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Antioxidative Constitution of the Mistletoe, *Viscum capitellatum* Smith

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Mistletoes are being used in the treatment and management of a number of diseases, both in the traditional and in the complementary medicines, in several cultures. Fresh aerial parts of the obligate epiparasite, *Viscum capitellatum* Sm., hyperparasiting on the mistletoe *Dendrophthoe falcata*, a parasite on the terrestrial host, *Albizzia lebbeck*, was found to possess *in vitro* antioxidant capacity, as determined by the ABTS$^{\bullet+}$ and NO scavenging, FRAP and β-carotene bleaching assays and capable of potentially chelating ferrous ions. Terpenes (lupeol and betulin), terpenic acids (betulinic and oleanolic acids), sterols (spinasterol and β-sitosterol) and carotenoids have been identified as the antioxidant metabolites of the lipophilic fraction and phenolic acids (chlorogenic acid, caffeic acid and *p*-coumaric acid), 7-hydroxy-3,5,6,3',4'-pentamethoxyflavone, eriodictyol and its 7-O-β-D-glucopyranoside as the predominant antioxidant phytoconstituents of the hydrophilic fractions of the mistletoe extract.

Key Words: Mistletoe, *Viscum capitellatum*, Antioxidant, Flavonol, Flavanone, Phytosterol, Terpene, Terpenic acid, Total carotenoids.

INTRODUCTION

The practice of utilizing the products of nature to combat ailments is as old as the human evolution. Its significance has assumed greater dimensions during the past few decades, owing largely to the understanding of the multiple roles of the diverse array of secondary metabolites, which exhibit potent antioxidant properties, elaborated in them. The therapeutic efficacies of several plants that are used in traditional medicines are now attributed to their antioxidant constitution. Oxygen uptake, inherent to cell metabolism, generates reactive oxygen and nitrogen species (RONS). An imbalance in their production and their quenching by cellular antioxidant defence systems results in oxidative/nitrosative stress (ONS). Thus, ONS is a pervasive condition of accumulation of RONS, which is now recognised to be a prominent feature of several acute and chronic diseases and even of the process of normal aging. Though definitive evidence for this association has often been lacking because of recognized shortcomings with biomarkers and/or methods available to assess ONS status in humans, ONS has been implicated as a potential contributor to the pathogenesis of a number of ailments. Notable among these are cancer[1], aging[2] and age-related neurodegenerative disorders such as Alzheimer's[3] and Parkinson's diseases[4], arthritis and inflammation[5,6], diabetes[7,8], atherosclerosis[9], liver diseases[10] and AIDS[11] and several other chronic and age-related disorders. Oxidative reactions are also concerned with food industry since lipids, natural constituents of cellular membranes, are oxidized during peroxidation, producing partial or total changes in food sensorial properties and in its nutritional value due to the loss of vitamins, essential fatty acids and protein. Plant-based antioxidants are now preferred to preserve food quality because of safety concerns. These factors have inspired the widespread screening of plants to exploit their medicinal and antioxidant potentials. A profile of the chemical composition of a plant together with the knowledge of its antioxidant capacity may provide a fair estimate of its therapeutic potential.

Parasitism by means of haustorial connections to a host to complete their life cycle is widespread in the angiosperms and comprise about 900 species belonging to 65 genera occurring in approximately 20 plant families that are mainly distributed in Africa, Asia, Australia and South America (representing more than 1 % of all angiosperms)[12,13]. Mistletoes are the largest and the most diverse group of aerial hemi-parasitic plants, which belong to two taxonomically related families, *i.e.*, Loranthaceae and Viscaceae which share the order Santalales. Unlike other plants, mistletoes do not follow a 12-month vegetation period, never touch the earth and remain ever green and flourished, posing to possess ever-lasting life. This has attached sanctity to the mythical plant since ancient times and the Celtic Druids believed that it possessed miraculous powers and considered it as an 'all-heal' herb that ensured fertility and an antidote for poisons[13]. Mistletoes are being used in the treatment and management of a number of diseases, both in the traditional and in the complementary

medicines in several cultures[13,14]. In traditional Chinese and other East Asian medicines, mistletoes, for instance, *Viscum alniformosanae* Hay., *V. angulatum* Heyne in DC., *V. articulatum* Burm., *V. coloratum* (Komar) Nakai, are being commonly used as a curative for a number of ailments. These include atherosclerosis, epilepsy, gout, spasms of the heart, haemorrhage, hypertension, pleurisy, rheumatism and arthritis, neuralgia, threatened abortion and locally to treat frostbite[15-17]. Antioxidative and apoptosis-inducing activities of plants belonging to the genus *Viscum* have also been reported[18,19]. The European mistletoe (*V. album*) preparations are today counted among the heavily advertised array of unproven methods in oncology. Their applications in complementary cancer therapy have been perceived in its entirety by the spiritual intuition of Rodulf Steiner (1904), without any clinical support. *V. album* is reported to be cytotoxic to tumours and endothelial cells and is used for cancer, epilepsy, headache and some inflammatory diseases and as a cardiotonic[13,14,20-22]. The New Zealand mistletoes are reported to be used as cardiotonics and in the form of poultices for treating itches[23]. In Africa, many mistletoes are known as 'all-healers' and 'bone-setting' drugs and a number of biological effects, including antimutagenic, anticancer, antimicrobial, antiviral, apoptosis-inducing and immunomodulatory activities have been reported[14]. Mistletoe teas and infusions are recommended ethnomedicinally for the prevention and management of stroke in parts of Nigeria and they are also believed to improve the circulatory system and heart function in traditional medicine. They are also reported to be effective in the management of chronic metabolic disorders such as diabetes and as a cure for epilepsy and infertility. In Southeast Asia they are used against malaria and cancer and as a tonic, while in South America, their uses as cardiotonics and cancer remedies reflect their adoption from European settlers. Mistletoes are also claimed to be an antiseptic against erysipelas[24], a remedy for headache, a diuretic and a contraceptive[25].

Mistletoe therapy has recently been reported (i) to reduce therapy-associated toxicity, (ii) to result in a non-specific stimulation of the immune system, especially cell-mediated immunity in immune-compromised chemoradiation-received patients, (iii) to accelerate bone marrow regeneration after cytostatic therapy and in general result in marked improvement in the quality of life[26-28]. It is now well accepted that mistletoe lectins, viscotoxins, oligo- and poly-saccharides, phenylpropanoids, triterpenoids and flavonoids are the biologically active components that potentially contribute to the pharmacological characteristics of the mistletoe extracts. In view of the fact that mistletoes find extensive application in the traditional medical practices of various cultures and ONS and its amelioration by the antioxidant phytoconstituents have now come to occupy an amazingly central role in the chemoprotection against several chronic and age-related human ailments, investigation of the antioxidant constitution of the mistletoe, *V. capitellatum* has been undertaken.

V. capitellatum Smith (Vernacular: 'netty pulluruvi'[29]) of Viscaceae is a small, usually pale green, densely branched epiparasitic shrub that commonly parasites only on Loranthaceae, particularly *Dendrophthoe falcata* L.f. (Vernacular: 'periya pulluruvi') and *D. neelgherrensis*, with distribution in India and Srilanka[30-32]. The outcome of the investigation of the fresh aerial parts of *V. capitellatum* for its antioxidant capacity and the phytometabolites potentially responsible for the activities determined are reported in this paper.

EXPERIMENTAL

2,2'-Azino*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) diammonium salt, 2,4,6-*tris*(2-pyridyl)-*s*-triazine (TPTZ), 3-(2-pyridyl)-5,6-di(4-phenylsulphonic acid)-1,2,4-triazine (ferrozine) sodium salt, anhydrous ethanol, ferrous chloride, (±)-eriodictyol, quercetin and β-carotene were obtained from Sigma-Aldrich Inc. and Sephadex LH-20 from Pharmacia. Silica gel (60-120 mesh for column chromatography), acetone and methanol for chromatography/spectroscopy were procured from Merck Specialities Private Limited. All other chemicals/reagents were of analytical/laboratory grades from Himedia/Merck/Loba Chemie. Shimadzu UV-160 spectrophotometer was used for electronic spectral measurements and NMR spectral recordings were performed on Bruker DRX-500 spectrometer, using DMSO-d_6 solutions.

Plant material and processing: Fresh aerial parts of the obligate epiparasite, *V. capitellatum*, hyperparasiting on the mistletoe *D. falcata*, a parasite on the terrestrial and non-parasitic host, *Albizzia lebbeck*, (Fig. 1) were collected from Urani sacred grove, belonging to Marakkanam Panchayat Union of Villupuram District, Tamil Nadu, India (12°9'N and 79°55' E), during the month of September. After establishing its identity, 620 g of the fresh material (FM) was extracted with boiling 80 % aq. C_2H_5OH (4 × 6 L, 6 h). The combined extract was evaporated to dryness under reduced pressure and the residue (75.00 g of the total 86.23 g) was washed successively with CH_2Cl_2 (6 × 0.4 L), $(CH_3)_2CO$ (4 × 0.4 L) and CH_3OH (6 × 0.4 L) to get the fractions soluble in the respective solvents (MEFs). After evaporating the solvents *in vacuo*, aliquots of each fraction as well as the aq. C_2H_5OH residue (ME) were dissolved in CH_3OH and quantitatively made up to 25 mL in volumetric flasks for quantitative determinations.

Fig. 1. *V. capitellatum*, hyperparasiting on the mistletoe *D. falcata*, a parasite on the terrestrial host, *A. lebbeck*

Determination of *in vitro* antioxidant capacity: Determination of ABTS radical cation scavenging capacities were assessed according to the improved $ABTS^{\bullet+}$ decolourising assay of Re *et al.*[61] and ferric-reducing/antioxidant power

(FRAP) assay by the procedure of Benzie and Strain. The *in vitro* antioxidant capacities of the mistletoe extracts (ME and MEFs), determined by the two assays, were expressed as mg vitamin C equivalents (VCE)/100 g FM as described previously[33]. Percentage inhibition of β-carotene bleaching by the ME, MEFs and the controls, (2,6-di-*tert*-butyl-4-methylphenol/ BHT and eriodictyol) were measured by the method described by Pratt and percentage chelating efficiencies were evaluated based on the method of Dinis *et al.*[33] in relation to disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA) and eriodictyol standards[33]. The percentage inhibition of NO and hence NO_2^- (NOx) was determined according to the method of Marcocci *et al.*[34], using eriodictyol as positive control.

Determination of antioxidant constitution: Fresh aerial parts (25 g) were homogenized with acetone (100 mL), vacuum-filtered and concentrated. The residue was dissolved in 20 % aq. acetone and made up to 25 mL. The total carotenoid content of the mistletoe in mg/100 g FM was determined by the protocol described by Lichtenthaler and Buschmann[35]. Phenolic and flavone/flavonol contents of the ME and MEFs were determined by the spectrophotometric method using standard gallic acid and quercetin curves, respectively[33] and the flavanones/ dihydroflavonols contents by the colorimetric assay[36] using a standard (±)-eriodictyol calibration curve. The mean ± SD of three replicate measurements were expressed, respectively as mg gallic acid equivalent (GAE), mg quercetin equivalent (QE) and mg (±)-eriodictyol equivalent (EE)/100 g FM.

Isolation of the predominant antioxidant constituents: CH_2Cl_2 fraction was subjected to normal phase open SiO_2 column chromatography (CC) and separated by gradient elution using binary mixtures of *n*-C_6H_{14}:C_6H_6, followed by *n*-C_6H_{14}:$CH_3COOC_2H_5$. Fractions eluted with *n*-C_6H_{14}:C_6H_6 were found to be a complex mixture, containing predominantly carotenoids. From the fractions eluted with *n*-C_6H_{14}: $CH_3COOC_2H_5$ = 9:1 and 8:2, respectively, lup-20(29)-en-3β-ol[37] (lupeol) and stigmasta-7,22-dien-3β-ol[38] (spinasterol) were isolated. The fraction eluted with 6:4 yielded stigmast-5-en-3-ol[39] (β-sitosterol). The more polar fractions were combined and then separated by preparatory SiO_2 TLC using C_6H_6: $CH_3COOC_2H_5$:HCOOH = 36:12:5 to separate lupeol (R_f 0.77), 3β-lup-20(29)-en-3,28-diol[40] (R_f 0.65, betulin), 3β-hydroxy-lup-20(29)-en-28-oic acid[41] (R_f 0.59, betulinic acid) and 3β-hydroxyolean-12-en-28-oic acid[42] (R_f 0.51, oleanolic acid). The $(CH_3)_2CO$ fraction was subjected to Sephadex LH-20 gel filtration CC (90 % aq. CH_3OH) and the eluates monitored by PC (Whatman No. 1, 15 % HOAc, ascending, 28 ºC). Fractions containing similar compositions were pooled together and rechromatographed (Sephadex LH-20, 90 % aq. CH_3OH) to separate three fractions, F_1-F_3. F_3 was subjected to PC (Whatman No. 3, H_2O, descending, 28 ºC) to separate 3-O-(3,4-dihydroxycinnamoyl)-D-quinic acid (R_f 91, chlorogenic acid, 3-O-caffeoylquinic acid[43]), (E)-3-(4-hydroxyphenyl)-prop-2-enoic acid (R_f 83, *p*-coumaric acid[44]), (E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid (R_f 66, caffeic acid[44]) and (2S)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromanone (R_f 12, eriodictyol[45,46]). F_2 was subjected to PC (Whatman No. 3, 30 % HOAc, descending, 28 ºC) to isolate 7-hydroxy-3,5,6,3',4'-pentamethoxyflavone (R_f 14, quercetagetin 3,5,6,3',4'-pentamethylether[47]). F_1 and the concentrate of CH_3OH fraction, which also contained the phenolics reported above, upon gel filtration as before yielded 7-O-β-D-glucopyranosyl eriodictyol[46].

RESULTS AND DISCUSSION

Determination of *in vitro* antioxidant capacity: The use of herbal medicines to preserve human health is a well established tradition in several cultures and it is now duly recognized by international organizations[48]. Despite the increasing scientific interest in this field, comprehensive data on the composition, therapeutical applications and risks associated with the consumption of herbal medicines are still deficient in scientific literature. All plants, including fruit-, nut- or spice-bearing plants, synthesize a vast array of chemical compounds that are not necessarily involved in the plant's metabolism but instead serve a variety of functions that enhance the plant's survivability, including their ability to combat ONS. At present there is increasing interest in the role of free radical-mediated damages in the aetiology of human illness. The significance of RONS in the aetiology and pathophysiology of a broad array of human health issues, ranging from aging, endocrinal and neuronal disorders to cardiovascular, cataract and cancers, has attracted increasing attention over the past two decades or more. Various mechanisms have been proposed to contribute to the generation of these RONS in living organisms. The sources of exogenous RONS include several pollutants, tobacco smoke, organic solvents and pesticides. A balance normally prevails between the generation of RONS during normal physiological events and their inactivation by the endogenous antioxidant defence mechanisms of an organism. When the endogenous antioxidant defences turn inadequate ONS results, wherein excessive levels of these reactive species tend to accumulate, causing damage to cellular proteins, membrane lipids, nucleic acids and carbohydrates and eventually to cell death. Ample studies support that natural antioxidants, endogenous to plants, can scavenge RONS and current evidences strongly favour the significance of the ubiquitous carotenoids, tocopherols and polyphenols in preventing or in delaying the onset of a number of chronic human diseases. These compounds have been investigated to prevent oxidative damage to important biological membranes and to lipid-rich foods. Consequently, they tend to delay, inhibit or even prevent oxidative reactions by a variety of mechanisms that include (i) chain breaking by donation of hydrogen atoms or electrons that convert free radicals into more stable species, (ii) chelating metal ions that are involved in the generation of RONS, (iii) decomposing lipid peroxides into stable final products and (iv) inhibiting the deleterious action of prooxidant enzymes.

As a part of the pursuit for sources of potentially antioxidant natural substances, a systematic phytochemical analysis of the peninsular Indian mistletoe, *V. capitellatum*, obligate epiparasiting on the parasite *D. falcata* was undertaken to evaluate its antioxidant capacity and to explore the metabolites potentially responsible for the same. In order to facilitate the analysis of the composition of the antioxidant constitution of the mistletoe, ME was subjected to solvent extraction and fractionated into CH_2Cl_2 (DCM), $(CH_3)_2CO$ (acetone) and CH_3OH (methanol) soluble fractions (MEFs). The antioxidant capacities

and the chemical composition of each of these MEFs have been determined. The diverse mechanisms by which the low-molecular weight antioxidant metabolites bring about their activities obviously demand more than one kind of antioxidant capacity measurement.

One of the most commonly employed antioxidant capacity assays involves the generation of the coloured radical cationic oxidant, $ABTS^{\bullet+}$ and determining the ability of an extract/a metabolite to scavenge the same[33]. Though the radical used in the assay is not found in mammalian biology, the decolourization assay is widely recommended for plant extracts since (i) the λ_{max} used for monitoring the stable blue-green chromogen (734 nm) eliminates colour interference in the extracts, (ii) the absorbance reduction tends to become a constant in < 10 min and (iii) $ABTS^{\bullet+}$ is soluble in both aqueous and organic phases and is not affected by ionic strength, thus capable of reacting with both lipophilic tocopherols, carotenes and flavonoids and the hydrophilic polyphenolic glycosides and phenolic acids. Hence, this operationally simple assay using the sensitive $ABTS^{\bullet+}$ has been preferred to determine the scavenging capacities. The determination has shown that ME could potentially scavenge $ABTS^{\bullet+}$ generated *in situ* (Table-1). 67.4 % of the total antioxidant capacity of 407 mg/100 g VCE is found to be concentrated in the more polar CH_3OH fraction while the less polar $(CH_3)_2CO$ fraction contributed only 18.4 % and the lipophilic CH_2Cl_2 fraction shared about 11.0 %. Thus, the free radical scavenging capacity of the CH_3OH fraction was 3.67 and 6.12 folds greater than those of $(CH_3)_2CO$ and the CH_2Cl_2 fractions, respectively. FRAP assay[33], though originally developed to measure the ferric reducing ability of plasma, is conveniently adapted for the assay of antioxidants in botanicals too, since it is simple, rapid, versatile and inexpensive. It also provides reproducible results apart from its applicability to both aqueous and alcoholic media. The redox potentials of both $[Fe(TPTZ)_2]^{3+}$ and $ABTS^{\bullet+}$ are quite comparable. However, the medium required for stable iron salts is acidic as against the neutral medium that is sufficient for the latter. In this method, the antioxidant capacity is determined based on the ability of the antioxidants to reduce the yellow $[Fe(TPTZ)_2]^{3+}$ to blue $[Fe(TPTZ)_2]^{2+}$ and can also be expressed as VCEAC. In the present study, the FRAP assay also measured the highest capacity for the CH_3OH fraction (74.8 %) with the residual capacity distributed among the other two MEFs. The $(CH_3)_2CO$ fraction exhibited approximately one-fifth (16.8 %) while the CH_2Cl_2 fraction about one-seventh (10.99 %) of the total antioxidant capacity. The FRAP assay underestimated the total antioxidant/reducing capacity of the extract by about 22 % (Table-1), probably because (i) FRAP cannot detect compounds that act by radical quenching and (ii) the reducing power appears to be more related to the degree of hydroxylation and the extent of conjugation in polyphenols.

Autoxidation of an aqueous emulsion system of β-carotene and linoleic acid is another test model to assay the antioxidant activities of plant extracts as well as isolated phytoconstituents[49]. A measure of the loss of the yellow colour of β-carotene, caused by its reaction with radicals that are formed by linoleic acid oxidation in the emulsion, forms the basis of this method. When antioxidant constituents are also present in the system, the rate of bleaching is delayed and this concept is used in the evaluation of the antioxidant capacity. Hence, free-radical scavenging activities of the antioxidants are believed to be responsible for the inhibition of lipid peroxidation. It may be inferred from Fig. 2 that except the lipophilic CH_2Cl_2 fraction, which exhibited only 47 % of the activity of the standard eriodictyol, the other MEFs were of comparably similar order ($(CH_3)_2CO$ *ca.* 90 % and CH_3OH *ca.* 81 %) and the total antioxidant capacity was about 1.2 times the standard. However, ME, MEFs and the control eriodictyol were all less potent in inhibition of lipid peroxidation (Fig. 2) compared to the commercial BHT (ranging from 27 % for CH_2Cl_2 to 70 % for the ME).

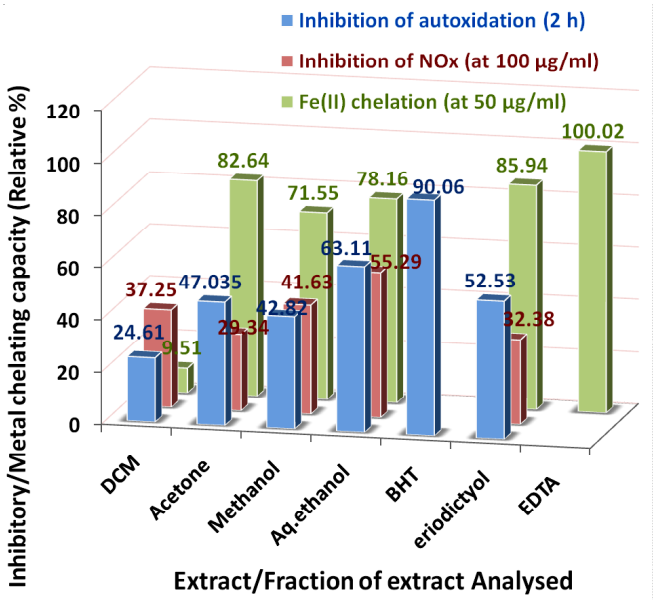

Fig. 2. Per cent inhibition of β-carotene bleaching, NO scavenging and Fe(II) chelating capacity

NO is a defence molecule with cytotoxic, microbiocidal and microbiostatic activities and act as a physiological

TABLE-1
ANTIOXIDANT CONSTITUTION OF *V. capitellatum* EXTRACTS

Determinations	Mistletoe extracts (mg/100 g FM[a])			
	CH_2Cl_2	$(CH_3)_2CO$	CH_3OH	aq. C_2H_5OH
VCEAC-$ABTS^{\bullet+}$	44.815 ± 03.596	74.831 ± 06.634	274.278 ± 09.406	407.109 ± 12.153
VCEAC-FRAP	34.813 ± 01.761	53.275 ± 04.192	237.037 ± 10.066	316.850 ± 07.059
Carotenoid content[b]	na	na	na	29.508 ± 02.467
Phenolic content[c]	2.387 ± 00.441	69.173 ± 07.962	290.447 ± 13.293	372.024 ± 09.572
Flavanone content[d]	na	17.058 ± 03.778	25.502 ± 01.886	51.917 ± 08.131
Flavonol content[e]	na	23.546 ± 02.147	5.047 ± 00.661	37.246 ± 01.787

[a]Mean ± SD of three determinations, [b]Total carotenoids as β-carotene equivalent, [c]GAE, [d]EE, [e]QE, na = not analyzed.

messenger mediating various physiological functions, including immunological, neuronal and cardiovascular tissues[50]. However, large amounts of reactive nitrogen intermediates (RNS), such as NO, peroxynitrite ($ONOO^-$) and other reactive nitrogen oxide species, play significant roles in inflammatory process and are considered to be potentially cytotoxic, causing injury to the surrounding cells. It has been reported to cause mutagenesis and deamination of DNA bases and, more importantly, to form carcinogenic N-nitroso compounds[51]. In addition, NO reacts rapidly and spontaneously with O_2^- to form $ONOO^-$, which is more toxic than both NO and O_2^- to biological systems and thereby cause modification of proteins[52] or nucleic acids[53]. Therefore, studies on terminating the reaction between NO and O_2^-or scavenging RNS become important. In the present study, the scavenging of NO and its products of oxidation by ME and MEFs was investigated according to the method of Marcocci *et al.*[34]. Sodium nitroprusside in aq. solutions at physiological pH spontaneously produce NO, which under aerobic conditions, reacts with oxygen to form stable NO_2^- that can be determined using Griess reagent. Scavengers of NO and other RNS compete with oxygen, leading to the decrease in the production of NO_2^-. ME was found to be 1.7 times more efficient in scavenging RNS compared to the eriodictyol standard (Fig. 2) and the capacity decreased in the order ME > CH_3OH > CH_2Cl_2 > $(CH_3)_2CO$ (respectively 1.7, 1.3, 1.15, 0.9 times the eriodictyol capacity).

Though scavenging of RONS by antioxidant metabolites is the generally accepted mechanism of their antioxidant activity, mechanisms involving metal binding have also been proposed and have gained due consideration. Fe(II) chelating activities of the ME, MEFs, Na_2EDTA and eriodictyol, determined according to the method of Dinis *et al.*[33], are shown in Fig. 2. Ferrozine quantitatively forms complex with Fe(II). However, in the presence of other potentially competing chelating agents, including antioxidant phytoconstituents, formation of ferrozine complex is disrupted, thereby impeding the formation of the red colour imparted by the complex as well. Measurement of this colour reduction, therefore, allows for the estimation of the metal chelating capacity of the coexisting chelator. ME constituents were found to potentially chelate Fe(II) as indicated by its chelating capacity, which is *ca.* 78 % of that of the versatile hexadentate ligand, Na_2EDTA. Standard eriodictyol (85.9 %) and the $(CH_3)_2CO$ (82.6 %) fraction were the active components inferred from this study (Fig. 2). Present knowledge of the essential role of transition metals in the living system is much clear compared to a few decades back. Issues relating to their adverse effects such as the initiation of oxidative processes in lipids, proteins and other cellular components have also emerged as a major concern in the past few decades. Free iron catalyzes the conversion of O_2^- and H_2O_2 into OH^-, which promote ONS leading to subsequent cell apoptosis. Chelation of Fe(II) not only prevents the Fenton reaction but have also been reported to account for the prevention of nuclear DNA damage of human cancer cells exposed to peroxides. Considerable evidence has emerged from clinical studies to show that increases in cellular free iron concentrations have been associated with ONS and that genetic and non-genetic iron misregulations in the brain contribute to neuronal death in certain neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's diseases and Hallervorden-Spatz syndrome. Even mildly elevated iron levels have been linked to increased cardiovascular disease and cancer incidences in humans and hence should be maintained within the optimum level. Moreover, in chronic anaemia associated with iron overload such as thalassemia major, Fe-chelating therapy is the only method available for preventing early death, caused predominantly by myocardial and hepatic iron toxicity or to prevent endocrinal abnormalities like diabetes and hypothyroidism. Persuasive epidemiological evidences, today, have brought to light that regular intake of bioactive polyphenolic compounds promises a wide range of benefits, including the regulation of transition metals such as iron.

Analysis of the antioxidant constitution: Endogenous antioxidant defence mechanisms of a living system involve both enzymatic and non-enzymatic strategies. Vitamins A, C and E, glutathione and the enzymes superoxide dismutase, catalase, glutathione peroxidise and glutathione reductase are the more familiar antioxidants. Others include α-lipoic acid, mixed carotenoids, coenzyme Q_{10}, polyphenols, certain organometallics containing copper, zinc, manganese and selenium and the cofactors. Consequently, several antioxidants, endogenous to plants, can scavenge RONS and current evidences strongly favour the significance of the ubiquitous carotenoids, polyphenols and other low-molecular weight metabolites in preventing or in delaying the onset of a number of chronic human diseases.

Polyphenols are among the most widespread class of metabolites in nature and are derived from the C_6-C_3 phenylpropanoid unit. According to this pathway, biosynthesis produces a variety of plant phenols, such as cinnamic acids (C_6-C_3), benzoic acids (C_6-C_1), flavonoids (C_6-C_3-C_6), proanthocyanidins [$(C_6$-C_3-$C_6)_n$], coumarins (C_6-C_3), stilbenes (C_6-C_2-C_6), lignans (C_6-C_3-C_3-C_6) and lignins [$(C_6$-$C_3)_n$]. The total phenol assay using Folin-Ciocalteu reagent (FCR) is such a convenient, simple and reproducible procedure that it has become the assay of choice in evaluating the phenolic contents of biological materials ever since its extension to the analysis of total phenols in wine by Singleton and Rossi in 1965. Phenolic compounds react with FCR only under basic conditions (pH 10) adjusted by adding aq. Na_2CO_3. The optical density of the blue complex, formed by transfer of electrons from phenolic compounds/other reducing species to Mo, is monitored at a wavelength of 765 nm, which also minimize interferences from the coloured sample matrix. The total amount of phenolics present in 100 g of ME was found to be 372.024 mg (Table-1). About 78 % of these are the more polar components extracted into the CH_3OH fraction and the others, about 19 % were determined from the $(CH_3)_2CO$ fraction. The CH_2Cl_2 was estimated to contain an infinitesimally small amount of phenolics (0.64 %).

The flavonoids are built upon a C_6-C_3-C_6 flavone skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen. Several classes may be differentiated according to the degree of unsaturation and the extent of oxidation of the three-carbon segment. Within the various classes, further distinction is possible based on the number and nature of substituents attached to the rings. Their remarkable antioxidant properties arise from the redox and

chelating capabilities of the phenolic groups conjugated to the stable delocalized π-electron clouds of the aromatic system. Reduction, formation of alkali metal phenolates and metal chelation results in the extension of the conjugation of the cinamoyl π-electron system of flavones and flavonols, leading to their absorption maxima (*ca.* 510 nm) in the visible spectrum. Quantitative determinations of these classes of flavonoids are generally carried out by exploiting this process[33] and the total flavones/flavonols content has been found to be about 37 mg QE/100 g FM (10 % of the total phenolics). More than 63 % of these were concentrated in the $(CH_3)_2CO$ fraction (Table-1). Flavanones/dihydroflavonols content was determined by employing 2,4-dinitrophenylhydrazine that reacts with the carbonyl function to form dinitrophenylhydrazones[36], absorbing at 486 nm. The total flavonoids of these classes of compounds were about 14 % of the total phenolics. Of this, 49 % was found to be in the CH_3OH fraction and 33 % in the $(CH_3)_2CO$ fraction.

The potent antioxidant capacity associated with the more polar, $(CH_3)_2CO$ and CH_3OH, fractions appears to be apparently contributed by the phenolic acids, flavonols, flavanones and other polymeric phenols elaborated in the mistletoe. In addition to its antibacterial, antiviral, antifungal, antioxidant, antiinflammatory, anticarcinogenic and antigenotoxic activities, reported in literature and offering protection in hepatic, renal, cardiovascular and neurodegenerative disorders, sources rich in phenolic acids, particularly, chlorogenic acid and caffeic acid appear to have a promising future in the most prevalent type-2 diabetes[43,44,54,55]. The flavanone, eriodictyol and its derivatives are known radical scavenging antioxidants that have also been reported to induce prooxidative macromolecular damage and cytotoxicity in cancer cells[45]. Carotenoids, terpenes, terpenic acids and phytosterols have been isolated as the metabolites contributing to the lipophilic antioxidant capacity of the ME. Betulinic acid and its reduced congener, betulin, are naturally occurring pentacyclic triterpenes that are known to exhibit a variety of biological activities, including antioxidant, antitumor, antibacterial, antimalarial, antiinflammatory and anthelmintic and are capable of inhibiting human immunodeficiency virus[56,57]. A recent review illustrates the antiprotozoal, antimicrobial, antiinflammatory, antitumour and chemoprotective characteristics, specifically cancer, cardio and hepato protective characters of lupeol[37]. The hypoglycemic, antiulcer, antihyperlipidemic, hypotensive, cardiotonic, antidysrhythmic, anticancer, antiinflammatory, antimycobial, antifertility and hepatoprotective activities of oleanolic acid have also been reviewed[42]. Further, oleanolic acid is also reported to offer protection against renal toxicity and aggregation of blood platelet. Phytosterols, including β-sitosterol, have been found to reduce intestinal cholesterol absorption, leading to decreased blood LDL-cholesterol levels and lowered cardiovascular disease risk and also to increase the activity of antioxidant enzymes, thereby reducing ONS[58]. Considerable emerging evidences also support the inhibitory actions of phytosterols on lung, stomach, as well as ovarian and breast cancer cells. They appear to act through multiple mechanisms of action, including inhibition of carcinogen production, cancer-cell growth, angiogenesis, invasion and metastasis and through the promotion of apoptosis of cancerous cells[58,59]. The carotenoids may participate in the propagation step of the oxidation process as chain-breaking antioxidants that scavenge reactive peroxyl radicals. A recent publication offers a wealth of information on structural, chemical and electrochemical properties, including relevant information on the antioxidant properties and the actual functioning of carotenoids in nature[60].

Conclusion

Mounting evidences support the involvement of free radical-mediated damages in the pathophysiology of a number of human illnesses. The capacity of the ubiquitous carotenoids, phytophenols, steroids and other low-molecular weight metabolites, endogenous to plants, to scavenge these reactive species and thereby prevent or delay the onset of these diseases is also well documented. Health benefits of the natural products are generally the outcome of the additive and synergistic combinations of the scores of metabolites elaborated in the taxon. From the present study, it may be learnt that the sanctity attached to the mistletoes may be the consequence of the realization of their potential curative characteristics by the traditional communities and the same may be attributed to the antioxidant phenolics, terpenoids and steroids accumulated in their vegetative parts.

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