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Peroxyl Radical Scavenging Capacity of the Flavonolignan Silybin, Ginkgo Biloba Extract EGb 761, American Green Tea and a Series of Germacranolides

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ABSTRACT *: We report on the applicability of a method recently developed in our laboratory for measuring the antioxidant potential of isolated chemicals and extracts derived from natural products. Peroxyl radicals generated by thermal homolysis of 2,2'-azobis-amidinopropane (ABAP) oxidize α-keto-γ-methiolbutyric acid (KMBA) to ethylene, which is monitored by gas chromatography. Inhibition of ethylene formation in the presence of antioxidants that compete with KMBA for peroxyl radicals is the basis of the Total Oxyradical Scavenging Capacity Assay (TOSCA; Winston et al., 1998). Antioxidative activities of water-soluble extracts of American green tea, the anti-hepatotoxic flavonolignan from milk thistle (Silybum marianum) silybin, Ginkgo biloba extract EGb 761, and a series of naturally occuring sesquiterpene lactones (all germacranolides found in in fungi, liverworts, and plants) were studied. The specific TOSC value per μM silybin was 5.2, which is essentially comparable to that of Trolox®, a water-soluble vitamine E analog. Tea and Ginkgo extracts exhibited potent peroxyl radical scavenging capacity with values, respectively of ≅1700 and 1000 μmols Trolox® equivalent per gram dry matter. The known anti-inflammatory activity of some germacranolides prompted study of their antioxidant capacity. None of the lactones exhibited antioxidant capacity toward peroxyl radicals comparable to Trolox®; costunilide, the most lipophilic, had a TOSC value ≅ to glutathione. The potential role of peroxyl radicals in lipid peroxidation, other cellular damage, and various disease states suggest a possible preventive role for silybin, green tea and Ginkgo biloba in oxidative stress caused by these free radical species.*

Key Words *: Peroxyl radical, Antioxidant, Ginkgo biloba, Silybin, Green tea, Sesquiterpene lactones*

I. INTRODUCTION

Formation of reactive oxygen species (ROS) in aerobic organisms is an unavoidable consequence of the coupling of oxidative phosphorylation of ADP with the reduction of molecular oxygen by four electrons to water. Other well recognized sources of ROS production include microsomal and mitochondrial electron transport, active phagocytosis, and the activity of several enzymes, e.g. xanthine oxidase, tryptophan dioxygenase, diamine oxidase, prostaglandin synthase, guanyl cyclase and glucose oxidase, which produce different ROS as intermediates (Cadenas *et al.*, 1984; Winston and Cederbaum, 1983a,b; Asada *et al.*, 1974; Nakagawara and Minakami, 1975; Babior, 1984; Fridovich, 1974; Fridovich, 1978; Halliwell, 1978). Xenobiotics and environmental pollutants may increase the intracellular formation of ROS for example through the Fenton reaction involving trace metals such as iron and copper (Halliwell and Gutteridge, 1984; Winston *et al.*, 1984) or redox cycling of several classes of organic compounds (Kappus, 1986). During redox cycling certain molecules may be reduced to their corresponding free radical, which rapidly donates its free electron to molecular oxygen producing the superoxide anion radical ($O_2^{\cdot}$) and regenerating the parent compound to undergo another cycle (Winston *et al.*, 1991).

To counteract the biological potential of ROS formation cells have evolved complex antioxidant defenses

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of specially adapted enzymes (e.g., superoxide dismutase, catalase and glutathione peroxidase) and smaller molecules such as vitamin E and β-carotene (as free radical scavengers in membranes), ascorbic acid, uric acid and glutathione (for the aqueous phase). In this respect, the intracellular production of ROS does not necessarily imply cellular toxicity, but oxidative stress will occur when the balance between ROS formation and antioxidant defenses is exceeded.

Oxidative stress has been implicated in several cellular toxicity processes, such as damage to proteins, enzyme inactivation, peroxidation of lipid membranes, DNA alteration (Cohen and d'Arcy Doherty, 1987) and various pathologies including chemical carcinogenesis, heart disease, reperfusion injuries, reumathoid arthritis, inflammation and aging (Gey *et al.*, 1991; Cutler, 1991).

Recently there has been substantial interest in antioxidant chemicals of foods, and the advertising of certain comestible products often extols the virtues of the antioxidants they contain. Commercial vitamins are often marketed as antioxidant formulas and health food stores promote many products for their antioxidant potential. Antioxidants found in red wine have been linked to the so-called French paradox, in which substantial consumption of red wine seems to lower the rates of coronary heart disease despite a diet high in saturated fats (Soleas *et al.*, 1997; Serafini *et al.*, 1997). Green tea is one of the most widely consumed beverages worldwide, and is now recognized for its extraordinary polyphenol content, which includes various catechins and flavonoids (Graham, 1992). These constituents are noted for their antioxidant properties (Valcic *et al.*, 1999; Dreosti, 1996; Terao *et al.*, 1994), and particular interest has been in their anticancer effects (Yang and Wang, 1993; Katiyar and Muktar, 1996).

The flavonoid (a flavonolignan) sylibin is used in the treatment of human liver pathology (Gyorgy *et al.*, 1992). It has been shown to prevent tert-butylhydroperoxide-induced lipid peroxidation and cell mortality in isolated rat hepatocytes (Fernandes *et al.*, 1995), decrease the accumulation of malondialdehyde protein adducts in iron-filled periportal hepatocytes in vivo during long-term iron overload in rats (Pietrangelo *et al.*, 1995), significantly reduce reperfusion-induced arrythmia and ischemia (Chen *et al.*, 1992), and inhibit iron-ADP-dependent microsomal lipid peroxidation in vitro (Valenzuela and Guerra, 1986). Each of these activities of sylbin were ascribed to its antioxidant capacity.

Ginkgo biloba is probably the most studied herb in the world with over 300 publications appearing in the literature. It is the most commonly used herbal remedy in Germany and France for numerous disorders, including circulatory disorders, and in 1997 sales exceeded $163 million in Germany alone (Gorman, 1997). In the United States Ginkgo biloba is marketed only as a dietary supplement. EGb 761 is a standardized extract of dried leaves of this plant and contains 24% flavone glycosides, including three major bioflavonoids (quercetin, kaemferol, isorhamnetin) and 6% terpene lactones, including diterpene Ginkolides and the sesquiterpene bilobilide (Kleijnen and Knipschild, 1992; Cott, 1995). This extract of Ginkgo biloba was reported to protect brain neurons from hydrogen peroxide-induced oxidative stress (Oyama *et al.*, 1996) and arrest many of the cardiomyopathic symptoms developed during streptozotocin-induced diabetes in rats (Fitzl *et al.*, 1999). Diabetic damage has been partly attributed to oxidative stress. Moreover, in vivo treatment of rats with 100 mg/kg extract EGb 761 significantly reduced skin flap necrosis, a condition in which a role for free radicals has been proposed (Bekerecio *et al.*, 1998). In this regard, EGb 761 has been reported to be a potent scavenger of hydroxyl, peroxyl, NO, and superoxide anion radicals (Boveris and Puntarulo, 1998). Further, this Ginkgo biloba extract catalyzes the dismutation of superoxide anion (Pincemail *et al.*, 1989) and modulates the glutathione redox cycle in vascular endothelial cells (Rong *et al.*, 1996). These antioxidative activities have been ascribed mainly to the flavonoid content of Ginkgo biloba extract.

Among naturally occurring plant products the sesquiterpene lactones represent one of the largest groups with demonstrated cytotoxic and anti-tumor activity (Picman, 1986). In a study of 54 sesquiterpene lactones, the germacranolides were the most active as inhibitors of serotonin release (Marles, 1995). The presence of an α,β-unsaturated moiety, in all cases, was required for this activity, as is true for their anti-

tumor activity (Picman, 1986). Parthenolide, a germacranolide, is purported to have antitumor, antibacterial, antifungal properties (Picman, 1986), antiinflammatory activity (Heptinstall *et al.*, 1985) and antiarthritis activity (Pattrick *et al.*, 1989). Parthenolide and other sesquiterpene lactones have been found to inhibit the expression of inducible cyclooxygenase (COX-2) and proinflammatory cytokines in macrophages (Hwang *et al.*, 1996). The activities of many antiinflammatory agents have been ascribed to their ability to act as antioxidants.

Herein, we report on the applicability of a simple and reliable assay of antioxidant activity, which is based on the reaction between peroxyl radicals and α-keto-γ-methiolbutyric acid (KMBA) which is oxidized to ethylene (Winston *et al.*, 1998) upon reaction with various reactive oxygen species. In light of the above, it was of interest to study the peroxyl radical scavenging capacity of the flavonolignan Silybin, *Ginkgo biloba* extract Egb 761, American green tea aqueous extracts, and some sesquiterpene lactones in this assay system and attempt to assign quantifiable values to this scavenging capacity.

II. MATERIALS AND METHODS

Reagents: α-keto-γ-methiolbutyric acid (KMBA) was purchased from Sigma Chemical Co. (St Louis, MO, USA); 2,2'-azobis-amidinopropane (ABAP) was obtained from Wako Chemicals (Richmond, VA, USA);

Germacranolides: parthenolide was isolated from leaves of *Magnolia grandiflora* as described previously (el-Feraly and Chan, 1978), 11βH,13-dihydroparthenolide was obtained from the common ragweed *Ambrosia artemisiifolia* L. (Fischer *et al.*, 1981), costunolide was isolated from costus resin oil purchased from Pierre Chauvet S. A., France (Lu and Fischer, 1996); enhydrin was obtained from *Polymnia uvedalia* (Tak *et al.*, 1994), melampodin A was isolated from *Melampodium leucanthum* (Fischer *et al.*, 1972) and 6-*epi*-desacetyllaurenbiolide from *Montanoa grandiflora* (Quijano *et al.*, 1984) was kindly provided by Dr. Leovigildo Quijano (Instituto de Química, Universidad Nacional Autónoma de Mexico).

Silybin and Ginkgo biloba: Silybin (Silibinin; 2,3-dihydro-3-[4-hydroxy-3-methoxyphenyl]-2-[hydroxymethyl]-6-[3,5,7-trihydroxy-4-oxobenzopyran-2-yl]benzodioxin) was kindly donated by Dong-Kuk Pharmaceuticals (Seoul, Korea). *Ginkgo biloba* extract (EGb 761) was obtained from Yu-Yu Industrial Co. (Seoul, Korea).

Green Tea: Tea bags (Lipton® green tea and Bigelow® green tea) were purchased from a local merchant and prepared according to package instructions for consumption. Essentially, one tea bag was steeped in 250 ml of near boiling water (≅ 99°C) for 1.5 min. The dry tea content of the bags were 2.0±0.2 grams. After the tea was prepared the solutes were evaporated to dryness and the residue weighed to quantify the total amount of solid matter in a typical serving of the tea and for determination of solute amounts used in the assays.

Oxyradical Scavenging Capacity (TOSC) Assay: The TOSC assay of Winston *et al.* (1998) was used to evaluated antioxidant behavior of the various antioxidants studied herein. Essentially, peroxyl radicals were generated by the thermal homolysis of ABAP at 39°C. The assay conditions used were 0.1 mM KMBA and 10 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4. Reactions were carried out in 10-ml vials sealed with Mininert® valves (Supelco) in a final reaction volume of 1 ml. The reactions were initiated by the injection of 100 μl of 100 mM ABAP in water directly through the rubber septum. Ethylene production was measured by gas-chromatographic analysis of 1-ml aliquots taken directly from the headspace of the reaction vials. Samples were monitored in sequence at 12-min intervals. Analyses were performed with a Hach-Carle (Series 100 AGC) gas chromatograph equipped with a 6 foot Poropack N column (Supelco) and a flame ionization detector (FID). The oven, injection and FID temperatures were respectively, 60°, 280° and 190°C. Helium was used as the carrier gas at a flow rate of 30 ml/min.

Quantification of TOSC: The area under the kinetic curve was calculated from the integral of the equation that best defines the experimental points for the control and sample reactions. TOSC is then quantified according to equation 1, where $\int$SA and $\int$CA are the integrated areas from the curve defining the sample and control reactions, respectively. Thus, a sample that displays no oxyradical scavenging capacity would

give an area equal to the control making

$$TOSC = 100 - \left(\frac{\int SA}{\int CA} \times 100\right) \quad (1)$$

the ($\int SA/\int CA$) equal to one and hence a corresponding TOSC value of zero. Conversely, as $\int SA$ approaches 0 the hypothetical TOSC approaches 100. Specific TOSC values (sTOSC) were calculated from the slope of the linear regression lines for the TOSC curves. Relative TOSC values (rTOSC) were calculated as shown in equation 2 by dividing the

$$rTOSC = \frac{sTOSC(antioxidant)}{aTOSC(Trolox)} \quad (2)$$

rTOSC of the antioxidants tested by the rTOSC obtained for Trolox®, a water-soluble analog of α-tocopherol (vitamin E), thus establishing a scale based on Trolox® equivalents. Statistical analysis was performed using the spreadsheet program Excel by Microsoft and Mathematica 3.0 for students by Wolfram. A specific micromolar TOSC may be obtained by interpolation of linear plots of rTOSC vs concentration curves (see Fig. 3).

III. RESULTS

Green Tea: Figure 1 shows the effects of Bigelow® green tea on peroxyl radical-dependent ethylene production from KMBA. Here it can be see that this green tea preparation inhibited ethylene production in a concentration-dependent manner. When prepared in accord with the package instructions (Methods) the tea contained 1.01 mg/ml of solute or in a typical serving of 200 ml, 202 mg. Another brand, Lipton® green tea gave similar inhibition curves (not shown). According to the Lipton® green tea package each serving contains approximately 135 mg of flavonoids, which would account for about 6.2~7.5% of the dry content of the tea bag. Bigelow® tea does not specify antioxidant content on their package. A specific TOSC value of 9.8 per mg for total solute content of a "serving" of Bigelow® tea was calculated from the linear portion of TOSC vs tea solute concentration plots (Fig. 1 insert). Similarly, the specific TOSC for Lipton® green tea was 11.6 per μg. Both brands of green tea displayed similar hyperbolic curves in the kinetic

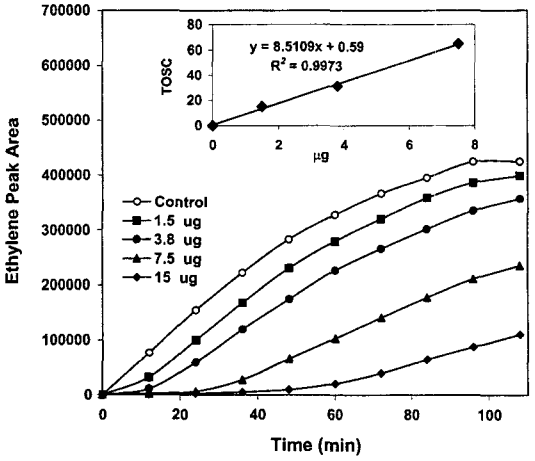

Fig. 1. Time-course of ethylene generation from KMBA oxidation upon thermal homolysis of ABAP for reactions with the different amounts of Bigelow® green tea aqueous extract indicated in the graph. The amount values are mg of dried residue equivalents that were present in the final reaction mixture of 1 ml. Reactions were carried out for 110 min at 39°C. The reaction was initiated by addition of ABAP. The final reaction volume was 1.0 ml as described in Methods and Materials.

profiles of the inhibited reactions. These were characterized by a finite period of complete inhibition followed be an apparent recovery period of ethylene production. These data are indicative of antioxidants that are exhaustible upon quenching of oxidizing radicals (Winston *et al.*, 1998; Cao *et al.*, 1993).

Silybin: The ability of the flavonolignan silybin to protect KMBA from oxidation by peroxyl radicals is shown in Fig. 2. Very potent inhibition is seen at the highest concentration (100 μM) of silybin; ethylene production was suppressed to greater than 99% of controls by this concentration throughout the time course studied. Even at concentrations as low as 2 μM inhibition was observed. A specific micromolar TOSC was calculated from the linear portion of plots of TOSC vs concentration curves (Fig. 2 insert) and was found to be 5.2 per μM. This value was comparable to that of Trolox® (5.6 per μM).

Ginkgo biloba extract: A representative inhibition curve for an extract of *Ginkgo biloba* is shown in Fig. 3. This extract was also shown to afford potent antioxidant protection against KMBA oxidation by peroxyl radicals generated during thermolysis of ABAP (data not shown). Ethylene production was inhibited by about 50% in the presence of 8~10 μg *Ginkgo biloba* extract EGb 761. Nearly total inhibition was

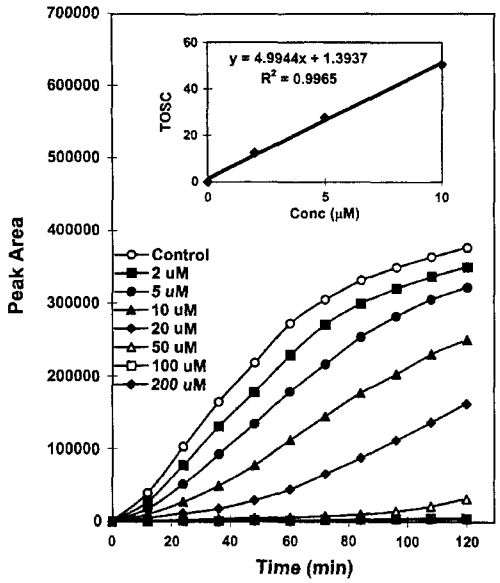

Fig. 2. Time-course of ethylene generation from KMBA oxidation upon thermal homolysis of ABAP for reactions with the different concentrations of silybin indicated in the graph. Reactions were carried out for 120 min at 39°C. The reaction was initiated by addition of ABAP. The final reaction volume was 1.0 ml as described in Methods and Materials.

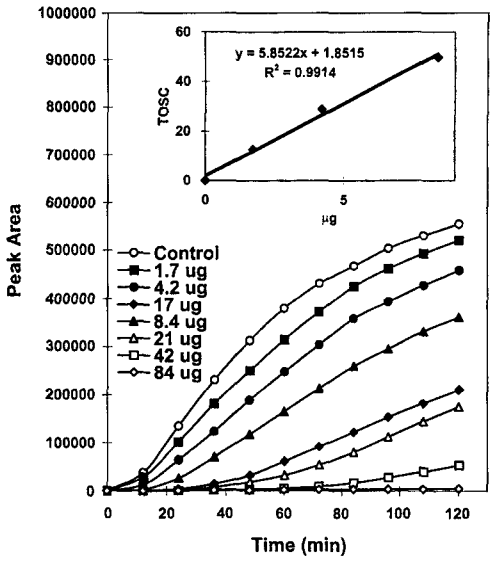

Fig. 3. Time-course of ethylene generation from KMBA oxidation upon thermal homolysis of ABAP for reactions with the different amounts of Ginkgo biloba extract indicated in the graph. Reactions were carried out for 120 min at 39°C. The reaction was initiated by addition of ABAP. The final reaction volume was 1.0 ml as described in Methods and Materials.

observed over the entire time course of 120 min in the presence of 84 μg of this extract. As was noted for the green tea extracts, the kinetic curves for the antioxidant capacity of *Ginkgo* extract show a finite period of complete protection in the reaction; after a certain time, ethylene production is seen to increase in a trend that indicates exhaustion of the antioxidative components. A specific TOSC value generated from linear plots of TOSC vs *Ginkgo* (Fig. 3 insert) extract content was estimated at about 5.4 per μg. Thus, on a per weight basis, the TOSC of Ginkgo extract is approximately 47% that of the Bigelow® green tea and about 55% that of Lipton® green tea.

Germacranolides: The structures of the germacranolides tested are shown in Fig. 4. This group was subdivided into two subgroups, depending on the configuration of the 1,10- and 4,5- double bond in the cyclodecadiene ring. Compounds 1~4, contain a *trans,trans*-cyclodecadiene skeleton and are called germacrolides (Fischer, 1990), whereas compounds 5 and 6, with a cisoid arrangement of the 1,10-double bond, are melampolides. Five of the germacranolides, are 12,6-lactonized and one of them, 6-*epi*-desacetyllaurenobiolide, 4, is lactonized in the 12,8 position.

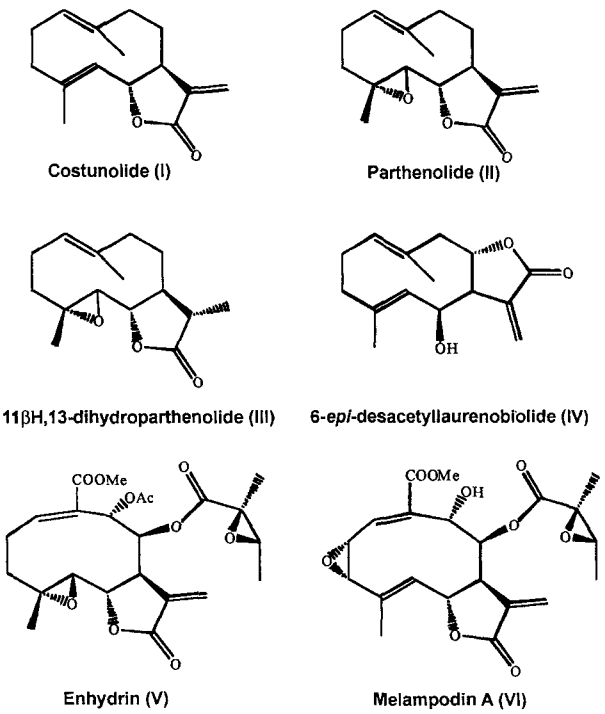

Fig. 4. Chemical structure of the germacranolides studied.

In the presence of the germacrolides, ethylene production was reduced in a concentration-dependent manner, indicating their capacity to compete for per-

Table 1. Linear regression slopes and intercepts of TOSC curves of the germacranolides

Compound	y-int	Slope	R^2
Costunolide	8.71	0.91	0.9987
Parthenolide	12.32	0.20	0.9966
Dihydroparthenolide	8.16	0.21	0.9907
6-*epi*-desacetyllaurenobiolide	15.23	0.71	0.9950
Enhydrin	0.50	0.06	0.9897
Melampodin A	1.68	0.11	0.9659

Table 2. Specific (per μM) and relative TOSC values for the sesquiterpene lactones tested

Compound	sTOSC	rTOSC
Trolox	5.62	1
GSH	1.01	0.18
Costunolide	0.91	0.162
Parthenolide	0.20	0.036
Dihydroparthenolide	0.21	0.037
6-*epi*-desacetyllaurenobiolide	0.71	0.126
Enhydrin	0.06	0.011
Melampodin A	0.11	0.020

oxyl radicals with KMBA (data not shown). The melampolides did not exhibit significant antioxidant activity up to 200 μM (data not shown). Specific TOSC (sTOSC) values were calculated from the slope of the regression line within the compound's linear range of TOSC values. The slopes and intercepts of the TOSC regression lines are listed in Table 1. The specific TOSC (sTOSC) values for all of the germacranolides are shown in Table 2. In general, the germacrolides (compounds I-IV) have higher sTOSC values than the melampolides (compounds V and VI). For the former, sTOSC values fall into two clusters; sTOSC for parthenolide (II) and its analog dihydroparthenolide (III) were 0.2. Costunolide (I) and 6-epi-desacetyllaurenobiolide (IV) display sTOSC values, respectively 4.5 and 3.5 times greater than the melampolides. The relative TOSC values (rTOSC) are relative to the benchmark antioxidant, Trolox®. The rTOSC values for the germacrolides are 0.16 for costunolide, 0.04 for parthenolide, 0.04 for 11βH,13-dihydroparthenolide and 0.13 for 6-*epi*-desacetyllaurenobiolide (Table 2). Thus, relative to Trolox®, the germacranolides are weak antioxidants.

IV. DISCUSSION

The TOSC assay is a simple and reliable method for measuring the antioxidant potential of various chemicals and biological samples. It has proven robust in the determination of the peroxyl radical scavenging capacity of a homologous series of bioflavonoids (Dugas *et al.*, 2000), apple tissue extracts (Eberhardt *et al.*, 2000), various vitamins, biogenic and synthetic antioxidants, including ascorbate, melatonin, urate and BHA (Winston *et al.*, 1998), and cytosolic and microsomal fractions of rat and marine organisms (Winston *et al.*, 1998; Regoli *et al.*, 2000). The present report shows the strength of this assay in the assignment of a quantifiable parameter (the TOSC value) for the relative peroxyl radical scavenging capacity of mixtures of antioxidants in extracts of Ginkgo biloba and green tea, as well as additional purified natural chemicals such as silybin and germacranolides. The generation of peroxyl radicals is common to organisms and may be especially high in oxidatively stressed organisms. The scavenging of peroxyl radicals is a key step in the prevention of lipid peroxidation by breaking the chain of propagation of free radical reactions; thus, prompting the study of many compounds with respect to their ability to scavenge these radicals (Winston *et al.*, 1998; Wayner *et al.*, 1986; Cao *et al.*, 1993). The assay has been shown to compare very favorably with results obtained from other common assays used for similar purposes in- cluding the ORAC assay (Cao *et al.*, 1993) and the TRAP assay (Wayner *et al.*, 1986).

The role of dietary antioxidants in disease prevention is a topic of increasing research interest. In particular, the flavonoids and polyphenolic compounds in various tea commercial blends have received significant attention (Graham, 19992; Yang and Wang, 1993; Cao *et al.*, 1996; Dreosti, 1996; Katiyar and Muktar, 1996; Valcic, *et al.*, 1999). In an ongoing program in our laboratory, we have been studying the antioxidative activity of several tea blends and herein, report on two popular American brand green teas produced by two of the largest manufacturers of tea worldwide, namely Lipton® and Bigelow® green teas. Both of these teas, when prepared in accord with package instructions showed strong peroxyl radical scavenging activity in the TOSC assay. Presumably, the antioxidant activity measured reflects the presence of flavonoid content of the teas. A very potent antioxidant found only in green tea is epigallocatechin gallate (EGCG), albeit a TOSC value has not been

obtained. Yang and Wang (1993) reported on the inhibition of tumorigenesis by tea polyphenols in various animal models. A detailed analysis of the precise flavonoid content of the two teas tested is beyond the scope of this study, nevertheless, in a single serving of both of the green teas that we did study there are approximately 200 mg of water-extractable solute. For 1 μg of solutes the specific TOSC values were about 10±1, a value that is roughly equivalent to 1.8 μM of Trolox®. Expression of this value as mmol of Trolox® equivalent per gram of dry solute permits a reasonable comparison with the antioxidative capacity of the dry matter of some fruits, vegetables and tea studied by Cao *et al.* (1996) in the ORAC assay. A caveat in this comparison is that Trolox® equivalents were based on specific TOSC values calculated only from the range of concentrations in which TOSC is linear, which might not be the case with the ORAC assay. Further, the teas were prepared differently between the two laboratories. Nevertheless, relative TOSC values of various antioxidants to Trolox® compare very favorably between the two assays. Thus, the Lipton® and Bigelow® green tea blends have values of about 1,800 μmol Trolox® equivalent per gram dry tea residue as compared to the green tea used by Cao *et al.* (Chin Chu oriental blend), which had a value of about 820 μmol Trolox® equivalent per gram. In either case, our data are consistent in that they show the very potent antioxidant activity as compared to dry weight matter of numerous fresh fruits and vegetables. Despite studies cited that indicate possible preventative effects of tea consumption towards cancer (Yang and Wang, 1993; Katiyar and Muktar, 1996), the precise implications for human health of tea consumption remain unclear. Epidemiological studies have not indicated a definitive trend of prevention of cancer risk in humans (International agency for Research on Cancer, 1991).

The μM specific TOSC for the flavonoid silybin was essentially that for Trolox® (5.2 vs 5.6, respectively), which indicates its high potency as a peroxyl radical scavenging agent. As alluded to above in our discussion of teas, the antioxidant activity of flavonoids is an area of intensive study. Three functional groups that are attributed to an increase in oxyradical scavenging potential among the flavonoids are: 1) the *o*-dihydroxy structure of the B ring; 2) the C_2-C_3 double bond in concert with a 4-oxo functionality of the C ring; and 3) the additional presence of both a 3- and 5-hydroxyl moiety of the C and A ring, respectively (Bors *et al.*, 1990). A quantum chemical explanation has been proposed to explain the increase in oxyradical scavenging potential of these functional groups (Van Acker *et al.*, 1996). We note that silybin does not meet either the first and second criteria. However, it does have hydroxyl groups at positions C5 and C7 of the A ring and a C3 hydroxyl on the C ring, in essence meeting criteria 3. It is noted that the OH group at position 3 of the C ring is not an SP^2-OH (phenolic). Typically, nonphenolic OH groups do not contribute to antioxidant action (Dugas *et al.*, 2000). The fact that only one of the three aforementioned criteria for antioxidant potency are met in silybin may account for its relatively low activity as compared to quercetin (Dugas *et al.*, 2000). Quercetin meets all three criteria and is 7 times more potent as a peroxyl radical scavenger in the TOSC assay than silybin. Hydroxylation of both the A and B rings enhances the antioxidant potential of flavonoids. There is no OH substitution in the C ring of the flavone moiety of silybin. The C4' and C5' OH groups of silybin have been derivatized through dioxo linkage of the lignan moiety, which contains an *o*-methoxy-hydroxyphenyl group. This seems to play a role in the activity of this molecule as evinced by the fact that 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone with a hydroxy group in the B ring *ortho* to a methoxy group scavenges peroxyl radicals with similar efficiency (TOSC = 4.5) as does silybin, whereas an analogue of this flavonoid that contains a 4' hydroxy group but without an ortho methoxy group has a TOSC value with respect to peroxyl radical of 1.2 (Dugas *et al.*, 2000).

The specific TOSC value for Ginkgo biloba EGb 761 was 5.4 per μg dry matter, a value very close to the TOSC of 1 μM Trolox® or about 1,000 umols Trolox® equivalent per gram Gink biloba dry matter. As stated under Introduction, Ginkgo EGb 761 is standardized to 24% bioflavonoids, which undoubtedly account for the preponderance of the antioxidant activity toward peroxyl radical exhibited by this extract. If this is the case, the specific TOSC is indicated to be 22.5 per μg of bioflavonoids. Quercetin, a major flavonoid of Ginkgo extract has a TOSC value that is 7~10 times greater than that of Trolox®, and its glycoside is about 4~5

times greater. The presence of this flavonoid probably plays a significant role in the expressed activity of this extract, but apparently does not account for all of the activity. The antioxidant capacity of the sesquiterpene lactones reported herein (see below) are quite weak as peroxyl radical scavengers and presumably play less of a role in this activity of Ginkgo, albeit the ability of Ginkgo EGb 761 to scavenge superoxide anion has been ascribed to the terpenoids that it contains (Gardes-Albert *et al.*, 1990).

Finally, the germacranolides presented in this study contain highly constrained rings (Tak *et al.*, 1994; Fischer *et al.*, 1972; Quijano *et al*, 1984) and both subgroups, germacrolides and melampolides, are stable. As alluded to above, the antioxidant activity of the germacrolides appears to be related to the number of double bonds in the molecule. Compounds I and IV contain two double bonds in the cyclodecane ring and the less active compounds II and III contain only one. Comparing the two melampolides, compound VI, with double bonds at the 1,10 and 4,5 positions is consistently more active the compound V, which is 4,5-epoxidated. The conjugated 11,13-exomethylene double bond is not likely to contribute to antioxidant behavior since compound II and its 11,13-dihydro derivative III, are equally as potent. Such double bonds appear to only impart antioxidant capacity when not in conjugation to a carbonyl group. Thus, the relatively weak antioxidant capacity of the melampolides compared to the germacrolides may be due to the substitution of the 1,10-double bond, which is conjugated in both cases to an ester carbonyl at C-14. The greater protection against KMBA oxidation by costunolide and 6-*epi*-desacetyllaurenobiolide could be accounted for by the presence of an additional double bond at the C4-C5 position, which is absent in parthenolide and 11βH,13-dihydroparthenolide. Nevertheless, the lower TOSC value for parthenolide indicates that its biological role as an anti-inflammatory agent is not due to its ability to act as an effective antioxidant, and this is likely the case for the antiinflammatory activity of the terpenoids of Ginkgo biloba (Boveris and Puntarulo, 1998).

Taken as a whole, the green teas, silybin and Ginkgo extract EGb 761 are all indicated to be reasonably strong peroxyl radical scavenging agents. In light of the potential role of peroxyl radicals as mediators of tumor initiation and promotion (Marnett, 1987), the antioxidative activity of these natural herbal substances suggests their possible therapeutic role in cancer prevention as well as in other diseases that may be linked to oxidative stress.

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