Free Radicals and Antioxidants in Health and Diseases: A Case for EM-X as an Antioxidant Prophylactic Agent

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The general wellness of individuals and management of human clinical conditions in which a role for free radicals and oxidants are implicated continues to underpin the search for non-toxic antioxidants. Free radical reaction of lipid peroxidation is an important issue in the food industry where manufacturers minimize oxidation in lipid containing foods by use of antioxidants during the manufacturing process-foods are produced that maintain their nutritional quality over a defined shelf life. Biomedical scientists and clinicians are interested in antioxidants because they protect the body against damage by reactive oxygen species. EM-X is a novel antioxidant drink derived from rice bran and seaweed extracts that were fermented with Effective Microorganisms (EM). EM-X contains over 35 minerals, *a*-tocopherol, lycopene, ubiquinone, saponin and flavonoids (includingquercetin, quercetin-3-O-glucopyranoside, quercetin-3-O-rhamnopyranoside). Interest in plant phenolics has increased greatly because of their roles as antioxidants and scavengers of free radicals and their potential effects on human health. In the same vein, there is research interest directed towards the understanding the role of free radicals, and antioxidants in nutrition, health and disease. These research efforts are being complemented with the development and validation of biological markers with which scientists can begin to delineate the efficacy of dietary antioxidants.

INTRODUCTION

A free radical is any chemical species (capable of independent existence) possessing one or more unpaired electorn, an unpaired electorn being one that is alone in an orbital. Radicals (-often denoted by the insertion of the radical dot (?) to indicate that one or more unpaired electons is present) are generally less stable than non-radicals, although their reactivities vary. Food lipids have different susceptibility to free radical dependent oxidation, an outcome that depends on whether one is dealing with bulk food lipids (e.g. oils), dispersed food lipids (e.g. membranes and emulsions), dispersed lipids in living organisms (membranes and organelles) and/or the lipids found in watery fluids in organisms (e.g. cytoplasm, plasma). It is important to note that the outcome of antioxidant assessment will have different implications for each of the groups. A good antioxidant activity towards bulk food lipids may be relevant for the dispersed food lipids and biological fluids in organisms but a good antioxidant activity towards biological

fluids may be irrelevant for bulk food lipids (see below). This under pins the need to make use of the right substrate and more importantly, to restrict the interpretation of the data accordingly.

Antioxidants may protect in one system but fail to protect, or even sometimes to cause damage, in others. Antioxidant inhibitors of lipid peroxidation may not protect other targets (such as DNA and protein) against damage, and sometimes can even aggravate such damage. This may not matter much in the food matrix, since damage to DNA and proteins, unless very excessive, will not alter the taste or texture of food (unlike lipid peroxidationn). For example, butylated hydroxyanisole (BHA) is a powerful inhibitor of lipid peroxidation, and yet huge dietary doses of it can induce cancer of the rat forestomach, Also, it has been suggested that oxidative DNA damage could be involved.

The interestttt in the antioxidant indications of plant extracts continues. The active principles in extracts from rosemary, sage, cocoa shells, oats, tea, olives, garlic, ginger, red onion skin, grapes, apple cuticle, wheat gliadin, korum rind, licorice, nutmeg, clove, oregano, thyme, mustard leaf seed chia seed, peanut seed coat, birch bark,carob pod, tempeli, yam, mango, ginseng, mangostum, and vanilla are widely reported. Vitamin E, Vitamin C, ß-carotene, flavonoids and other polyphenols found in some of these extracts are widely discussed as potential antioxidant prophylactics. Flavonoids are naturally occurring benzo-?-pyrone derivatives which are ubiquitous in plant cells and are therefore accessible to consumers through diet. In order to establish this it is prudent to measure 'markers' of baseline oxidative damage *in vivo* and examine how they are affected by changes in diet, such as alterations in fruit and vegetable intake, changes in consumption of saturated/polyunsaturated fats or supplementation with antioxidants (e.g. pure compounds or complex plant extracts). EM-X is a novel antioxidant drink derived from rice bran and seaweed extracts that were fermented with Effective Microorganisms (EM). EM-X contains over 35 minerals, a-tocoherol, lycopene, ubiquinone, saponin and flavonoids (including quercetin, quercetin-3-O-glucopyranoside, quercetin-3-O-rhamnopyranoside).

A course of research on the biopotency of EM-X is currently being conducted under respective themes:

- ?? [1] Antioxidant profile using established assays
- ?? [2] Effects on the release of pro-inflammatory cytokines
- ?? [3] Protection of lipid and low density lipoprotein oxidation
- ?? [4] Assessment of the neuroprotectory effects of EM-X
- ?? [5] Modulation of oxidative stress by EM-X in a Fenton chemistry-animal model

the multifunctional nature of flavonoids makes them ideal candidates for further investigations into the possible beneficial effects in neurodegenerative diseases associated with an aging population. Figure 1 shows the functional relationship between health status and disease state and the role the antioxidants and

functional foods can play in their management (Aruoma (1999) *Asia Pacific Journal of Clinical Nutrition*).

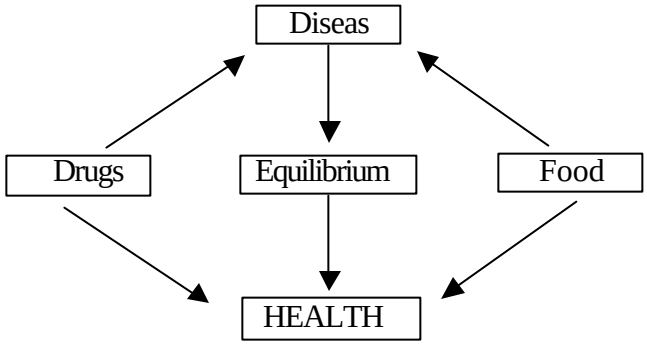

Fig.1. Functional relationships between health status and disease state and the role that food and drugs might play in the management of health.

We need to study and understand the absorption, distribution and metabolism of bioactive components of extracts or functional antioxidant formulations, especially whether they cross the blood brain barrier and whether they are neuroprotectory. This is one of the key research area proposed for EM-X (**research theme [4]**). However such products may need to be administered over long periods of time and hence need to be free of any toxicological effects. Indeed extensive studies on the acute and chronic toxicity of EM-X (by EMRO, Bin et al 1999) have shown that the beverage is safe for oral administration.

ANTIOXIDANT CHARACTERIZATION

A compound might exert antioxidant actions *in vivo* or in food by inhibiting generation of ROS, or by directly scavenging free radicals. Although this 'screening' approach in vitro can be used to rule out direct antioxidant activity *in vivo*, direct reproduction of in vitro effectiveness *in vivo* are often hard to achieve. Similarly, the question as to the ability of natural antioxidants to exert antioxidant or prooxidant actions has been asked for some years. So has uncertainly as to their *in vivo* relevance. The ability of antioxidants to inhibit low-density lipoprotein oxidation is often performed *in vitro* by use of copper ions. The tendency of the antioxidant to promote the Cu^{2+} reduction suggests that the use of the Cu^{2+}-dependent LDL oxidation may not be an accurate method for assessing the antioxidant action of compounds that can redox cycle metal ions. This has led to equivocal results in the literature. For example, ferulic acid can be prooxidant when LDL oxidation is induced by copper at concentrations of the phenolic acid which are protective when the LDL oxidation is mediated by metmyoglobin. Caffeic acid but not ferulic acid has strong antioxidant acitivty towards LDL. Green tea catechins, (-)-epicatechin and (-)-epigallocatechin (depending on concentrations used), accelerate the Cu^{2+}-dependent LDL oxidation. Thus and "antioxidant" could have both antioxidant and prooxidant activities depending on the test system.

Metal chelation, its stabilization and redox activity are critical. Wee have compared the abilities of hydoxyrosol (an antioxidant molecule found in extra virgin olive oil) and ascorbic acid to reduce copper ions. Hydroxytyrosol rapidly reduces copper ions with a stoichiometry of 1:2. This first rapid reduction probably retains Cu^{2+} ions in a complex with a partial bond between the 3'OH function and the C-2 OH on the ethanolic moiety of hydroxytyrosol. This is then followed by a second slower reduction at lower concentrations of hydroxytyrosol and only when Cu^{2+} ions are still available. Ascorbic acid on the other hand reduces copper ions with a stoichiometry of 2 molecules of copper ions being reduced by one molecule of ascorbic acid. For each point at t=0, ascorbic acid is fully consumed and no further increases in Cu^{2+} reduction could be seen although it is not clear if this is due to full consumption of the copper ions. Interestingly, the abilities to reduce Cu^{2+} ions could not translate to the extent of oxidative damage to DNA. That is, the high non-physiological levels of hydroxytyrosol (5mM) achieved similar level of damages as caused by 0.25 mM ascorbate using the model system copper-1, 10-phenanthroline dependent DNA oxidation. The amount of Cu^{2+} during the early phase was dependent on the concentration of Cu^{2+} and of the concentration of a-tocopherol within the native LDL, Artificial enrichment of the LDL with a-tocopherol led to increased Cu^{2+} reduction and as well as to its rate of reduction. Thus antioxidant molecules capable of reducing Cu^{2+} will confound the copper-dependent LDL oxidation simply by interfering with the assay rather than acting as prooxidants. The complex relationships between the structure actions of plant-derived antioxidants and their radical scavenging, inhibition of lipid peroxidation, prooxidant and metal ions chelating abilities, have implications for the consideration of the antioxidants as prophylactic agent *in vivo*. For EM-X, it is of interest to delineate potential inhibition of LDL on the one hand and in an extension project to investigate the interactions of the component flavonoids on the metal chelation properties that may determine overall efficacy, as outlined above. This is in line with **research theme [3].**

? -Tocopherol delays lipid peroxidation by reacting with chain-propagating peroxyl radicals faster than these radicals can react with proteins or fatty acid side-chains. In theory, ß-Carotene can interact with a free radical to form a ß-carotene-derived radical which in the presence of oxygen forms a peroxyl radical. The abilities to recycle vitamin E- and ß-carotene derived radicals may vary *in vivo*. Thus the accumulation of the ß-carotene radicals may present a prooxidant status *in vivo*. This contrasts with the dehydroascorbate (DHA) radical levels. DHA is much less reactive than are many of the radicals that can be scavenged by ascorbate. Intracellular enzymic system exist *in vivo* to reduce this radicl back to ascorbate suing NADH (the NADH-semidehydro-ascorbate reducctase enzymic) or GSH (the dehydroascorbate reductase enzyme) as sources of reducing power. So unlike the ß-carotenederived radical, DHA does not accumulate. However, in foods fortified with ascorbic acid (orange juice for example), the accumulation of DHA in the matrix contributes to the oxidation dependent off-flavour.

DIFFERNET APPROACHES TO ASSESSING DIETRAY ANTIOXIDANT ACTION

The extent to which oxidation of fatty acids and their esters occurs depends on the chemical structure of the fatty acid. Measureemnts of lipid peroxidation using rat live or cardiac microsomes, ox-brain phospholipids liposomes, arachidonic acid, and other lipid model systems (e.g. bulk oil and emulsified oil systems) should be the first line of tests to establish the potential antioxidant action of dietary antioxidant compounds. Antioxidant index based on ability to scavenge peroxyl radicals provides support for antioxidant efficacy in *in vitro* system.

Antioxidant actions of nutrient components have been described in the literature suing several other methods. For example, the spectrophotometric technique, total antioxidant activity (TAA) or the Trolox equivalent antioxidant activity (TEAC). The method involves the generation of the long-lived specific radical cation chromophore of 2,2' –azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) by controlled chemical oxidation. The ABTS?$^{+}$ radical cation has absorption maxima in the near-infrared region at 645, 734 and 815 nm. The TEAC reflects the ability of hydrogen- or electron-donating antioxidants to scavenge the ABTS?$^{+}$ radical cation compared with that of Trolox. The antioxidant suppresses the A_{734} to an extent and on a time scale depend on the antioxidant activity. The peroxyl radical species trichloromethylperoxyl (CCl_3O_2?) is a "clean" lipid-soluble radical for *in vitro* studies of antioxidant actions. The (CCl_3O_2? can be generated by radiolysis of an aqueous mixture of propan-2-01 and CCl_4. the rate constants calculatedfrom the kinetics of the radical antioxidant interactions are good indicators of antioxidant propensities *in vitro* albeit for a very reactive free radical. Reactions involving the free radical 2,2 disphenyl-1-picrylhydrazyl (DPPH?) are also in use . The reduction of DPPH? is followed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction. In its radical form, DPPH? absorbs at 515 nm, but upon reduction by an antioxidant (AH) or a radical species (R?), the absorption disappears. The representative data from these methods are shown in Tables 1 and 2. Existing data from Dr. Higa and colleagues has shown that EM-X doses scavenge the DPPH radical; nevertheless the efficacy cannot at present be ascribed to a particular component. The application of in vitro tools to examine the antioxidant action of EM-X forms part of **research theme [1].**

ANTIOXIDANTS AND FENTON CHEMISTRY

Antioxidants that protect lipids against free radical damage may actually accelerate damage to other molecules such as NDA, carbohydrates, and proteins under certain conditions. The deoxyribose assay allows determination of rate constants of reactions with OH? radicals, assessment of abilities to exert prooxidant action, and assessment of abilities to chelate metal iron. In using this test to assess pro-oxidant actions *in vitro*, it is the ability of the compound to mediate a reacton similar to that of ascorbate that constitutes the basic of the evaluation. The Fe (III)-EDTA complex has a tested propensity to be reduced by

the pro-oxidantt (if indeed it is able to do so). The redox potentials of other metal complexes (which may be physiologically relevant) would vary. In the absence of EDTA, iron ions are equally available to both the deoxyribose and the compound under test. Thus compounds that are able to preferentially chelate iron and present the resulting metal-complex in a less redox-active form compared with EDTA-metal complex will protect deoxyribose against damage in the presence of ascorbate and H_2O_2. Substances that inhibit in the assay are also those that are able to bind iron ions strongly enough to remove them from deoxyribose. Thus EDTA removes iron ions from deoxyribose, but iron-EDTA chelates are very effective ingenerating OH? so that the deoxyribose is still degraded-this time by OH? in "free" solution, rather than by OH? formed on the deoxyribose molecule. While it is important to understand the mechanism of EM-X-induced protection in this system, direct demonstration of *in vivo* protection of oxidative stress remain critical (see below).

The ability of to inhibit deoxyribose degradation under these reaction conditions is a measure of its ability to interfere with site-specific Fenton chemistry. When ascorbate is omitted from the deoxyribose reaction mixture, the ability of added compounds to reduce the Fe^{3+}-EDTA complex can be tested. "The Fe^{3+}-EDTA complex has a tested propensity to be reduced by the pro-oxidant (if, indeed, it is able to do so). It follows that the redox potentials of other metal complexes (which may be physiologically relevant) would vary". This simple idea led to the proposal to use assays involving DNA damage to specifically test for the abilities of dietary antioxidants to exert pro-oxidant actions, different from their intended abilities to minimize oxidation of lipids. The tests involve measurement of DNA damage in the presence of bleomycin-iron and copper-1, 10-phenanthroline complexes.

ANTIOXIDANTS AND CELLULAR RESPONSES

TNF-a is a pleiotrophic protein that mediates a multitude of inflammatory events in the lung. The induction of inflammatory mediators can be regulated by the activation of redox-sensitive transcription factors Activator Protein-1 (AP-1), (c-Fos/c-Jun) and nuclear factor-kappa B (NF-?B. TNF- aincreases AP-1 binding via the MAP kinase pathway. TNF-a has also been shown to affect the local tissue oxidant/antioxidant balance. Binding of AP-1 and NF-?B leads to the transcription of genes for both the protective antioxidants such as ? –glutamylcysteine synthetase (? –GCS), as well as several inflammatory mediators and chemokines, including IL-8.

Interleukin-8 (IL-8) is a major chemotactic and activating mediator for polymorphonuclear leukocytes (PMN) in the lungs. Thus modulation of its production may provide a therapeutic target in inflammatory lung diseases. Transcription factors such as NF-?B and AP-1 have been shown to be involved in the transcriptional activation of IL-8. These transcription factors may be activated by a variety of extra cellular signals, acting via surface receptors, and leading to kinase-mediated phosphorylation cascades. The transcription factors NF- ?B and

AP-1, which are activated by inflammatory stimuli, switch on inflammatory genes, including IL-8 leading to increasedsynthesis of inflammatory proteins. Moreover, these transcription factors are redox-sensitive. I have sought to provide preliminary data to show modulation by EM-X in these processes, in line with the **research theme [2]**. Indeed EM-X (100μ1/ml) inhibited both H_2O_2 and TNF-a-mediated activation of NF-?B and AP-1. Both H_2O_2 and TNF-asignificantly increased IL-8 release, which is associated with decreased AP-1 and NF-?B activation. Please refer to the accompanying poster presentation at this conference. This is important and more work is required to fully understand the molecular effects of EM-X on the signal transduction pathways and how this could apply to modulation of diseases of inflammation.

MEASURING OXIDATIVE DAMAGE *IN VIVO*

There are several indicators of the extent of oxidative damage upon which the antioxidant efficacy *in vivo* can be assessed, by virtue of their modulation of the levels of the biomarkers.

Oxidative DNA damage and its measurement: Oxidative damage to DNA appears to occur continuously *in vivo,* in that low levels (presumably a 'steady state' balance between DNA damage and repair) are detectable in DNA isolated from human cells and tissues. The pattern of damage to the purine and pyrimidine bases bears the chemical fingerprint of OH? attack, suggesting that OH? formation occurs within the nucleus *in vivo*.

Lipid peroxidation and its measurement: Lipid peroxidation is important *in vivo*. It contributes to the development of cardiovascular diseases such as pre-eclampsia and atherosclerosis and the end products of this process [particularly cytotoxic aldehydes suich as malondialdehyde (MDA) and 4-hydroxynonenal, (HNE)] can cause damage to proteins and to DNA. Levels of isoprostanes and hydroxyeicosatetraenoic acids (HETC) *in vivo* may represent clinically relevant oxidative lipid damage. HETEs are chemotactic for neutrophils and may facilitate calcium uptake and protein kinase C mobilization. Isoprostanes are a series of prostaglandin-like compounds formed during peroxidation of arachidonic acid although probably similar products are formed from other PUFAs. Because they are structurally similar to prostaglandin F_{2a}, the compounds are collectively reffered to as F_2-isoprostances. These compounds can be measured in plasma (35 ± 6 picograms/ml) and urine (1600 ± 600 pg/mg creatinine) of healthy volunteers, indicative of ongoing lipid peroxidation even in healthy human subjects. The majority of plasma isoprostanes are esterified to phospholipids, but some are 'free'. Isoprostanes and their metabolites can be measured in human urine and this may prove to be valuable assay of whole body lipid peroxidation relevant in assessing the antioxidant efficacy of endemic plants.

We have developed an animal model of Fenton chemistry involving the assessment of the profile of polyunsaturated fatty acids induced by ferric-nitrilotriacetic acid. The method has just been published be Deiana et al 2001 in

Toxicology Letters. This model has been adopted to test the modulation of oxidative stress under the **research theme 5.** briefly, intraperitioneal injection of the iron- complex, ferric-nitrilotriacetate (Fe-NTA), induces renal proximal tubular damage associated with oxidative damage *in vivo.* Fe-NTA induced a time-dependent decrease of several polyunsaturated fatty acids (PUFA), together with increased conjugated diene values and decreased cellular levels of a-tocopherol and glutathione. At the time of maximum detectable oxidation (3 hours), after the injection of a sublethal dose of Fe-NTA there were clear reductions in the peak values over the controls for several fatty acids notably, 20:5 (eicosapentaenoic acid) 36%), 22:6 (docosahexanoic acid) (30%), 20:3 n 6 (eicosatrienoic acid) (30%) and 20:4 (arachidonic acid) (28%) in the kidney. Fewer fatty acids showed a reductin in their residual values in the liver. 20:% was reduced by 45% and for the 18:3 n3 and 18:3 n6, reductions of 35% respectively. The profile of PUFAs is sensitive to the oxidative damage due to Fe-NTA and thus a novel oxidative biomarker model. EM-X is being tested in this model. The data will be important in delineating the *in vivo* protection of EM-X under oxidative stress conditions.

Protein oxidation and its measurement: Free radical attack upon protein generates radicals from amino acid residues, and electrons can be transferred between different amino acids. The levels of *ortho*-tyrosine and dityrosine in human lens proteins have been reported in relation to age. ROS can also attack amino acid residues (particularly histidine, arginine, lysine and praline) to produce carbonyl functions that can be measured after reaction with 2,4-dinitrophenylhydrazine. This can be complemented by Western-blotting assays based on the use of anti-DNPH antibodies to identify oxidative-damaged proteins in tissues and bodies fluids. Current advances in the measurement of protein oxidation products using monoclonal antibody HNEJ-2 which selectively recognizes histidine-4-hydroxy-2-nonenal adducts. Hydroxynonenal levels decreases rapidly after an oxidative stress insult, thus the more stable HNE-modified protein represents a more sensitive marker for oxidatnve stress than the aldehyde itself.

ANTIOXIDANT AND NEUROPROTECTION

Neurodegenerative disorders are a heterogenous group of chronic progress that may chare a common mechanism with the aging process. Alzheimer's disease (AD) is achronic neurodegenerative disorders with characteristic neuropathological hallmarks including senile plaques, neurofibrillary tangles and neuronal loss. Senile plaques are rich in amyloid precursor protein (APP). Parkinson's disease (PD) is a progressive and disabling neurodegenerative disorder, characterized by a primary loss of dopaminergic neurons in the substantia nigra, resulting in a reduction in striatal dopamine levels.

Although laboratory data suggests a role for oxidants in the pathogenesis of many age-related diseases, the clinical use of antioxidant therapy is at its infancy. It has also produced equivocal results. For example, vitamin E is not protective in early Parkinson's disease whilst being beneficial in Alzheimer's disease. The

antioxidant N-acetyl cysteine did not effect survival in amyotrophic lateral sclerosis (ALS). However, reducing oxidative burden or boosing host defence mechanisms are the best prophylactic approach. Given that there may be genetic basis for the diseases, the genetic link between longevity and oxidative stress resistance needs to be understood. Of present importance is the understanding of the biopharmacy of the antioxidants in the brain and how this compares with systemic responses.

The flavonoid tangeretin can cross the blood brain barrier, thus gaining access to the brain, and is neuroprotectory in the 6-hydroxydopamine model of Parkinson's disease. Thus it is of interest to investigate whether flavonoids and the bioactive components in plant extracts with antioxidant indications, and EM-X, can penetrate into the brain, whether they are neuroprotectory and whether they can halt the progression of a lesion in an animal model of Parkinson's disease. Tangeretin was administered as a daily oral injection (20mg/kg) for three days prior to a 6-hydroxygopamine (6-OHDA) lesion to the medial forebrain bundle. In the control animals that received the drug vehicle only, the 6-OHDA produced an approximate 50% loss in tyrosine hydroxylase (rate limiting enzyme in dopamine synthesis) immunopositive neurons in the substantia nigra, while in the tangeretin treated animals the same dose of 6-OHDA produced a significantly smaller lesion (approximately a 13% loss in the number tyrosine hydroxylase +ve neurons. Datla et al. will publish this work, in the journal Neuroreport (December 2001 issue).

We have applied this method to EM-X and are currently processing sample from our experiments. This of course is in line with the **research theme [4]** in our remit. It is my belief that the work should be extended to research into Alzheimer's disease, research funds permitting.

Table 1 Rate constant for the reaction antioxidants with CCl_3O_2? and Trolox equivalent antioxidant capacity for EM-X, ergothioneine and a variety of naturally occurring flavonoids, phenylpropanoids, carotenoids and vitamins.

Antioxidant	Sources	Rate constants for CCl_3O_2?, ($M^{-1}s^{-1}$)	TEAC (mM)*
Hydroxytyrosol	Extra virgin olive oil, olives	8.37×10^6	Not available
Vitamin C	Fruit and vegetables	1.3×10^8	1.0 ± 0.02
Vitamin E	Grains, nuts, oils	4.9×10^8	1.0 ± 0.03
Vanillin	Vanilla	3.97×10^6	Not available
Vanillic acid	Vanilla	9.54×10^7	Not available
Oenin	Black grapes/red wine	Not available	1.8 ± 0.02
Cyanidin	Grapes, raspberry, strawberry	Not available	4.4 ± 0.02
Delphinidin	Aubergine skin	Not available	4.4 X 0.11
6-Gingerol	Ginger	4.67×10^6	Not available
Zingerone	Ginger	5.63×10^6	Not available
Quercetin	Onion, apples, berries, grapes, Tea, broccoli	3.9×10^7	4.7 ± 0.10
Kaemprerol	Endive, broccoli, grapefruit, tea	Not available	1.3 ± 0.08
Trolox C	Synthetic vitamin E analogue	2.23×10^6	1
Rutin	(Rutinoside of quercetin)	Not available	2.4 ± 0.12
Luteolin	Lemon, olive, celery, red pepper	Not available	2.1 ± 0.05
Chrysin	Fruit skin	9.86×10^7	1.4 ± 0.07
Apigenin	Celery, parsley	Not available	1.5 ± 0.08
Fisetin		4.09×10^6	Not available
(Epi)catechin	Black grapes/red wine	7.3×10^6	2.4 ± 0.02
Morin		3.10×10^6	Not available
Naringin	Peel of citrus fruit	Not available	0.24 ± 0.02
Taxifolin	Citrus fruit	Not available	1.9 ± 0.03
Carvacrol	Oil of thyme	3.92×10^5	Not available
Ferulic acid	Grains, tomatoes, seeds, vegetables	7.5×10^6	1.9 ± 0.02
Carnosic acid	Rosemary	2.7×10^7	Not available
Carnosol	Rosemary	$1\text{-}3 \times 10^6$	Not available
Lycopene	Tomatoes	Not available	2.9 ± 0.15
ß-Carotene	Carrots, sweet potato, tomatoes, Paprika, green vegetables	Not available	1.9 ± 0.10
a-Carotene	Tomatoes, carrots, green vegetables	Not available	1.3 ± 0.04
ß-Cryptoxanthin	Mango, papaya, peaches, paprika, oranges	Not available	2.0 ± 0.02
Lutein	Banana, egg yolk, vegetables	Not available	1.5 ± 0.10
Zeaxanthin	Paprika, orange peels	Not available	1.4 ± 0.04
Astaxanthin	Salmon, crab	Not available	0.03 ± 0.03
Canthaxanthin	Carrots, kale, red peppers	Not available	0.02 ± 0.02
Ergothioneine	Various plant	Not available	0.60 ± 0.03
EM-X	Herbal extract drink	Not available	0.10 ± 0.005

*Some values abstracted from ref 15. **Some values abstracted from ref 12

Table 2 Classification of anti-radical efficiencies of antioxidants

Compound	ARP	Number of reduced PPH?
Isoeugenol	1.94	0.97
Ascorbic acid	3.70	1.85
Isoascorbic acid	3.70	1.85
d-tocopherol	4	2
Rosmarinic acid	6.90	3.33
Phenol	0.002	<1
Coumaric acid	0.02	<1
Vanillin	0.05	<1
Vanillic acid	0.17	<1
?-resorcylic acid	0.36	<1
Ferulic acid	2.33	1.16
Eugenol	3.7	1.85
Zingerone	3.7	1.85
Guaiacol	4	2
BHA	4	2
BHT	5.30	2.63
Protocatechuic acid	7.14	3.6
Caffeic acid	9.1	4.54
Gentisic acid	11.1	5.6
Gallic acid	12.5	6.25

Data abstracted from ref 11

CONCLUSION

Current research directed towards understanding the role of free radicals, plant extracts, plant-derived antioxidants in food and in nutrition and in human health is being complemented with the development and validation of biological markers with which scientists could being to delineate the efficacy of dietary antioxidants. The projects **theme [1] – [5]** involving EM-X form part of this endeavour. The next stage however is to consider clinical trials where the efficacy of the herbal tea extract can be targeted to a particular disease condition, e.g. asthma, Parkinson's disease or Alzheimer's disease, diabetes etc.

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