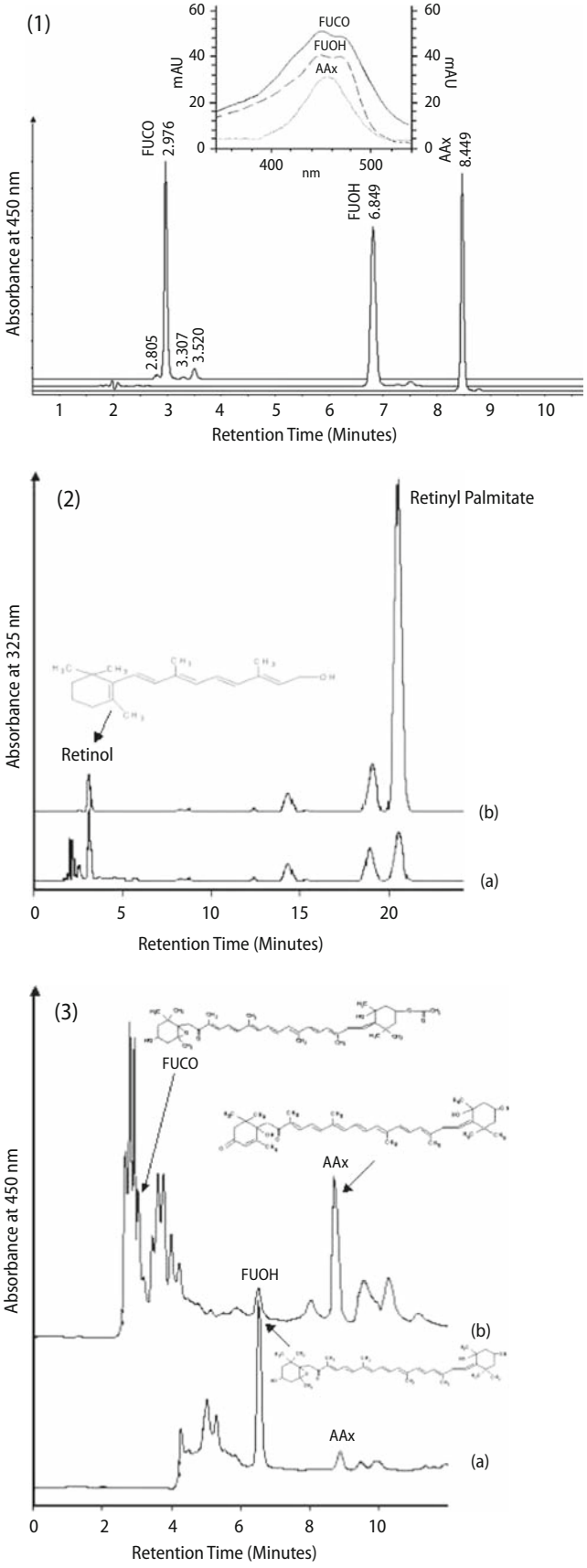

Fig. 1 HPLC profile and UV–visible spectrum of purified FUCO, FUOH and AAx ▶ (1), HPLC profile of retinol (2) and FUCO metabolites (3) from plasma (**a**) and Liver (**b**) of ROH deficient rats intubated with either ROH or FUCO. Extraction and HPLC analytical details are given in methodology section. *FUCO* fucoxanthin, *FUOH* fucoxanthinol, *AAx* amarouciaxanthin, *ROH* retinol

[15]. In brief, FUCO was extracted by homogenisation with cold acetone (4 times). The extracts were pooled and filtered. The filtrate was evaporated to dryness and dissolved in methanol. The extract was partitioned in methanol: water: hexane (10: 1: 10, v/v/v) and washed several times with hexane. The lower methanol/water phase was subjected to extraction by diethyl ether. FUCO was purified by open column chromatography, on a silica (60–120 mesh size) column equilibrated with hexane. Chlorophylls and carotenoids other than FUCO were eluted with hexane (100%) followed by hexane: acetone (9:1, 4:1, v/v). The extract was further purified by preparatory HPLC using a TSK-gel ODS 80Ts column with methanol as mobile phase and the purity was checked by HPLC. The purified FUCO was used for obtaining fucoxanthinol (FUOH) and AAx as described by Asai et al. [5]. The HPLC profile and spectrum of the purified FUCO, FUOH and AAx is shown in Fig. 1.

Induction of ROH deficiency and intubations with FUCO & ROH

ROH deficiency was induced in a group (n = 45) of rats by feeding a semi-synthetic diet devoid of ROH [2, 18] for a period of 8 weeks. For comparison, a separate group of rats (n = 5) was fed on a diet with sufficient ROH and this group was designated as control. The composition of ROH sufficient diet (g/Kg) is as follows: vitamin A free casein (200), methionine (3), cellulose (50), corn starch (325), glucose (324) mineral mix (35), vitamin A free vitamin mix (10), choline chloride (2), ascorbic acid (1) groundnut oil (50) and ROH (400,000 IU) whereas, the composition of the ROH deficient is identical except with no added ROH. The ROH deficiency was confirmed by estimating its level in blood (0.53 μmol/l) drawn from the orbital plexus. Feed intake and gain in body weight of animals were recorded during the experiment. During the course of this study, a group of ROH deficient rats (n = 5) received only peanut oil equivalent to the volume given to treatment groups and these animals were sacrificed to get the base line data. The remaining ROH deficient rats were divided in to two groups (n = 20/group). Each group was further divided in to 4 sub-groups (n = 5/sub-group) to study time course effect of ROH or FUCO. The first group was administered orally a dose of ROH (0.87 μmol) while the second group received FUCO (0.83 μmol), both solubilized in peanut oil (0.5 ml). Rats in each sub-group (n = 5) of ROH or FUCO were sacrificed

using anesthetic at 2, 4, 6 and 8 h after intubations, for collection of blood and tissue.

Preparation of plasma, liver homogenate and liver microsomes

At the end of each pre-determined interval, rats were sacrificed; blood was sampled into heparin-coated tubes. Liver from each animal was separated immediately, rinsed in saline and weighed. All the samples were processed under a dim yellow light on ice (<5°C) to minimize isomerization and photooxidation of FUCO. The plasma was separated from blood by centrifugation (Remi India Ltd., Mumbai) at 1,000×*g* for 15 min at 4°C. Liver samples (1 g) were homogenized at 4°C in buffered saline for ROH assay or in 120 mM KCl and 30 mM phosphate buffer (pH 7.2) for LPx and enzyme assay. The suspension was centrifuged at 10,000×*g* for 10 min at 4°C to remove nuclei and cell debris. The supernatant was used for enzyme assays [26]. Liver microsomes (hereafter referred as microsomes) were prepared according to the procedure of Yehuda and Shamir [33] from a 10% homogenate in 0.25 M sucrose, 0.03 M histidine and 0.001 M EDTA (pH 7.4). Protein in plasma, liver and microsomes was measured by the method of Lowry et al. [20].

HPLC analysis

ROH, retinyl palmitate and FUCO and its metabolites were extracted from the plasma and liver samples as previously described [5, 31] with slight modification. Briefly, plasma (0.8 ml) was diluted with 3 ml of dichloromethane: methanol (1:2; v/v) containing 2 mM α-tocopherol, mixed for 1 min using a vortex mixer, followed by the addition of 1.5 ml hexane. The mixture was centrifuged at 5,000×*g* for 3 min at 4°C. The upper hexane-dichloromethane layer was withdrawn. The extraction procedure was repeated for the lower phase twice using dichloromethane: hexane (1:1.5, v/v). The pooled extract was evaporated to dryness under a stream of nitrogen, re-dissolved in dichloromethane: methanol (2:1; v/v) and used for analysis of ROH, retinyl palmitate and FUOH by HPLC. ROH extraction in 10% liver homogenate (0.8 ml) was by the same procedure as described for plasma.

ROH, retinyl palmitate and FUOH in plasma and liver extracts were quantified with an HPLC system (LC-10Avp; Shimadzu, Kyoto, Japan) equipped with Shimadzu photodiode array detector (SPD-M20A). All the components were separated on a Phenomenex C_{18}-ODS column (250 mm × 4.6 mm; 5 μm) by isocratically eluting with 1 ml/minute of acetonitrile: dichloromethane: methanol (60:20:20; v/v/v) containing 0.1% ammonium acetate as mobile phase. ROH, retinyl palmitate and FUOH were monitored at 325 and 450 nm respectively using Shimadzu Class-VP version 6.14SP1 software. The peak identity of each component was confirmed by their characteristic spectrum. Quantification of individual compounds was evaluated by comparing their peak area with authentic standards.

Analysis of LPx

Lipid peroxidation in plasma, liver homogenate and microsomes was estimated using TBA as described previously [23]. Briefly, 20% acetic acid (1.5 ml), 8% sodium dodecyl sulphate (0.2 ml) and 0.8% TBA (1.5 ml) were added to the sample in that order and the reaction mixture were incubated for 1 h in constantly boiling water (97 ± 2 °C). The reaction mixture was allowed to cool followed by the addition of *n*-butanol (5 ml) and centrifuged at 3,000×*g* for 15 min. The upper butanol phase containing TBA-reactive substances (TBARS) was read spectrofluorometrically (Hitachi, Japan) with an excitation at 515 nm and emission at 553 nm. 1,1,3,3-Tetramethoxypropane (TMP) was used as a standard to estimate the TBARS.

Assay for antioxidant enzymes

Activity of CAT in plasma and liver homogenates was determined by measuring the decrease in absorption at 240 nm in a reaction mixture containing phosphate buffer (PB) (0.1 mM, pH 7.0) and H_2O_2 (8.8 mM) according to the procedure described by Aebi [1]. One CAT unit is defined as the amount of enzyme required to decompose 1 μM of H_2O_2/min. Similarly, GST activity in liver homogenates and microsomes was determined following the formation of conjugate of reduced glutathione (GSH) and 1-chloro-dinitrobenzene (CDNB) at 340 nm in a reaction mixture containing GSH (20 mM), CDNB (20 mM), PB (0.1 mM, pH 6.5) [12].

Na^+K^+-ATPase assay

Na^+K^+-ATPase activity in microsomes was estimated by the method of Kaplay [17]. Briefly, buffer composition used for the assay was as follows—$MgCl_2$ (3 mM), KCl (14 mM), NaCl (140 mM), EDTA (0.2 mM) and Tris–HCl (20 mM, pH 7.4). Samples were simultaneously run in two batches, one containing inhibitor ouabain (2 mM) and the other without. The sample blank containing no assay standard and experimental sample (microsomes) were also run simultaneously. The reaction was stopped by the addition of trichloroacetic acid (10%). Inorganic

phosphate (P_i) liberated was determined in aliquots (0.7 ml) of incubated mixtures by the addition of ascorbic acid-ammonium molybdate solution (0.3 ml) prepared according to the method of Ames [3]. Then, the reaction mixture was mixed well and incubated at 45°C for 20 min. Extinction at 820 nm was measured by UV–visible spectrophotometer (Shimadzu 1601, Kyoto, Japan). Specific activity was expressed as μmol P_i/h/mg protein.

Analysis of fatty acids

Plasma and liver microsomal fatty acids were methylated using boron triflouride (BF_3) in MeOH as described by Morrison and Smith [22] to obtain fatty acid methyl esters (FAME). FAME were analyzed by GC (Shimadzu 14B, Shimadzu, Kyoto, Japan) fitted with flame ionization detector (FID) and a fused silica capillary column (25 m × 0.25 mm; Konik Tech, Barcelona, Spain) to identify individual fatty acids by comparing the retention times with that of authentic standards. The injector, column and detector temperatures were 220, 230 and 240°C respectively with nitrogen as carrier gas at 1 ml/min.

Statistical analysis

The experimental data obtained for different parameters ($n = 5$) were subjected to analysis of variance (ANOVA). In case of significant difference, mean separation was accomplished by Tukey's highest significant difference test using STATISTICA software [30]. The level of statistical significance was set to $P < 0.05$ for all the tests.

Results and discussion

Changes in food intake and body weight during ROH deficiency

Although the food intake was not different between rats fed on diet with or devoid of ROH, the gain in body weight of rats fed on diet devoid of ROH was 25% lower ($P < 0.05$). Deprivation of ROH for 8 weeks did not affect liver weight, while the ROH stores were depleted significantly in liver and plasma as compared to ROH fed (control) group. The ROH deficient rats did not exhibit abnormal morphological and behavioral signs during the experimental run.

HPLC analysis of ROH and FUCO

Typical HPLC chromatograms of ROH, retinyl palmitate, FUOH and AAx (hydrolytic products of FUCO) extracted from plasma and liver of ROH deficient rats after administration of FUCO is presented in Fig. 1. FUCO or its metabolites were not detected in the plasma and liver of ROH deficient group (base line). Both in the case of plasma and liver, FUOH and AAx peaks appeared 2 h after administration of FUCO and were confirmed from their UV–Vis spectra, and mass spectra at m/z 617 $(M + H)^+$, 599 $(M + H\text{-}18)^+$ ions and 615 $(M + H)^+$, 596 $(M + H\text{-}18)^+$, 579 $(M + H\text{-}36)^+$ respectively (6 h sample; MS spectra not shown). Plasma and liver levels of FUCO metabolites (FUOH + AAx) in ROH deficient rats 8 h after administration of FUCO was 0.12 pmol/ml and 14.8 pmol/g, respectively. FUOH + AAx concentration reached maximum at 6 h after administration of FUCO in case of plasma and remained at detectable levels up to 8 h (Fig. 2). FUCO was not detected in plasma in its native, non-metabolized form at any time point during the time-course studies. Sugawara et al. [31] and Asai et al. [5] also identified FUOH as the major metabolic product of FUCO, and AAx in mice.

ROH levels in liver and plasma after ROH deficiency

The ROH and retinyl palmitate levels in plasma, liver and microsomes of ROH deficient rats along with their levels after administration of either ROH or FUCO are shown in Fig. 2b–d. The plasma and liver ROH concentration of ROH deficient group (base line value) was significantly lower (5.3 nmol/ml and 1.0 μmol/g respectively) than that of control (59.3 nmol/ml and 306.4 μmol/g respectively). On administration of ROH to ROH deficient rats, its plasma and liver levels (ROH + retinyl palmitate) reached maxima after 4 (70.5 nmol/ml) and 8 h (11.1 μmol/g) of intubation, respectively (base line corrected). However, no significant difference was noticed in the ROH level in plasma and liver of ROH deficient rats that received FUCO indicating that even under deficient conditions rats fail to accept FUCO as a retinoid precursor.

Modulation of LPx by FUCO and ROH

ROH deficiency is reported to affect tissue membrane lipids through initiation of LPx [18] and hence it was of interest to elucidate LPx status in plasma, liver and microsomes due to ROH deficiency. Results showed that LPx in plasma was significantly higher (81.1 TMP nmol/ml) in ROH deficient group as compared to control group (53.3 TMP nmol/ml) that received ROH. The LPx level in plasma decreased significantly 8 h after administration of either ROH (62.3%) or

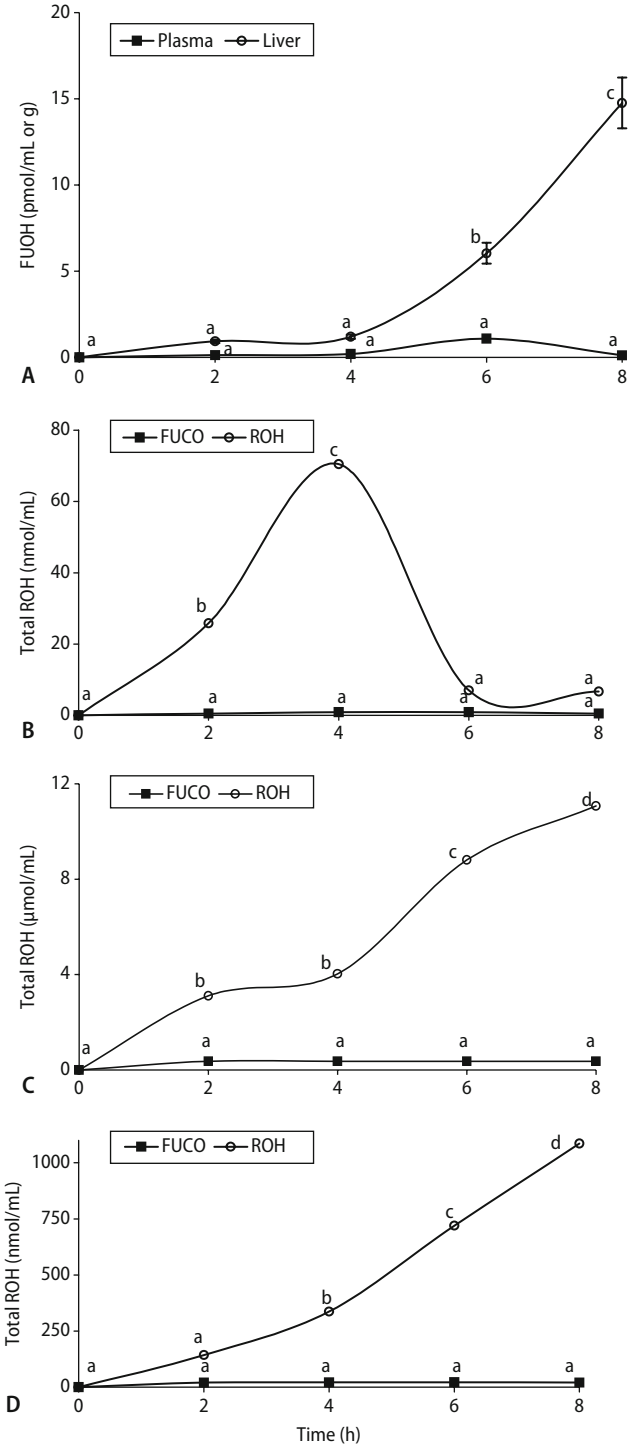

Fig. 2 FUOH (FUOH + AAx) level in the plasma and liver 6 h after intubation (**a**), total retinol levels in plasma (**b**), liver (**c**) and liver microsomes (**d**) of rats after FUCO or ROH administration to ROH deficient rats. Data represent mean ± SD (n = 5/time point). Data represented after correcting base line values. Values not sharing a common letter at each time point are significantly different as determined by one-way ANOVA followed by Tukey's test. Fucoxanthin, *FUOH* fucoxanthinol, *AAx* amarouciaxanthin, *ROH* retinol

FUCO (84.9%) to ROH deficient rats compared to ROH deficient (Fig. 3a). LPx levels in liver tissue and microsomes of control group were 2.08 ± 0.22 µmol/g and 3.09 ± 0.23 µmol/ml, respectively. Corresponding values in treatment groups are presented in Fig. 3. As can be seen in the figure, similar to ROH intubated group, there is significant ($P < 0.05$) decrease in LPx over a period of 8 h after intubation of FUCO to ROH deficient rats in plasma (7–85%), liver (24–72%) and microsomes (61–87%). Due to relatively high concentration of PUFA and close proximity to pro-oxidative factors (like oxygen, metal

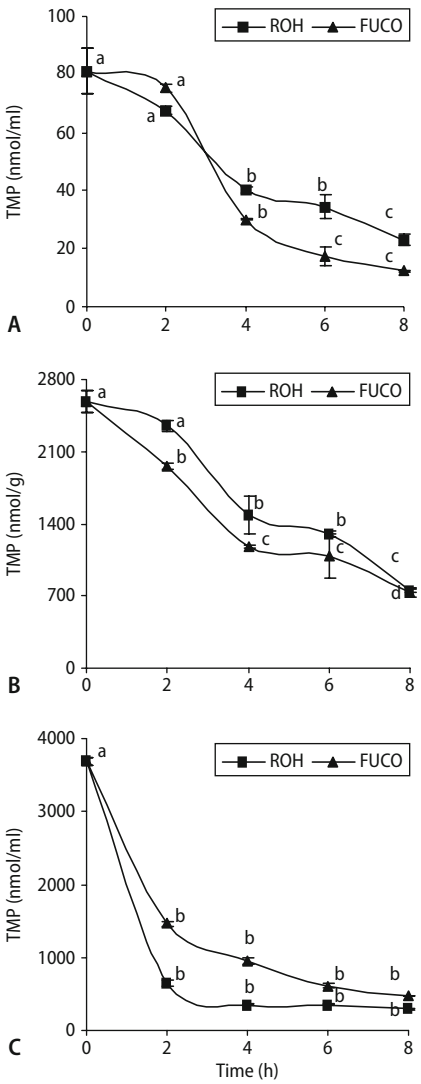

Fig. 3 Lipid peroxidation level in plasma (**a**), liver homogenate (**b**) and liver microsomes (**c**) after intubation with either ROH or FUCO to ROH deficient rats (0 h). Data represent mean ± SD (n = 5/time point). Values not sharing a common letter at each time points are significantly different as determined by one-way ANOVA followed by Tukey's test. *ROH* retinol, *FUCO* Fucoxanthin

ions, peroxidases etc), the cellular and subcellular membranes are highly susceptible to LPx [24]. The present results corroborate well with other works related to ROH deficiency in rats [4, 18], which indicated that ROH deficiency results in elevated levels of LPx in liver (63.87 ± 3.15 pmol MDA/mg protein) as well as its microsomes (2.47 ± 0.09 nmoles MDA/mg/h). It is clear from the results that increased LPx resulting from ROH deficiency was greatly decreased on administration of ROH or FUCO, which can possibly be attributed to their anti-oxidative properties. Further, FUCO is a non-provitamin A carotenoid that can suppress oxidative stress [28] as it gets embedded in bi-layers of lipid membrane due to its poor aqueous solubility [32]. FUCO and its metabolites are known antioxidants found in marine brown algae [31] whose antioxidant properties have been proven through in vitro studies [8, 28]. Although several marine carotenoids and xanthophylls do not possess pro-vitamin A activity, they have been reported to be similar or even greater in antioxidative potency as compared to β-carotene [7, 21, 28]. As FUCO possesses a similar acyclic carbon skeleton ($C_{40}H_{56}$) in its structure like the one in ROH and β-carotene, it is presumed that it may produce free radical scavenging effects in tissues which in turn decrease oxidative stress.

Effect of FUCO and ROH on fatty acid profile

Tables 1 and 2 illustrate the composition of fatty acids (FAs) in plasma and microsomes of ROH deficient group and after administration of either ROH or FUCO. The result shows that ROH deficiency decreased ($P < 0.05$) C18:1, C18:2 and 20:4 levels in

Table 1 Effect of ROH deficiency and subsequent intubations with ROH or FUCO on fatty acid profile of plasma of rats

Fatty acids (% w/w)	ROH deficient	Control	ROH fed group				FUCO fed group			
			2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
12:0	0.60	0.42	1.00	1.50	0.80	0.80	1.25	0.80	0.75	0.90
14:0	0.45	0.27	0.80	0.60	0.85	0.55	0.75	0.50	0.40	0.35
16:0	34.25	22.30	27.10	26.50	30.60	26.25	28.10	23.10	23.20	24.10
18:0	17.45	9.50	11.15	12.90	13.05	11.15	12.05	11.05	10.55	10.95
16:1	2.20	1.95	2.90	2.25	2.55	1.95	2.60	2.10	2.40	2.40
18:1	17.55	31.00	22.00	25.55	23.00	26.85	20.35	23.65	26.50	26.3
18:2	6.20	19.27	11.75	10.55	9.85	12.70	9.00	10.80	12.90	11.30
20:4	7.40	10.95	18.20	18.35	17.15	17.35	22.35	17.90	17.20	16.35
Unidentified	13.90	4.34	5.10	1.80	2.15	2.40	3.55	10.10	6.10	7.35
Total SFA	52.75^{a}	32.49^{b}	40.05^{c}	41.50^{c}	45.30^{c}	38.75^{c}	35.45^{c}	34.90^{c}	42.15^{c}	36.30^{c}
Total MUFA	19.75^{a}	32.95^{b}	24.90^{c}	27.80^{c}	25.55^{c}	28.80^{c}	25.75^{c}	28.90^{c}	22.95^{a}	28.70^{c}
Total PUFA	13.60^{a}	30.22^{b}	29.95^{b}	28.90^{b}	27.00^{b}	30.05^{b}	28.70^{b}	30.10^{b}	31.35^{b}	28.65^{b}

Data represent mean ± SD ($n = 5$/time point). Values not sharing a similar superscript within the row in a group are significantly different ($P < 0.05$) as determined by one-way ANOVA followed by Tukey's test

Table 2 Effect of ROH deficiency and subsequent intubations with ROH or FUCO on fatty acid profile of liver microsomes of rats

Fatty acids (% w/w)	ROH deficient	Control	ROH fed group				FUCO fed group			
			2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
12:0	3.45	1.87	2.40	2.25	1.95	2.50	3.15	2.95	3.00	3.00
13:0	30.1	21.08	23.90	26.20	22.65	25.9	22.45	21.70	23.70	22.00
14:0	4.85	3.85	4.90	3.70	3.95	4.25	5.25	4.30	3.60	3.75
15:0	2.60	1.97	2.20	2.05	2.35	2.1	2.65	2.00	2.20	2.25
16:0	30.8	27.65	27.45	28.00	28.60	25.45	23.85	28.30	22.90	21.65
18:0	10.25	9.50	10.10	9.70	10.05	9.95	8.50	9.25	10.80	8.25
16:1	2.10	4.55	3.55	3.50	2.75	2.75	4.30	2.75	2.80	2.25
18:1	8.20	13.33	11.85	10.90	12.60	12.10	9.65	12.45	12.9	10.20
18:2	2.05	5.30	3.95	4.65	3.10	3.60	2.95	4.50	4.20	3.35
Unidentified	5.60	10.90	9.70	9.95	12.00	11.40	17.25	11.80	13.90	23.30
Total SFA	82.05^{a}	65.92^{b}	70.95^{c}	71.00^{c}	69.55^{c}	70.15^{c}	65.85^{b}	68.50^{c}	66.20^{b}	60.90^{c}
Total MUFA	10.30^{a}	17.88^{b}	15.40^{c}	14.40^{c}	15.35^{c}	14.85^{c}	13.95^{c}	15.20^{c}	15.70^{c}	12.45^{c}
Total PUFA	2.05^{a}	5.30^{b}	3.95^{c}	4.65^{b}	3.10^{c}	3.60^{c}	2.95^{c}	4.50^{c}	4.20^{c}	3.35^{c}

Data represent mean ± SD ($n = 5$/time point). Values not sharing a similar superscript within the row in a group are significantly different ($P < 0.05$) as determined by one-way ANOVA followed by Tukey's test

plasma (49, 68 and 32.4%). Reduction in unsaturates itself may be one of the contributing factors for increase in LPx [16] seen under ROH deficient condition. An increase in plasma levels of C18:1, C18:2 and 20:4 was observed on administration of either ROH (48.6, 70.2 and 5.5%) or FUCO (34.8, 74.2 and 2.9%). The percentage of saturated FAs (except 16:0) also improved with FUCO and ROH intubation (Table 1).

There was a slight elevation (4.8, 20.6, 10.2 and 7.3%) in 12:0, 14:0, 16:0 and 18:0 and a significant decrease(53.9, 38.5 and 61.3%) in 16:1, 18:1 and 18:2 in microsomes of ROH deficient group as compared to control group fed diet with ROH ($P < 0.05$). Intubating with FUCO significantly brought their levels towards control ($P < 0.05$). The decrease in saturates and increase in unsaturates in FUCO administered group can be attributed to the fact that FUCO might inhibit desaturase activity and thereby increase the content of α-linoleic acid, suggesting that the metabolism of FAs is affected [13, 14].

Effect of FUCO and ROH on the activities of antioxidant enzymes

Vitamins and carotenoids are known to scavenge reactive oxygen species and up-regulate the activities of antioxidant enzymes [10]. Results show a progressive increase in CAT and GST activities on feeding ROH or FUCO to ROH deficient rats (Table 3). In case of plasma, the activity of CAT significantly increased by 89.6% 90.5% in ROH and FUCO intubated groups respectively as compared to rats deficient in ROH ($P < 0.05$). Likewise, ROH and FUCO elevated the activity of GST in liver homogenates to maximum of 46% in ROH and 44% in FUCO intubated groups, compared to ROH deficient group (0.797 μmol/min/μg protein). Activity of CAT and GST in plasma, liver and microsomes of ROH and FUCO groups were enhanced when compared to ROH deficient group. At the end of 8 h after gavages, in case of microsomes of ROH and FUCO groups, the CAT activity increased significantly than ROH deficient group and reached the level as seen in control rats (fed diet with ROH). Likewise, GST activity in microsomes rose from 0.37 μmol/min/μg protein (ROH deficient) and 0.43 μmol/min/μg protein (ROH fed control) to 0.49 and 0.55 μmol/min/μg protein in ROH and FUCO fed groups, respectively.

The present results demonstrate that ROH deficiency alters the activities of CAT and GST, possibly resulting in increased LPx. However, administration of ROH or FUCO to ROH deficient rats resulted in decreased LPx levels (Fig. 3a–c) which concur with increased activities of CAT and GST (Table 3). Increased GST activity in both liver and its microsomes of FUCO and ROH groups is indicative of adaptability of the system against oxidative stress due to ROH deficiency [24]. Results of the study demonstrate the possibility of adaptive changes in antioxidant defense system due to ROH and FUCO. This possibly could be a mechanism to suppress LPx caused by ROH deficiency. Suppression of LPx by FUCO was similar in effect as ROH.

Na^+K^+-ATPase activity in microsomes

ATPases are very sensitive to peroxidation reactions and they are intimately associated with the plasma membrane and participate in the energy requiring translocation of Na^+ and K^+ ions [29]. Gavage of either ROH or FUCO significantly decreased the activity

Table 3 Effect of ROH deficiency and subsequent intubations with ROH or FUCO on the activity of antioxidant enzymes in plasma, liver homogenate, liver microsomes and Na^+K^+-ATPase in liver microsomes of rats

Group	Control	ROH deficient	2 h	4 h	6 h	8 h
Plasma catalase (nmol/min/μg protein)						
ROH group	7.46 ± 0.3^a	0.79 ± 0.2^b	17.75 ± 0.5^c	23.77 ± 0.5^d	8.96 ± 0.2^a	7.57 ± 1.4^a
FUCO group	7.46 ± 0.3^a	0.79 ± 0.2^b	8.41 ± 0.2^a	14.53 ± 1.0^c	13.82 ± 1.0^c	8.33 ± 0.5^a
Liver microsomes catalase (nmol/min/μg protein)						
ROH group	5.66 ± 0.3^a	0.53 ± 0.0^b	3.38 ± 0.0^c	5.07 ± 0.2^a	7.20 ± 0.2^d	6.03 ± 0.0^a
FUCO group	5.66 ± 0.3^a	0.53 ± 0.0^b	3.55 ± 0.1^c	5.76 ± 0.1^a	7.23 ± 0.2^d	5.65 ± 0.4^a
Liver total homogenate glutathione transferase (μmol/min/mg protein)						
ROH group	1.01 ± 0.02^a	0.80 ± 0.07^b	1.31 ± 0.05^c	1.23 ± 0.07^d	1.31 ± 0.03^c	1.48 ± 0.03^e
FUCO group	1.01 ± 0.02^a	0.80 ± 0.07^b	1.42 ± 0.03^c	1.42 ± 0.08^c	1.26 ± 0.05^d	1.30 ± 0.02^d
Liver microsomes glutathione transferase (μmol/min/mg protein)						
ROH group	0.43 ± 0.02^a	0.37 ± 0.0^b	0.56 ± 0.2^c	0.47 ± 0.01^a	0.63 ± 0.01^d	0.49 ± 0.02^a
FUCO group	0.43 ± 0.02^a	0.37 ± 0.0^b	0.61 ± 0.01^c	0.38 ± 0.03^b	0.53 ± 0.04^d	0.55 ± 0.03^d
Liver microsomes Na^+K^+-ATPase (μmol of Pi /h/mg protein)						
ROH group	2.27 ± 1.01	24.39 ± 0.2^a	13.69 ± 1.8^b	15.33 ± 2.2^b	7.63 ± 0.03^c	9.36 ± 0.07^c
FUCO group	2.27 ± 1.01	24.39 ± 0.2^a	12.01 ± 1.4^b	11.88 ± 0.5^b	10.59 ± 0.2^b	10.46 ± 0.05^b

Data represent mean ± SD (n = 5/time point). Values within the same row with similar superscript are not significantly different ($P > 0.05$) as determined by one-way ANOVA followed by Tukey's test

of Na^+/K^+- ATPase from the high levels seen in ROH deficient group (Table 3). The decreased ATPase activity in FUCO and ROH groups may be due to increased CAT and GST activity in those groups, thereby affording protection against LPx in the membranes. Higher Na^+/K^+-ATPase activity recorded in ROH deficient group might therefore be due to increased LPx at membrane level. The results indicate that FUCO and ROH tend to restore the ATPase activity towards normalcy by modulating the activity of GST and/or CAT thereby protecting membranes against LPx due to ROH deficiency. Long-term feeding trials may provide more information to support the ameliorating effect of FUCO on biochemical changes caused by ROH deficiency.

In conclusion, ROH deficiency increases LPx in tissues leading to alteration in membrane lipids and structure, which become further susceptible to oxidative stress with changes in antioxidant and membrane bound enzymes. Results demonstrate that administration of ROH and FUCO suppresses the LPx induced by ROH deficiency possibly by modulating CAT, GST and Na^+K^+-ATPase activities. It was found that FUCO is slightly more effective than ROH in reducing LPx in plasma and liver homogenate resulting from ROH deficiency. FUCO being a non-provitamin A carotenoid, it would be very interesting to understand the mechanism by which it helps in protecting the tissue against oxidative stress induced by ROH.

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References

1. Aebi H (1984) Catalase in vitro. In: Packer L (ed) Oxygen radicals in biological systems. Methods Enzymol, Academic Press Inc., Orlando, 105:121–126
2. American Institute of Nutrition (1977) Report of the American Institute of Nutrition Ad. Hoc Committee on standards for nutritional studies. J Nutr 170:1340–1348
3. Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. In: Neufeld EF, Ginsburg V (eds) Complex carbohydrates. Methods Enzymol, Academic Press Inc., New York, 8:115–118
4. Anzulovich AC, Oliveros BL, Munoz E, Martinez LD, Gimenez MS (2000) Nutritional vitamin A deficiency alters antioxidant defenses and modifies the liver histoarchitecture in rat. J Trace Elem Exp Med 13:343–357
5. Asai A, Sugawara T, Ono H, Nagao A (2004) Biotransformation of fucoxanthinol into amarouciaxanthin in mice and HEPG2 cells: formation and cytotoxicity of fucoxanthin metabolites. Drug Metabol Disp 32:205–211
6. Barber T, Borras E, Torres L, Garcia C, Cabezuelo F, Lloret A, Pallardo FV, Vina JR (2000) Vitamin A deficiency causes oxidative damage to liver mitochondria in rats. Free Rad Biol Med 29:1–7
7. Bhosale P, Bernstein PS (2005) Synergistic effects of zeaxanthin and its binding protein in the prevention of lipid membrane oxidation. Biochem Biophys Acta 1740:116–121
8. Chandini SK, Ganesan P, Bhaskar N (2008) In-vitro antioxidant activities of three selected brown seaweeds of India. Food Chem 107:707–713
9. Ciaccio M, Valenza M, Tesoriere L, Bongiorno A, Albiero R, Livrea MA (1993) Vitamin A inhibits doxorubicin-induced membrane lipid peroxidation in rat tissues in vivo. Arch Biochem Biophys 302:103–108
10. Fang YZ, Yan Wu G (2002) Free radicals, antioxidants, and nutrition. Nutrition 18:872–879
11. Gatica L, Alvarez S, Gomez N, Zago MP, Oteiza P, Oliveros L, Gimenez MS (2005) Vitamin A deficiency induces prooxidant environment and inflammation in rat aorta. Free Rad Res 39:621–628
12. Gluthenberg C, Alin P, Mannervik B (1985) Glutathione transferase from rat testis. In: Meister A (ed) Glutamate, glutamine, glutathione and related compounds. Methods Enzymol, Academic Press Inc., Orlando, 113:507–510
13. Grolier P, Cisti A, Danbeze M, Narbonne JF (1991) The influence of dietary vitamin A intake on microsomal membrane fluidity and lipid composition. Nutr Res 11:567–574
14. Hamm MW, Chan V, Wolf G (1987) Liver microsomal membrane fluidity and lipid characteristics in vitamin A deficient rats. Biochem J 245:907–910
15. Haugan JA, Akermann T, Jensen LS (1992) Isolation of fucoxanthin and peridinin. In: Packer L (ed) Carotenoids part A: chemistry, separation, quantitation and antioxidants: Methods in enzymology. Academic Press Inc., Orlando, 213:231–245
16. Kang HW, Bhimidi GR, Odom DP, Brun PJ, Fernandez ML, McGrane MM (2007) Altered lipid catabolism in the vitamin A deficient liver. Mol Cell Endocrinol 271:18–27
17. Kaplay SS (1978) Erythrocyte membrane Na^+ and K^+ activated adenosine triphosphatase in PCM. J Clin Nutr 31:579–584
18. Kaul S, Krishnakantha TP (1997) Influence of retinol deficiency and curcumin/turmeric feeding on tissue microsomal membrane lipid peroxidation and fatty acids in rats. Mol Cell Biochem 175:43–48
19. Kotake-Nara E, Kushiro M, Zhang H, Sugawara T, Miyashita K, Nagao A (2001) Carotenoids affect proliferation of human prostate cancer cells. J Nutr 131:3303–3306
20. Lowry OH, Rosebrough WJ, Farr AL, Randall RS (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265–275
21. Matsuno T (1991) Xanthophylls as precursors of retinoids. Pure Appl Chem 1:81–88
22. Morrison WR, Smith LM (1964) Preparation of fatty acid methyl esters and dimethylacetals from boron fluoride-methanol. J Lipid Res 5:600–608
23. Okhawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358
24. Oliveros L, Vega V, Anzulovich AC, Ramirez D, Giminez M (2000) Vitamin A deficiency modifies antioxidant defenses and essential element contents in rat heart. Nutr Res 20:1139–1150

25. Palacios A, Piergiacomi VA, Catala A (1996) Vitamin A supplementation inhibits chemiluminescence and lipid peroxidation in isolated rat liver microsomes and mitochondria. Mol Cell Biochem 154:77–82
26. Rauchora H, Redvinkora J, Kalous M, Drahota Z (1995) The effect of lipid peroxidation on the activity of various membrane bound ATPase in rat kidney. Int J Biochem Cell Biol 27:251–255
27. Ross AC (1999) Vitamin A. In: Maurice S, James O, Moshe S Catharine Ross A (eds) Modern nutrition in health and disease, 9th edn. Williams & Wilkins, Baltimore, p 305–313
28. Sachindra NM, Sato E, Maeda H, Hosokawa M, Niwano Y, Kohno M, Miyashita K (2007) Radical scavenging and singlet oxygen quenching activity of marine carotenoid fucoxanthin and its metabolites. J Agric Food Chem 55:8516–8522
29. Selvendrian K, Singh PVJ, Krishnan BK, Sakthisekaran D (2003) Cytoprotective effect of piperine against benzo(a)pyrene induced experimental lung cancer in Swiss albino mice with reference to lipid peroxidation and antioxidant enzymes. Fitoterapia 74:198–205
30. Statsoft (1999) Statistics for Windows. Statsoft Inc., USA
31. Sugawara T, Baskaran V, Tsuzuki W, Nagao A (2002) Brown algae fucoxanthin is hydrolyzed to fucoxanthinol during absorption by caco-2 human intestinal cells and mice. J Nutr 132:946–951
32. Sujak A, Gabrielska J, Grudzinski W, Borc R, Mazurek P, Gruszecki WI (1999) Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: the structural aspects. Arch Biochem Biophys 371:301–307
33. Yehuda G, Shamir YK (1971) Effect of urea sodium and calcium on microsomal ATPase activity in different parts of the kidney. Biochem Biophys Acta 233:133–136