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Assessment of the ability of the antioxidant cocktail-derived from fermentation of plants with effective microorganisms (EM-X) to modulate oxidative damage in the kidney and liver of rats in vivo: studies upon the profile of poly- and mono-unsaturated fatty acids

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Abstract

The antioxidant cocktail EM-X derived from ferment of unpolished rice, papaya and sea weeds with effective microorganisms (EM) of lactic acid bacteria, yeast, and photosynthetic bacteria is widely available in South-East Asia. Oral administration of a EM-X to rats for 7 days inhibited the ferric-nitrilotriacetic acid (Fe-NTA)-dependent oxidation of fatty acids with protections directed towards docosahexanoic, arachidonic, docosapentanenoic acids, oleic, linoleic and eicosadieonoic acids in the liver and kidney. But only the protections of oxidation to docosahexanoic, arachidonic acid in the kidney were statistically significant. Treatment of rats with EM-X prior to the intraperitoneal administration of Fe-NTA led to a reduction in the overall levels of conjugated dienes (CD) measured in the kidney by 27% and in the liver by 19% suggesting inhibition of lipid peroxidation in these organs. The levels of glutathione and α-tocopherol were largely unaffected suggesting that the protection by the regular strength of EM-X was confined to the inhibition of lipid peroxidation in vivo, a point dependent on the concentrations of bioactive flavonoids.

Keywords: EM-X; Antioxidants; Iron toxicity; Nitrilotriacetic acid; Flavonoids; Glutathione; Vitamin E; Polyunsaturated fatty acids; Oxidative stress; Cancer

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1. Introduction

Antioxidant actions in vivo or in vitro may involve direct inhibition of the generation of ROS, or directly scavenging of free radicals. Although the in vitro outcome can be used to rule out direct antioxidant activity in vivo, direct reproduction of in vitro effectiveness in vivo are often hard to achieve. From prevention of the oxidative reactions in foods, pharmaceuticals and cosmetics to the role of ROS in chronic diseases and aging, there is great interest in the use of antioxidants in preventive strategies (Aruoma, 1998, 1999; Seddon et al., 1994; Pryor, 2000; Cantuti-Castelvetri et al., 2000). In order to establish bio-efficacy of antioxidants, it is prudent to measure 'markers' of baseline oxidative damage in vivo and examine how they are affected by changes in diet or by antioxidant supplements. We have focused on the development of an in vivo biomarker model of Fenton chemistry using the metal complex ferric-nitrilotriacetic acid (Fe-NTA) and have suggested the profile of polyunsaturated fatty acids including the monounsaturates in the kidney and liver of rats as a potential tool for the assessment of antioxidant actions in vivo (Deiana et al., 2001). The iron complex of the chelating agent nitrilotriacetic acid (NTA) is nephrotoxic. Intraperitoneal injection of ferric nitrilotriacetate (Fe-NTA), induces renal proximal tubular damage associated with oxidative damage that eventually leads to a high incidence of renal cell carcinoma in rodents after repeated administration (Ebina et al., 1986; Li et al., 1987; Ikeda et al., 1998; Okada et al., 1993; Toyokuni et al., 1996).

EM-X is an antioxidant drink derived from ferment of unpolished rice, papaya and sea weeds with effective microorganisms (EM) of lactic acid bacteria, yeast, and photosynthetic bacteria. 'EM-X' is the only name of this antioxidant drink and also its commercial name. EM-X contains over 40 minerals, α-tocopherol, lycopene, ubiquinone, saponin and flavonoids, such as quercetin, quercetin-3-*O*-glucopyranoside and quercetin-3-*O*-rhamnopyranoside, the structures of which are shown in Fig. 1. EM-X is widely available in South-East Asia as a beverage and is accepted in clinical practice. EM-X has been suggested as a prophylactic beverage in the treatment of cancer, hypertention, diabetes, rheumatism, various allergies and chemical substance hypersensitivity, HIV/AIDS, tuberculosis and other infectious diseases (Higa and Ke, 2001). Extensive study using high concentrations of EM-X up to 1800 ml/kg body weight of mice suggest that oral dosing is not toxic and this concentration corresponds to 1500 times the daily dose in the clinic (Zhong et al., 1999). The concentration of EM-X up to 1800 ml/kg body weight means that the daily dose of concentrated EM-X (20-fold) used in the toxicity studies was equated to1800 ml/kg of the original liquid ($0.3 \times 3 \times 100 \times 20$). Ke et al. (2001) reported that EM-X has anti-aging properties and was able to increase serum concentrations of superoxide dismutase and to decrease malondialdehyde levels as well as prolonging the life-span of fruit flies.

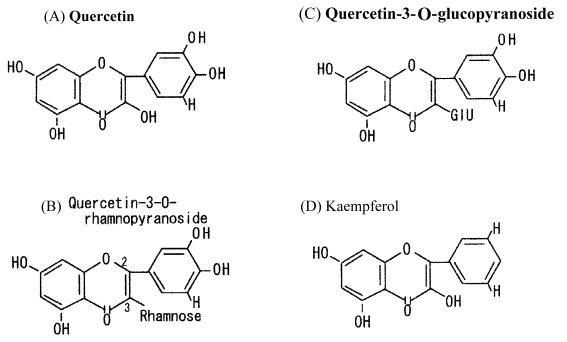

Fig. 1. Some of the flavonoid structures found in EM-X.

In the kidney, Fe-NTA can be filtered through the glomeruli into the lumen of the renal proximal tubule where Fenton chemistry mechanisms mediate oxidative damage. Intraperitoneal administration of sub-lethal dose of Fe-NTA to rats induces a time-dependent reduction in the levels of polyunsaturated fatty acid (PUFA), together with an increase of conjugated dienes (CD) value and a decrease of cellular antioxidants α-tocopherol and glutathione (Deiana et al., 2001). We studied the ability of EM-X administered orally to modulate the oxidative damage caused by the Fe-NTA in the liver and kidney of rats.

2. Experimental procedures

2.1. Chemicals and preparation of Fe-NTA

All solvent used were HPLC grade (Merck, Darmstadt, Germany). Ferric nitrate enneahydrate, NTA disodium salt and fatty acid standards were purchased from Sigma Chemical, St. Louis, MO, USA. Desferal (deferoxamine methanesulfonate) was purchased from CIBA–Geigy, Basel, Switzerland. α-Tocopherol was purchased from Fluka AG, Switzerland. All other reagents and chemicals were of the highest available purity. The Fe-NTA solution was prepared as described in Deiana et al. (2001), briefly, ferric nitrate enneahydrate and NTA disodium salt were dissolved in deionised water to form a 300 and a 600 mM solution, respectively. The two solutions were combined in a volume ratio of 1:2 with magnetic stirring at room temperature and the pH was adjusted to 7.4 with sodium bicarbonate.

2.2. Animals and treatment

Adult male Wistar rats were purchased from Charles River Italy, Calco, Italy. The rats were housed in solid bottom polycarbonate cages with wire tops in a room maintained at 22 ± 2 °C and fed a non-purified diet and tap water ad lib. Five or six animals per groups (body weight 180–200 g) were used for each trial after 1 week of acclimatisation. Rats treated with EM-X ($n = 5$) were given 1 ml/100 g body weight by gavage once daily for 1 week. About 24 h after the last treatment with EM-X or pure water in the controls ($n = 6$), animals were injected IP with either physiological saline or Fe-NTA solution (15 mg Fe per kg body weight) and sacrificed by decapitation after 3 h. Both kidneys and liver from each animal were immediately removed, snap-frozen in liquid nitrogen and stored at −80 °C for subsequent biochemical analyses.

2.3. Lipid extraction, preparation of fatty acids and their measurements

Total lipids were extracted from a portion of kidney or liver by the Folch et al. (1957) procedure and quantitated essentially as described in Chiang et al. (1957) and recovery of unsaturated fatty acids was calculated by using as an external standard a mixture of unsaturated fatty acids standards.

Free fatty acids were measured essentially as described in Deiana et al. (2001) and Banni et al. (1994, 1996). Separation of unsaturated fatty acids was carried out with a Hewlett–Packard 1050 liquid chromatograph equipped with a diode array detector 1040M (Hewlett–Packard, Palo Alto, CA). A C-18 Alltech Adsorbosphere column, 5 μm particle size, 250×4.6 mm, was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, v/v/v) at a flow rate of 1.5 ml/min. Unsaturated fatty acids were detected at 200 nm. Spectra (195–315 nm) of the eluate were obtained at 28 s intervals. Identification of sample fatty acids was carried out, as previously described (Banni et al., 1996), by comparing their retention time and UV spectra to those of reference fatty acids standards. The purity of the peaks was determined using the PHOENIX 3D HP Chemstation software. For the second derivative spectroscopic analysis, 2.5 mg of lipids from each sample were dissolved in cyclohexane and their simple and second derivative UV absorption spectra between 195 and 350 nm were taken using a Hewlett–Packard 8452A diode array spectrophotometer. The height of the two signals with a minimum at around 233 and 242 nm were measured and added together. The concentration of CD in the samples was determined by using a standard reference curve as previously described (Corongiu and Banni, 1994).

2.4. Extraction and measurement of α-tocopherol and glutathione

α-Tocopherol was extracted from the tissue by a worm saponification method (Taylor et al., 1976) and measured as described in Deiana et al. (2001). Reduced glutathione was assayed by the method of Jollow et al. (1974) with slight modification. A portion of kidney or liver was homogenised in metaphosphoric acid (0.1 M); the homogenate was filtered through a fibreglass filter and centrifuged at $10\,500 \times g$ for 15 min at 4 °C. The supernatant (1 ml) obtained was precipitated with 1 ml of

sulfosalicylic acid (4%). The samples were kept at 4 °C for 1 h and then centrifuged at 1200 × *g* for 15 min. The assay mixture contained 0.2 ml of filtered aliquot, 2.6 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (40 mg/10 ml phosphate buffer 0.1 M, pH 7.4) in a total volume of 3 ml. The absorbance of the yellow colour that developed was measured immediately at 412 nm on a Hewlett–Packard 8452A diode array spectrophotometer (Deiana et al., 2001).

2.5. Statistical analyses

Data are presented as mean ± S.D. of values obtained for each group in two independent experiments ($n = 10/12$ for each sample/condition). Statistical significance within sets of data was determined by one-way analysis of variance with the ANOVA program. It uses the Bonferroni method. The threshold for significance is the traditional value ($P < 0.05$) divided by the number of comparison.

3. Results

Feeding EM-X to rats over a 7 day period led to minimal increases in their respective weights (Fig. 2). The dosage of the Fe-NTA were calculated based on the final weight for each rat. Intraperitoneal injection of Fe-NTA at a sub-lethal dose of 15 mg/kg weight led to a time dependent enhancement of lipid peroxidation both in the kidney and liver of treated rats. In order to assess ongoing oxidative stress-dependent lipid peroxidation, the levels of CD were measured in the liver and kidney of rats. CD values with respect to the control level increases with a maximum peak occurring at 3 h after injection (Deiana et al., 2001). Treatment of rats with EM-X prior to the IP administration of Fe-NTA led to a reduction in the overall levels of CD measured in the kidney by 27% and in the liver by 19% if the levels are expressed as a function of total lipids (Fig. 3) and the protection was statistically different from the controls.

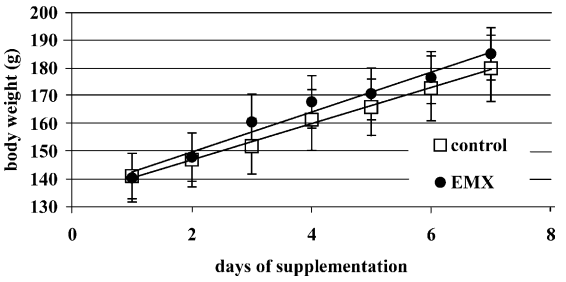

Fig. 2. The effect of EM-X daily feeding on the respective weights of rats.

Organ concentrations of the antioxidants α-tocopherol and GSH were measured (Table 1). Treatment of rats with EM-X led to a 10 and 15% increase in the levels of α-tocopherol and GSH when expressed as a function of the total lipids in the kidney. For the liver, α-tocopherol level fell by 15% and GSH level rose by 16%. However, these changes between the values of the controls and those found in the control treated with EM-X (Table 1) were not statistically significant.

The level of GSH was lower for the Fe-NTA treated rats indicating increased metabolism of GSH in the kidney. Fe-NTA treatment resulted in a 43% reduction of GSH level in the kidney and a reduction of 19% in the liver (Table 1 lower part). EM-X supplemntation did not affect the loss of GSH in both organs. Again the changes between the values in the Fe-NTA animals and the Fe-NTA + EM-X were not statistically significant. Thus EM-X at the regular strength tested neither protected against GSH consumption nor excerbated its utilisation in both the kidney and liver.

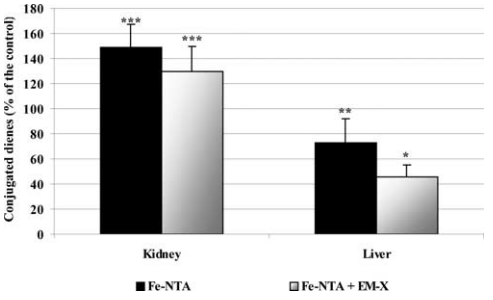

Fig. 3. The effect of EM-X on the levels of CD in the liver and kidney of Fe-NTA treated rats. Kidney control value: 2.09 + 0.16 ng/μg lipids; liver control value: 1.85 + 0.08 ng/μg lipids. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ versus controls (animals non-treated with Fe-NTA).

Table 1
The effect of EM-X on the levels of α-tocopherol, total lipids, and glutathione in the kidney of rats following the treatment with Fe-NTA at 3 h time point and the effects of EM-X

Sample sets	Total lipids (μg/mg tissue)	α-Tocopherol (ng/μg lipids)	Glutathione (ng/μg lipids)
Kidney values			
Control	33.80±3.53	0.46±0.03	57.89±6.04
Control+EM-X	29.93±1.33	0.51±0.07	66.43±2.96
Fe-NTA	26.14±1.56*	0.44±0.09	33.11±1.91***
Fe-NTA+EM-X	24.47±0.95*	0.43±0.02	29.54±1.10***
Liver values			
Control	32.25±1.78	0.73±0.10	74.55±4.12
Control+EM-X	31.28±5.95	0.62±0.04	76.86±14.63
Fe-NTA	27.47±2.32	0.54±0.14	60.46±4.93**
Fe-NTA+EM-X	32.01±0.75	0.49±0.07	54.10±1.31**

*, $P < 0.05$;**, $P < 0.01$; ***, $P < 0.001$ vs. control. The experiments were conducted as described in Section 2. For the control experiments and Fe-NTA treated animals, $n = 6$. For the EM-X treated animals (control and Fe-NTA), $n = 5$.

Total lipids were extracted from the organs and the profile of several fatty acids (Table 2) assessed. Emphasis was placed on the more unsaturated ones that are highly susceptible to oxidation. Fig. 4 (kidney) and Fig. 5 (liver) show the values of the fatty acids at 3 h following the administration of Fe-NTA. Treatment with the regular strength EM-X provided limited protection against the effects of Fe-NTA with notable protections directed to 22:6 (docosahexanoic acid, 9%), 20:4 (arachidonic acid, 3%), 22:5 n6 (docosapentanenoic acid, 15%) in the liver. For the kidney, the protections are directed to 18:1 (oleic acid, 5%), and 20:2 n6 (eicosadieonoic acid, 14%). The effect of EM-X on the levels of 22:6 and 20:4 were similar in the kidney (i.e. the levels were no different from Fe-NTA and Fe-NTA/EM-X treated rats). However, when we transformed the data to reflect the levels of total lipids, only protection to 22:6 and 20:4 in the kidney was statistically significant (Figs. 4 and 5). Total lipids were similar for the Fe-NTA and FeNTA/EM-X treated rats in the liver whilst EM-X protected the lipid in the kidney and this was statistically significant (Table 1). In order to

Table 2
Fatty acids measured in the kidney and/or liver and their values in the control animals

Fatty acid	Common name	Liver (ng/μg lipids)	Kidney (ng/μg lipids)
18:1	Oleic	94.02±17.00	78.65±12.10
18:2	Linoleic	198.20±21.64	130.50±27.97
18:3 n3	Linolenic n3	3.76±0.80	2.12±0.29
18:3 n6	Linolenic n6	1.61±0.33	0.40±0.10
20:2 n6	Eicosadienoic acid	5.65±0.80	2.72±0.59
20:3 n6	Eicosatrienoic acid n6	13.40±1.98	7.00±0.48
20:4	Arachidonic	261.58±20.63	163.93±14.04
20:5	Eicosapentaenoic acid	6.13±0.79	2.84±0.54
22:4	Docosatetraenoic acid	7.57±1.59	5.40±0.54
22:5 n3	Docosapentaenoic acid n3	18.50±2.94	0.96 ± 0.15
22:5 n6	Docosapentaenoic acid n6	3.20±1.11	–
22:6	Docosahexaenoic acid	93.96±10.86	17.03±0.79

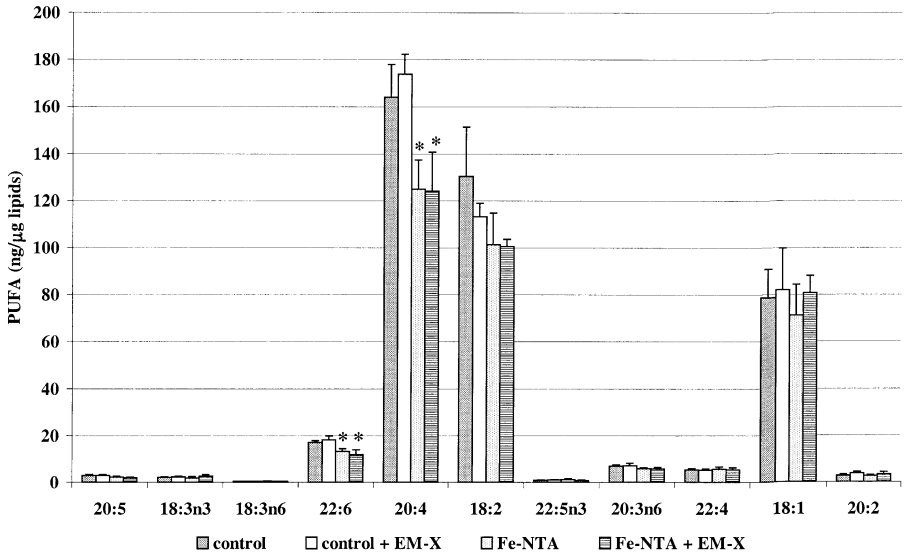

Fig. 4. PUFA measured in kidney at 3 h after intraperitoneal injection of Fe-NTA, as ng/μg lipids. PUFA common names are reported in Table 1. *, $P < 0.05$ vs. controls (animals non-treated with Fe-NTA).

exclude the possibility that the decreased levels of PUFA and antioxidant consumption were due to the effects of Fe-NTA or free iron during experimental procedure, some ancillary experiments were performed as discussed in Deiana et al. (2001). Essentially, Fe-NTA (up to 135 μl/500 mg kidney or liver of the solution used for the in vivo protocol) or the iron-chelating agent Desferal (40 mg/500 mg kidney or liver) were added to the control and treated samples. No differences were

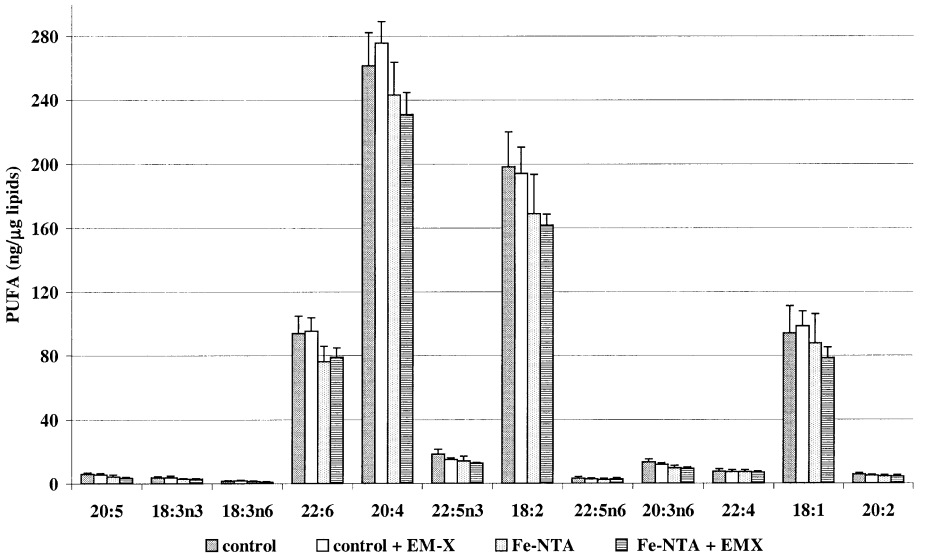

Fig. 5. PUFA measured in liver at 3 h after intraperitoneal injection of Fe-NTA, as ng/μg lipids. PUFA common names are reported in Table 1.

found between the respective values of PUFA and antioxidants in the normal samples and those where Fe-NTA or Desferal were added.

4. Discussions

Early detection of oxidative stress-dependent damage, use of antioxidants and the development of effective preventive strategies are critical in combating ROS-mediated diseases and in management of health (Aruoma and Halliwell, 1998; Bray and Schoene, 2000). It is becoming increasingly evident that responses to oxidative stress and antioxidant supplementations vary and are often determined by the nature and duration of exposure. We reported in this journal (Deiana et al., 2001), an in vivo biomarker animal model based on the assessment of the profile of unsaturated fatty acids arising from the oxidative stress induced by Fe-NTA. In the model, a strong reduction of PUFA concentrations, up to 35–45% of the control levels, were observed in the kidney and liver of rats at the time of the maximum detectable oxidation (3 h) after the injection of a sublethal dose of Fe-NTA. Intraperitoneal injection of an acute dose of Fe-NTA also produced a time dependent effect on the levels of glutathione and α-tocopherol (Deiana et al., 2001). Supplementation of rats with EM-X 7 days prior to the administration of Fe-NTA did not affect the decreased levels of glutathione and α-tocopherol at 3 h following the administration of Fe-NTA. The lowest levels of GSH following Fe-NTA administration was reached at 3 h after the injection as reported in our previous study (Deiana et al., 2001). Interestingly, whilst the levels of GSH rose at 4 h both in the kidney and liver, their respective levels of α-tocopherol remained at the same level (Deiana et al., 2001). The rise in GSH may reflect increased synthesis in response to the acute oxidative stress imposed by the acute dose of Fe-NTA in the liver and kidney. The level of CD measured in both the kidney and liver were modulated by the oral administration of EM-X (Fig. 3). Milchak and Bricker examined the effects of glutathione and vitamin E on acute iron toxicity in isolated rat hepatocytes and came to the conclusion that 'iron induced cell death may not be dependent upon lipid peroxidation, in short term iron exposures' and suggested that the interaction between GSH and vitamin to be critical (Milchak and Bricker, 2002). In our study, however, that the decreased levels of GSH and α-tocopherol due to Fe-NTA were not affected by EM-X administration was indicative that the effect of EM-X was confined to inhibition of lipid peroxidation, seen by the decreased levels of diene conjugation products reported here.

The PUFAs 20:5 (eicosapentaenoic acid), 22:6 (docosahexanoic acid), 20:3 n6 (eicosatrienoic acid), 20:4 (arachidonic acid) and the 18:3 n3 and 18:3 n6 isomers of linolenic acid, which are highly oxidised at 3 h following Fe-NTA administration, have been suggested as indices to be measured when using this model to assess antioxidant efficacy. In this study, treatment of rats with the regular strength EM-X led to a limited protection of 22:6, 20:4, 22:5 n6, 18:1, and 20:2 n6 in the liver and kidney. But only the protection to the levels of 22:6 and 20:4 were statistically significant in the kidney. The principal flavonoidic compounds found in EM-X are quercetin, quercetin-3-*O*-glucopyranoside, quercetin-3-*O*-rhamnopyranoside and kaempferol (Fig. 1). The molecular concentration of each of the bioactive compounds responsible for the observed effects is not known, however, the trend of the data suggests that optimising their concentrations within the cocktail could increase the level of protection observed. This of course could become evident from the outcome of future chronic studies using lower doses of Fe-NTA over a longer period to mimic a chronic disease state and testing antioxidant protection of regular and optimised EM-X. Nevertheless, the ability of EM-X to inhibit lipid peroxidation (decrease of diene conjugation) and limited fatty acid oxidation in vivo is consistent with the observation of Shiomi et al. (1997) that α-G rutin, a water soluble flavonoid containing 4^{G}-α-D-glucopyranosyl rutin and small amount of isoquercetin inhibits lipid peroxidation induced by Fe-NTA–H_2O_2 mixture. Similarly, Zhang et al. (1996) demonstrated that ginseng extracts was able to modulate thiobarbituric acid reactive species (TBARs) formation and loss of arachidonic acid

during lipid peroxidation by Fe-NTA complex. By studying the ability white, black and red rice species to protect against the Fe-NTA dependent oxidative damage, Toyokuni et al. (2002) found that black and red but not white rice modulated renal lipid peroxidation and suggested that this may be related to their contents of cyaniding 3-*O*-β-D-glucoside and protocatechuic acid. Our data shows the potential application of the PUFA profile model to assess antioxidant protection in vivo for extracts, and antioxidant cocktails. Indeed, the difficulties that need to be overcome in assessing in vivo protection of plant and seed extracts include the delineation of the molecular entity and concentrations of the bioactive compounds responsible for the observed effects. Whilst the regular strength EM-X used in this study had limited antioxidant protection, it is anticipated that the point of optimising the concentrations of the bioactive flavonoids in EM-X could afford greater antioxidant protection in vivo.

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