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Total Antioxidant Potential of Resinous Exudates from *Heliotropium* Species, and a Comparison of the ABTS and DPPH Methods

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Total reactive antioxidant potential (TRAP) of resinous exudates from *Heliotropium* species was evaluated by measuring the bleaching of stable free radicals. The antioxidant capacity of the resinous exudates in Trolox equivalents, evaluated from the bleaching of ABTS derived radical cations, ranged from 2.0 M (*H. huascoense*) to 5.2 M (*H. stenophyllum*), indicating a very high concentration of phenolic compounds. Considerably smaller values were obtained by measuring the bleaching of DPPH radicals. The ratio between the values obtained employing ABTS derived radicals and DPPH, ranged from 37 (*H. megalanthum*) to 4.5 (*H. chenopodiaceum* variety *typica*). The magnitude of the difference can be considered as an indication of the relative reactivity of the antioxidants present in the exudates. Similar ratios were observed when stoichiometric coefficients were evaluated for representative purified flavonoids obtained from the resinous exudates.

Keywords: *Heliotropium*, resinous exudates, DPPH, TRAP, flavonoids, antioxidants

INTRODUCTION

In search of an explanation of the role of resinous exudates, it has been proposed that they could constitute the first stage of protection against predators. This protection could be due both to a mechanical effect, associated with a sticky character which causes them to retain predators,[1] and to a chemical protection due to the presence of secondary metabolites with antimicrobial, antioxidant and cytotoxic properties.[2] On the other hand, the predominant occurrence of resinous exudates in plants growing in arid and semi-arid regions has been explained in terms of the extreme conditions of the environment[3] and, in particular, to the increased oxidative stress imposed upon them.[4] Also, it has been proposed that the presence of antioxidants in the exudates could prevent the oxidative degradation of other components of the resin that could lead to the loss of their physicochemical properties.[5]

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All the above proposed explanations emphasize the role that the presence of antioxidants could play in the resinous exudates. However, few studies have been carried out regarding the total antioxidants in exudates and the antioxidant capacity of their components. Among the procedures employed to evaluate the antioxidant capacity of pure compounds and/or complex extracts are those based on measurements of the consumption of stable free radicals. These methodologies assume that the consumption of the stable free radical ($X^{\bullet}$) will be determined by reactions such as

$$X^{\bullet} + YH \longrightarrow XH + Y^{\bullet} \qquad (1)$$

where YH is the additive and/or any antioxidant present in the added extract. The rate[6–8] and/or the extent of the process,[9–17] measured by the decrease in $X^{\bullet}$ concentration, would be related to the ability of the added compounds to trap free radicals. In the present communication we have applied this approach, employing 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the radical cation derived from 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as stable free radicals, to resinous exudates and purified flavonoids obtained from several species of the genus *Heliotropium*. These measurements provide an estimate of the total antioxidant potential (TRAP) of the exudates.[14,16,18]

MATERIALS AND METHODS

Resinous Exudates Extraction and Flavonoids Isolation

Samples were collected during the flowering season (October 1993). The species *H, huascoense*, *H. megalanthum*, *H. sinuatum*, *H. fili-folium*, *H. chenopodiaceum* v. *ericoideum* and *H. chenopodiaceum* v. *typica* were collected in Vallenar, Huasco and Carrizal Bajo (III region, Chile). *H. stenophyllum* was collected in Los Vilos (IV region, Chile). Voucher specimens of *H. filifolium* and *H. chenopodiaceum ericoideum* were deposited in the herbarium of the Biological Sciences Department of the Catholic University of Chile, Santiago, Chile (ST 2214 and ST 2229). The other specimens were deposited in the herbarium of Prof. Sebastián Teillier at the Natural History Museum, Santiago, Chile.

Resinous exudates were extracted by immersing the fresh plant between 15 and 20 s in dichloromethane.[5] The flavonoids were isolated by preparative column chromatography. The structures (**1–8**) (Figure 1) were determined by comparison of their spectral data (mass spectra, ^{1}H and ^{13}C NMR and UV) with those of authentic samples.[28]

Antioxidant Activities Determined by the Reaction with DPPH

Solutions of the resinous exudates and the purified flavonoids were prepared in ethanol at a concentration of 1 mg/mL. An aliquot of these solutions was added to a ethanolic solution of DPPH (1.29 mM). Changes in the absorbance of the solution, elicited by the addition of the solution containing the antioxidants, were measured at 517 nm as a function of the elapsed time employing a Shimadzu UV-160 spectrofotometer.

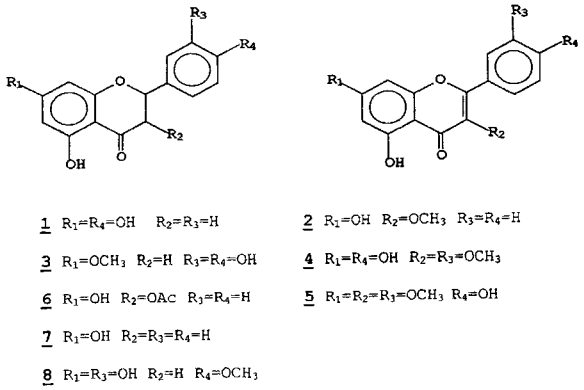

FIGURE 1 Structures of flavonoids isolated from resinous exudate from *Heliotropium*.

Antioxidant Activities Determined Employing the ABTS Derived Radical Cation

The procedure employed was similar to that described by Campos and Lissi.[14] ABTS derived radical cations were prepared by incubation of a phosphate buffer solution (50 mM, pH 7.4) containing ABTS (75 mM) and 2,2′-azobis(2-amidinopropane) (2 mM) at 45°C until an absorbance of ca. 0.5 at 734 nm was obtained. The solution was then cooled to room temperature, where the absorbance due to the ABTS derived radical cations remains unchanged up to more than 1 h. Aliquots of the ethanolic solution comprising the antioxidants were added to this solution. The decrease in the absorbance of the sample, resulting from the consumption of the pre-formed ABTS derived radical cations, was followed as a function of the elapsed time. Similar results were obtained when ABTS radicals were produced by other oxidation procedures (such as incubation with persulphate or MnO_2).

RESULTS AND DISCUSSION

Purified Flavonoids

Addition of purified flavonoids or whole resinous exudates to DPPH or ABTS radical cation solutions elicited a progressive decrease in the concentration of the free radicals, that can be followed by their absorbances at 734 nm (ABTS) or 517 nm (DPPH). Typical profiles are shown in Figures 2 and 3. The process can be characterized both by its stoichiometry (measured at a given time) and by the initial rate of the process.[13,15,18] Campos and Lissi have shown that bleaching of ABTS radical cations elicited by phenols is almost unrelated to the expected reactivity of the compounds towards free radicals.[14] Similarly, the reaction of DPPH with phenols is complex and involves several reversible reactions rendering difficult an interpretation of reaction rates in these systems.[6,21,22] Furthermore, for highly reactive substances the process takes place almost instantaneously (less than 10 s) and rate constants cannot be evaluated without employing stopped-flow devices. The following discussion will be carried out in terms of stoichiometric factors. These factors are defined as the number of free radicals consumed per molecule of additive, measured after an arbitrarily selected time such that the rate of the process had become negligible. This time was taken as

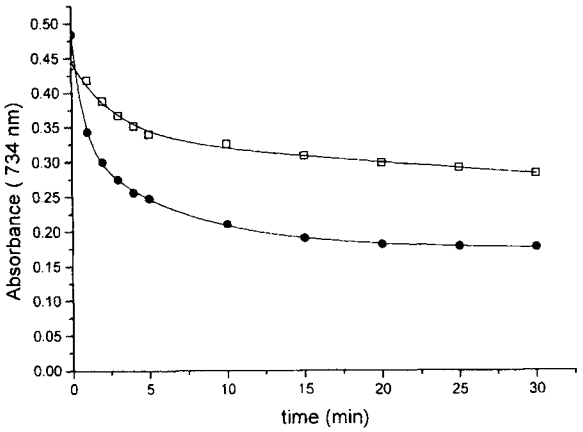

FIGURE 2 Profiles of the decrease in the concentration of the free radicals by addition of 5,7,4′-trihydroxyflavone (**6**) (□) and resinous exudate from *H. sinuatum* (•) to ABTS radical cation solution.

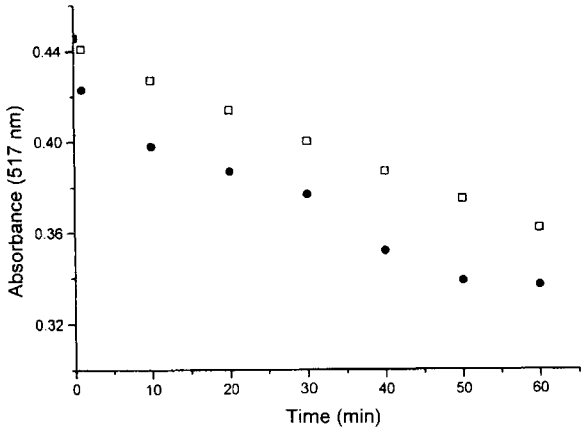

FIGURE 3 Profiles of the decrease in concentration of the free radicals by an addition of 5,7,4′-trihydroxyflavanone (**6**) (□) and resinous exudate from *H. sinuatum* (•) to DPPH radical cation solution.

30 min for ABTS and 1 h for DPPH. The stoichiometric coefficients (n) so obtained for several simple phenols are collected in Table I. The data presented in Table I for monophenol show that, while n values for ABTS range between one and two, the values obtained employing DPPH range from 0.012 (phenol) to 1.7 (α-tocopherol). Campos and Lissi have interpreted n values for ABTS in terms of the secondary reactions of the phenoxy radicals produced in reaction (1).[14] The fact that n values are close to one indicate that this process goes almost to completion. On the other hand, the low values of n obtained employing DPPH must be interpreted in terms of the reversibility of the reactions involving phenoxy and DPPH free radicals.[6,20–22] An interesting feature of these data is that there exist a fair correlation between the stoichiometric factor and the reactivity of the phenol.[13] This is stressed by the data given in Table I that indicate that substitution of the phenol increases the stoichiometric coefficient in parallel to the expected increase in reactivity.

TABLE I Stoichiometric coefficients for the reaction of DPPH free radicals and ABTS radical cations with simple phenols

Phenol	DPPH	ABTS*
Phenol	0.012	1.0
4-ethylphenol	0.027	2.0
4-tertbutylphenol	0.08	2.0
2,4,6-triterbutylphenol	0.20	—
2,6-ditertbutyl-4-methylphenol	0.60	2.0
α-tocopherol	1.7	
Propygallate	3.65	
Trolox	1.0	

*Data from Campos and Lissi, 1996.

The data obtained employing the purified flavonoids are presented in Table II. Similar values have been reported by Salah *et al.*[16] for a series of closely related flavonols such as quercetin, rutin and dihydroquercetin. However, in our data the effect of C2–C3 double bond and/or methylation of phenolic group in C3 were not so important. The values of n obtained employing ABTS, after taking into account the number of phenolic groups, lay predominantly between one and two. In fact, for all the compounds considered, the average number of ABTS radical cations bleached per phenolic group is 1.8 ± 0.4. The small range of n values obtained for the present compounds allowed an evaluation of the total charge of phenol groups in complex mixtures, such as the resinous exudates. On the other hand,

TABLE II Stoichiometric coefficients for the reaction of ABTS radical cations and DPPH free radicals with flavonoids

Compound	Number of phenolic groups	ABTS	DPPH	R
1	(3)	$4.3\ (1.4) \pm 0.10$	0.03 ± 0.002	143
2	(2)	$3.4\ (1.7) \pm 0.10$	0.5 ± 0	6.8
3	(3)	$4.8\ (1.6) \pm 0.13$	1.4 ± 0.4	3.4
4	(3)	$6.6\ (2.2) \pm 0.10$	1.0 ± 0.21	6.6
5	(2)	$5.0\ (2.5) \pm 0.10$	0.04 ± 0.004	125
6	(2)	$3.2\ (1.6) \pm 0.25$	0.02 ± 0.002	160
7	(2)	$2.6\ (1.3) \pm 0.10$	0.015 ± 0	170
8	(3)	$6.3\ (2.1) \pm 0.16$	0.05 ± 0.007	126
Vit E	(1)	1.7		
Trolox	(1)	1.0		
Quercetin	(5)	4.72 ± 0.10*		
Rutin	(4)	2.42 ± 0.12†		
Dihydroquercetin	(5)	1.88 ± 0.03‡		

*The data between parenthesis correspond to the number of radicals consumed per phenol group.
†Ratio between the values calculated employing ABTS and DPPH.
‡Data from Salah.[17]

stoichiometric coefficients evaluated by the DPPH procedure covered a considerably larger range, from 0.015 to 1.4. In this case, evaluation of the bleaching capacity of a complex mixture will not provide an estimate of the total amount of reactive phenolic groups. However, a comparison of the values obtained employing ABTS and DPPH could provide an insight regarding the average reactivity of the compounds present in the mixture. This approach was then employed in order to evaluate the total content of reactive groups (from ABTS values) and their average reactivity in the resinous exudates (from the response between both methodologies).

Antioxidant Capacity of the Resinous Exudates

The TRAP in the resinous exudates was obtained by monitoring their capacity to bleach ABTS and DPPH radicals. The equivalent antioxidant potential was obtained, in terms of TROLOX equivalents, with the formula

$$X_{ABTS} = f\Delta A_{sample}/\Delta A_{Trolox}$$

where f is a dilution factor equal to the ratio between the volume of free radical solution in the reaction cell and the exudate aliquot, ΔA_{sample} is the decrease in ABTS absorbance produced by the sample aliquot incorporation, and ΔA_{Trolox} is the decrease in absorbance elicited by a 1 mM Trolox concentration. A similar formula was employed in order to obtain the antioxidant capacity (in α-tocopherol equivalents) of the exudate (X_{DPPH}) from the decrease elicited by its addition to a DPPH solution. The levels of antioxidants in the resinous exudates, evaluated by those procedures, are given in Table III. As expected, the values obtained employing ABTS are considerably larger than those evaluated by the DPPH procedure. The ratios between both parameters, that can be considered as a measure of average reactivity of the phenols present in resinous exudates, are also included in this table.

There exists only a rather weak correlation ($r = 0.79$, Figure 4) between the charges of antioxidants evaluated by the ABTS and DPPH procedures. The poor correlation observed can be attributed to different average reactivities of the compounds present in the different resinous exudates, as shown by the wide range of R values shown in Table III. This assumption implies that the evaluation of total antioxidant charge in complex mixtures is extremely dependent upon the procedure employed, and that care must be excercised in the interpretation of the results. In particular, an analysis of the data given in Table III indicated that, while the largest concentration of antioxidants is present in *H. stenophyllum*, the highest average reactivity corresponds to the antioxidants present in *H. chenopodiaceum typica*.

TABLE III Concentration of Antioxidants present in the resinous exudates evaluated by the ABTS and DPPH procedures

Resinous exudates	X_{ABTS} (*) M Trolox	X_{DPPH} M α-tocopherol	R (†)
H. stenophyllum	5.2 (2.9) ± 0.10	0.86 ± 0	3.56
H. huascoense	2.0 (1.1) ± 0.10	0.058 ± 0.01	18.24
H. megalanthum	3.1 (1.7) ± 0.20	0.084 ± 0.001	21.76
H. sinuatum	2.8 (1.6) ± 0.17	0.19 ± 0.01	8.82
H. filifolium	2.4 (1.3) ± 0.07	0.28 ± 0.02	5.06
H. chenop. ericoideum	2.8 (1.6) ± 0.20	0.48 ± 0	3.41
H. chenop. typica	3.3 (1.8) ± 0.18	0.73 ± 0.01	2.65

* Values in parenthesis correspond to the concentration of phenolic groups in the resinous exudates, evaluated by considering an average stoichiometric factor of 1.8.
† $R = M_{ABTS}/M_{DPPH}$.

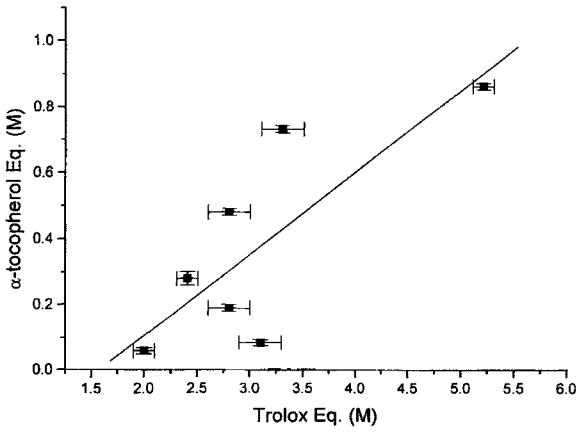

FIGURE 4 Correlation between the levels of antioxidants evaluated by DPPH in α-tocopherol M equivalent and ABTS in Trolox M equivalent procedures from resinous exudates.

It is interesting to note that reactivity ratios obtained in the resinous exudates fell in the range of those measured for the purified flavonoids. This is the expected result for a mixture of compounds of different reactivity. In any case, the large amount of antioxidants present in resinous exudates is remarkable, reaching values in the molar range. In fact, if the average value of 1.8 ABTS radicals scavenged by each phenolic group in the flavonoids is considered, the data of Table III indicate that the molar concentration of phenolic groups in the resinous exudates ranged from 1.3 M in *H. filifolium* to 2.9 M in *H. stenophyllum*.

The data obtained employing DPPH as stable free radical are different from those obtained employing ABTS derived free radicals. This difference can be related to the reversibility of the reaction between phenolic compounds and DPPH.[6,20–22] These results are interesting since it has been shown in several systems that apparent stoichiometric coefficients in these reactions are related to the phenol reactivity towards free radicals and to their antioxidant capacity.[8,10,11,13,23,24]

All flavonoids tested in the present work react at intermediate rates[13] and present apparent stoichiometric coefficients, on a phenolic group basis, considerably smaller than one. This decreased reactivity could be related to the lack of free hydroxyl groups at C3 position and the presence of a carbonyl group at C4, factors that reduce the reactivity of flavonoids towards DPPH radicals.[25] The compounds tested show widely different reactivities, ranging from 0.03 DPPH radicals consumed per additive molecule (5,7,4′-trihydroxyflavanone (**1**) to 1.4 radicals per additive (5,3′,4′-trihydroxy-7-methoxyflavanone (**3**)). The high reactivity of the latter compound can be explained in terms of the presence of two hydroxyl groups in the B ring, a feature that increases the flavonoid reactivity.[13,25] Other factors, such as the presence of unsaturation at C2–C3 bond could also strongly influence the reactivity of the compounds. Saturation at this bond would decrease the planarity of the produced phenoxyl radical, with a concomitant reduction in the reactivity of the parent phenol. This could explain the lower reactivity of 5,7,3′-trihydroxy-4′-methoxyflavanone. This type of effect has been previously reported in a comparative study of the antioxidant capacities of quercetin and taxifolin.[26] Similarly, it is interesting to note that the reactivity of 5,7,4′-trihydroxy-3,3′-dimethoxyflavanone (**4**) is larger than that of 5,4-dihydroxy-3,7,3′-trimethoxyflavanone (**5**), showing that methylation at C7 reduces the scavenging ability of the compound. A similar result has been previously reported in a study of the antioxidant capacity of flavonoids in non-enzymatic lipoperoxidation.[27]

In conclusion, we have shown that resinous exudates have very high concentrations of antioxidants. Furthermore, a comparison of the reactivity of these exudates with ABTS derived radicals and DPPH radicals allows an estimation of the average reactivity of the antioxidants present in the resinous exudate.

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References

[1] S. Eigenbrode, J. Trumble and K. White (1996) Trichome exudates and resistance to beet armyworm in *Lycopersicon hirsutum f. typicum* accessions, *Entomology* **25**, 90–95.

[2] J. Hoffman, B. Kingsolver, S. Mc Laughlin and B. Timmermann (1983) "Productions of resins by arid adapted Astereae" in *Recent advances in photochemistry phytochemical adaptations to stress*. (eds. B. Timmermann, C. Steelink and F. Loewus) Plenum Press, London and New York, pp. 251–255.

[3] K. Downum, J. Dole and E. Rodriguez (1988) Nordihydroguaiaretic Acid: Inter- and Intra populational variation in the Sonoran Desert Creasote bush (*Larrea tridentata*, Zygophyllaceae) *Biochemical Systematics and Ecology* **16**, 551–555.

[4] A. González-Coloma, C. Wisdom and P. Rundel (1988) Ozone impact on the antioxidant nordihydroguaiaretic acid content in the external leaf resin of *Larrea tridentata*, *Biochemical Systematics and Ecology* **16**, 59–60.

[5] A. Urzúa and L. Mendoza (1993) Composición química del exudado resinoso de *Haplopappus* velutinus, *Boletín de la Sociedad Chilena de Química* **38**, 89–90.

[6] T. Kurechi, K. Kikugawa and T. Kato (1980) Studies on the antioxidants XIII. Hydrogen donating capability of antioxidant to 2,2-diphenyl-11-picrylhydrazyl, *Chemical Pharmaceutical Bulletin* **28**, 2089–2093.

[7] J. Kruk, G. Schimd and K. Strzalka (1994) Antioxidant propiertes of plastoquinol and other biological prenylquinols in liposomes and solution, *Free Radical Research* **21**, 409–416.

[8] T. Dinis, V. Madeira and L. Almeida (1994) Action of phenolic derivates (acetaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers, *Archives of Biochemistry and Biophysics* **315**, 161–169.

[9] H. Masakis, T. Atsumis and H. Sakurai (1995) Peroxyl radical scavening activities of Hamamelitannin in chemical and biological systems, *Free Radical Research* **22**, 419–430.

[10] G. Yen and H. Chen (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity, *Journal of Agriculture and Food Chemistry* **43**, 27–32.

[11] J. Imai, N. Ide, S. Nagae, T. Morigushi, H. Matsuura and Y. Itakura (1994) Antioxidant and radical scavening effects of aped garlic extract and its constituents, *Planta Médica* **60**, 417–420.

[12] L. Horokova, R. Schaur, P. Balgavy and G. Egger (1995) Interaction of the pyridooindole stobadine with alkoxyl and stable free radicals, *Redox Report* **1**, 369–372.

[13] W. Brand-Williams, M. Cuvelier and C. Berset (1995) Use of a free radical method to evaluate antioxidant activity, *Lebensmittel-Wissenschaft und Technologie* **28**, 25–30.

[14] A. Campos and E. Lissi (1995) Evaluation of the antioxidant capacity of herbal teas by a procedure based on the bleaching of ABTS radical cations, *Boletín de la Sociedad Chilena de Química* **40**, 375–381.

[15] T. Unno, I. Sakane, T. Masumizu, M. Kohno and T. Kakuda (1997) Antioxidant activity of water extracts of *Lagerstroemia speciosa* leaves, *Bioscience Biotechnology and Biochemistry* **61**, 1772–1774.

[16] N. Salah, N. Miller, G. Paganga, L. Tijburg, G. Bolwell and C. Rice-Evans (1995) Poliphenolic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidant, *Archives of Biochemistry and Biophysics* **322**, 339–346.

[17] P. Plumb, K. Price, M. Rhodes and G. Williamson (1997) Antioxidant properties of the major polyphenolic compounds in Broccoli, *Free Radical Research* **27**, 429–435.

[18] C. Romay, C. Pascual and E. Lissi (1996) The reaction between Abts radical cations and antioxidants and its use to evaluated the antioxidant status of serum samples, *Brazilian Journal of Medicinal Biological Research* **29**, 175–183.

[19] A. Campos and E. Lissi (1996) Kinetics of the reaction between 2,2′-azinobis (3-ethyl)benzothiazoline-6-sulfonic acid (ABTS) derived radical cations and phenols, *International Journal of Chemical Kinetics*, 219–223.

[20] Ch. Hong, Ch. Wang, Sh. Huang and F. Hsu (1995) The inhibitiry effect of tannins on lipid peroxidation of rat heart mitochondria, *Journal of Pharmacy and Pharmacology* **47**, 138–142.

[21] P. Ayscough and K. Russell (1965) Spectroscopic studies of the reversible reaction between 2,2-diphrnyl-1-picrylhydrazyl and 2,4,6-tri-t-butylphenol, *Canadian Journal of Chemistry* **43**, 3039–3044.

[22] T. Kurechi and T. Kato (1982) Studies on the antioxidant XVI. Synergistic reaction between butylated hydroxyanisole and butylated hydroxytoluene in hydrogen donation 2,2-diphenyl-1-picrylhydrozyl, *Chemical Pharmaceutical Bulletin* **30**, 2964–2972.

[23] M. Tagashira, M. Watanabe and N. Uemitsu (1995) Antioxidative activity of hop bitteracids and their analogs, *Bioscience Biotechnology and Biochemistry* **59**, 740–742.

[24] H. Minami, M. Kinoshita, Y. Fukuyama, M. Kodama, T. Yoshizawa, M. Sugiura, K. Nakagawa and H. Tago (1994) Antioxidants xanthones from *Garcinia* subelliptica, *Phytochemistry* **36**, 501–506.

[25] J. Hu, M. Calomme, A. Lasure, T. Bruyne, L. Pieters, A. Vlietinck and A. Vanden Berghe (1995) Structure–activity relationship of flavonoids with superoxide scavening activity, *Biological Trace Element Research* **47**, 327–331.

[26] C. Rice-Evans, N. Miller, P. Bolwell, P. Bramley and J. Pridham (1995) The relative antioxidant activities of plant derived polyphenolic flavonoids, *Free Radical Research* **22**, 375–383.

[27] M. Montesinos, A. Ubeda, M. Terencio, M. Payá and M. Alcaraz (1995) Antioxidant profile of mono- and dihydroxylated flavone derivates in free radical generating systems, *Zeitschrift für Naturforschung* **50c**, 552–560.

[28] R. Torres, B. Modak, L. Villarroel, A. Urzúa, F. Delle Monache and F. Sanchez-Ferrando (1996) Flavonoides del exudado resinoso de *Heliotropium sinuatum*, *Boletín de la Sociedad Chilena de Química* **41**, 195–197.