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Antioxidant activity of procyanidin-containing plant extracts at different pHs

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Abstract

Anti-oxidant activity was measured for a *Pinus radiata* bark extract (bark 1), a *Pinus maritima* bark extract (bark 2), two grape seed extracts, three grape skin extracts, catechin, trolox, ascorbic acid (vitamin C) and calcium ascorbate. The activity was analyzed as ability to scavenge superoxide radicals in a hypoxanthine-xanthine oxidase system, using NBT and WST-1 assays. The 50% inhibition (IC_{50}) of formazan formation was estimated for the samples studied. This study demonstrated that, in basic solution, bark extracts were 13 times more effective antioxidants than vitamin C; however, in aqueous and acidic solutions, bark1 acted as a more potent antioxidant than the other antioxidants in the NBT assay. Grape seed extracts 1 and 2 acted as moderate antioxidants in aqueous and basic solutions in both NBT and WST-1 assays and, in most cases, were three times as efficient as vitamin C. Activities of grape skin extracts were in the same range as trolox, being about 10 times lower than that of the bark extracts. Statistical analysis showed that there was no significant difference between the two assay methods or pH of the solution.

Keywords: Antioxidant; Superoxide radicals; Procyanidins; *Pinus* bark; Grape seed; Grape skin

1. Introduction

Flavonoids are polyphenolic compounds, which are a major class of secondary metabolite biosynthesized throughout the plant kingdom and therefore found in foodstuffs derived from plant sources (Hollman & Katan, 1997; Middleton & Kandaswaml, 1993). The monomeric unit for these compounds consists of two substituted benzene rings A and B joined, in most cases, by a heterocylic ring C (Fig. 1; Hollman, Hertog, & Katan, 1996). Over 4000 of these compounds have been identified so far and it is estimated that there could be up to 20,000 different compounds. There is an array of different substitution patterns on A, B, and C rings, as well as differences in where the B ring is bonded to the C ring, providing possibility for the existence of numerous flavonoids, differing in their biological characteristics and chemical structures (Middleton & Kandaswami, 1993). The flavonoids are divided into several classes, which are chalcones, dihydrochalcones, aurones, flavonols, flavanols (catechins), flavan-3, 4,-diols, flavones, flavanones, anthocyanidins, isoflavones, isoflavanols, and isoflavanones. Some classes of the flavonoids occurring in foodstuffs, such as anthocyanidins (grapes, wine, berries, cherries, aubergine skin), flavonols (onions, leeks, broccoli, endive, kale, apple skin, cherries, tea, grapefruit, berries and grapes), flavones (lemons, olive, celery, red pepper and parsley), flavanols (grapes and tea) and flavanones (citrus fruit and tomatoes skin), possess anti-oxidant activity (Hollman & Katan, 1997; Rice-Evans & Miller, 1996). The greatest antioxidant activity appears to belong to the flavanol class, in particular the procyanidin group (Rice-Evans & Miller 1996). Procyanidins consist of compounds containing catechin, epicatechin and their esterified derivatives; the most commonly-occurring esters are *O*-gallates (Ricardo da Silva, Darmon, Fernandez, & Mitjavila, 1991).

Flavonoids act as an anti-oxidants by neutralizing oxidizing free radicals, including the superoxide and hydroxyl radicals (Bagchi, Garg, Krohn, Bagchi, Tran,

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& Stohs, 1997; Ricardo da Silva et al., 1991; Torel, Cillard, & Cillard, 1986). The redox properties of flavonoids also allow them to act as reducing agents and in some cases they chelate transition metals (Rice-Evans & Miller, 1996; Van Acker et al., 1996).

In the human body superoxide, hydroxyl radicals and hydrogen peroxide are produced by the reduction of oxygen (Proctor & Reynolds, 1984). The free radical-scavenging ability of flavonoids can protect the human body from free radical damage, which may cause many diseases, including cancer and coronary heart disease and lead to the aging process (Middleton & Kandaswami, 1993; Winterbourn, 1995). Studies have shown that increasing levels of flavonoids in the diet could decrease cancer and heart disease (Block, Patterson, & Subar, 1992; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993). Flavonoids have been shown to possess a number of biological effects, countering inflammatory, bacterial, viral, fungal, hormonal, carcinogenic, neoplastic and allergic effects in both in-vitro and in-vivo systems (Middleton, 1996).

This study examines a range of natural product concentrates of polyphenolics possessing antioxidant activity. Superoxide radical scavenging abilities were analyzed for a *Pinus radiata* bark extract (Bark1), *Pinus maritima* bark extract (Bark 2), two grape seed extracts 1 and 2, three grape skin extracts 1, 2 and 3, catechin, trolox, ascorbic acid (vitamin C) and calcium ascorbate. Phenolic contents of pinus barks are catechin, epicatechin, quercetin, dihydroquercetin, taxifolin, phenolic acids, and procyanidin dimers, trimers, oligomers and polymers formed from catechin and epicatechin (Markham & Porter, 1973; Packer, Rimbach, & Virgili, 2000; Porter, 1974). Grape seed phenolic contents are catechin, epicatechin, as well as their procyanidin dimers, trimers, oligomers, polymers and phenolic acids (Jayaprakasha, Singh, & Sakariah, 2001). The phenolic contents of grape skin are prodelphinidin, procyanidins; catechin and epicatechin monomers, dimers, oligomers and polymers, flavonols, anthocyanins (not in white grapes), leucodelphinidin, leucocyanidin and phenolic acids (Peynaud & Ribéreau-Gayon, 1971; Souquet, Cheynier, Brossaud, & Moutounet, 1996; Fig. 2).

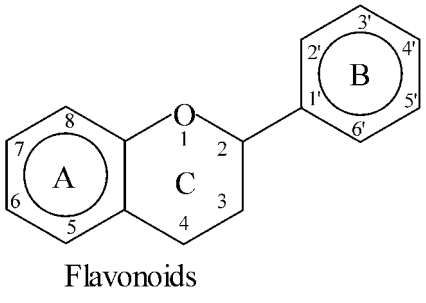

Fig. 1. Basic monomeric flavonoid structure.

2. Materials and methods

2.1. General

The antioxidant activities of the plant extracts, vitamin C, trolox, calcium ascorbate and catechin were measured by NBT and WST-1 methods. The 50% inhibition (IC_{50}) values (the antioxidant concentrations required for 50% reduction in superoxide radical concentration) were determined for all the samples.

2.2. Materials

The commercially available flavonoid-containing products, *P. maritima* bark extract (Bark 2), grape seed extracts 1 and 2 and calcium ascorbate, were obtained from the Health Product sales centre at Christchurch in New Zealand. *P. radiata* bark extract (Bark 1) Enzogenol® was supplied by ENZO Nutraceuticals Ltd. Bark 1 was prepared as described previously (Duncan & Gilmour, 1999). Hot 50% methanol-water was used to prepare three types of grape skin extracts and then these extracts were freeze-dried in our laboratory.

2.3. Chemicals

Hypoxanthine, xanthine oxidase, bovine Cu, Zn superoxide dismutase (Cu,ZnSOD), catalase, nitro blue tetrazolium, diethylenetriaminepentaacetic acid (DTPA), (+) catechin, gallic acid, Folin-Ciocalteau reagent, vitamin C and trolox used in this study were obtained from Sigma Chemical Company™ (St. Louis, MO). AnalaR grade chemicals, obtained from BDH™, were Na_2CO_3, $NaHCO_3$, KH_2PO_4, NaCl, KCl, HCl, NaOH, and Na_2HPO_4. WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate) was obtained from Dojindo Laboratories (Kumamoto, Japan).

2.4. NBT method for measurement of anti-oxidant activity

The nitroblue tetrazolium (NBT) method was based on a published method (Beauchamp & Fridovich, 1971; Sun, Oberley, & Li, 1988). In the NBT method, superoxide radicals were generated by a mixture of hypoxanthine plus xanthine oxidase. At pH 7.4, superoxide radicals reduce nitro blue tetrazolium chloride (yellow) to diformazan (purplish-blue), which is insoluble in water. If radical-scavenging compounds are present in the solution, less formazan blue will be formed, thus decreasing the absorption at 550 nm.

Aqueous solutions of 10 mmol/l NBT, 0.1 mmol/l DTPA and an aqueous solution of 10 mmol/l hypoxanthine, containing 10 μl 6 M HCl, were prepared. Xanthine oxidase (1μM; 13.8 mg protein/ml, 1.3 U/mg)

was prepared by dissolving 18 μl in 500 μl of phosphate-buffered saline and this solution was kept on ice. Phosphate-buffered saline solution (PBS) was prepared by dissolving 2 g KH_2PO_4, 80 g NaCl, 2 g KCl and 11.4 g Na_2HPO_4 in 1 water. This stock buffer solution is diluted 1:10 before using in the NBT assay.

In an absorbance cell, aqueous solutions of 10 μl 10 mmol/l NBT, 10 μl 10 mmol/l hypoxanthine, 10 μl 0.1 mmol/l DTPA and sample (concentration in cell ranged from 0.1–10 μg/ml) were mixed, followed by enough PBS buffer to make up to 990 μl, followed by 10 μl of xanthine oxidase solution. The absorbance was measured at 550 nm on a Shimadzu Multispec 1500^{TM} UV-visible spectrometer. The cell and buffer were kept at 25 °C.

The samples were dissolved in distilled water, an aqueous solution of HC1 (final pH of solutions 2.5), or an aqueous solution of $NaHCO_3$ (final pH of solutions 8).

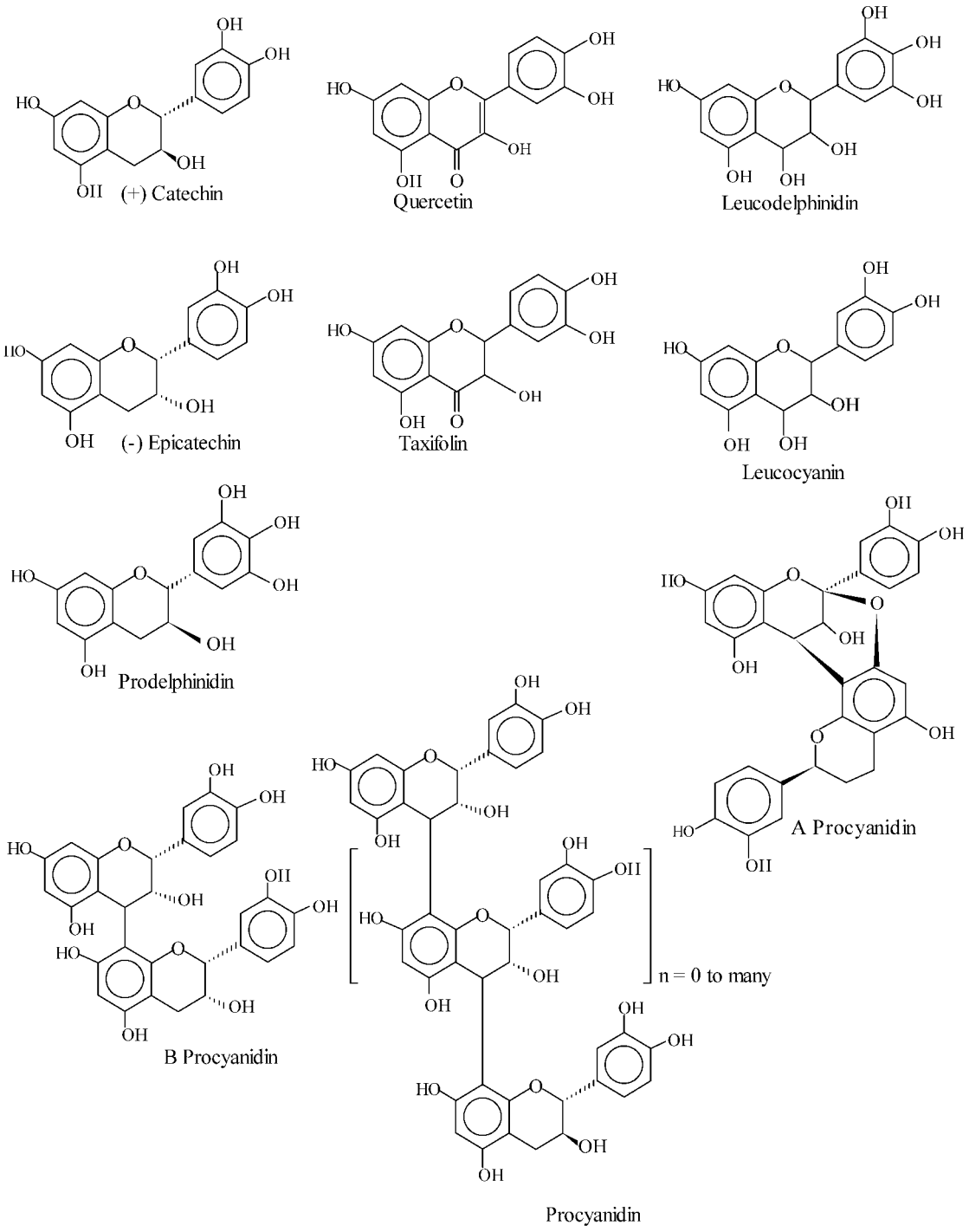

Fig. 2. Flavonoids present in grape skin, grape seed and pine bark extracts.

The acidic and basic solutions were used to mimic the pH encountered by the flavonoid extracts in the stomach and small intestine, respectively.

2.5. WST-1 method for measurement of anti-oxidant activity

Recently, a new assay for measuring superoxide radical-scavenging activity was developed (Peskin & Winterbourn, 2000). This method is similar to the NBT method; however, instead of using NBT, the colourless sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate was used. Since the end-product of this reaction is a water-soluble yellow formazan dye, the assay can be performed in microtitre plates.

The reagents used in this experiment were mixed immediately before use and are (for one 96-well plate): 19.3ml assay buffer (consisting of 50 mmol/l sodium phosphate buffer, pH 8, containing 0.1 mmol/l DTPA and 0.1 mmol/l hypoxanthine), 100 μl 10 mmol/l aqueous WST-1 solution, 100 μl 2 mg/ml aqueous catalase solution, and 5 μl xanthine oxidase (13.8 mg protein/ml (1.3 U/ mg).

In each plate, 190 μl of reagent mixture were added along with either 2, 4, 6, 8 μl of the standard 0.6 μg/ml bovine Cu, ZnSOD (prepared from 3100 U/mg bovine Cu, ZnSOD), or sample (final concentration range from 0.1 to 40 μg/ml). The solutions in the wells were made up to 200 μl by adding the appropriate amount of assay buffer. Three wells in each plate contained only the reagent mixture for estimation of the control rate of WST-1 reduction. The plates were then placed on a plate reader (SPECTRAmax® 190, Molecular Devices, California) at room temperature and shaken. After shaking briefly, the absorbance of the solution was measured at 438 nm.

2.6. Method to test xanthine oxidase inhibition by plant phenolics

Accumulation of urate, as the product of hypoxanthine oxidation, in the presence of xanthine oxidase was monitored at 295 nm. The basic procedure was the same as for the WST-1 assay, apart from the WST-1 which was excluded. The amounts of the aqueous polyphenolic extracts tested ranged from 0.1 to 40 μg/ml. The control rate of urate formation was measured in the absence of the phenolic substances.

2.7. Folin–Ciocalteu method for estimation of total phenols

The method used for this analysis followed a published method (Scalbert, Monties, & Janin, 1989; Singleton & Rossi, 1965). The Folin–Ciocalteau reagent was diluted 1:10 before use. All the polyphenolic samples were dissolved in distilled water and made up a 0.1 mg/ml solution. Diluted Folin–Ciocalteu reagent (2.5 ml) was added to 0.5 ml aqueous phenolic containing samples and allowed between 30 s and 8 min before adding 2 ml of sodium carbonate (75 g/l). The tubes were then kept in a water bath at 50 °C for 5 min before measuring the absorbance at 760 nm. For the correct analysis of the phenolic content of the unknown samples, their absorbances should be below 0.5. Calibration was achieved with aqueous gallic acid solutions (8–80 μg/ml). Total phenol values are expressed as gallic acid equivalents.

Table 1
Antioxidant activity (50% inhibition levels for each sample in μg/ml) for different samples dissolved in distilled water, basic and acidic solutions by NBT assay

Samples	Distilled water	Basic (pH = 8)	Acidic (pH = 2.5)
Vitamin C	2.5	3.79	1.74
Bark 1	0.35	0.28	0.27
Bark 2	0.96	0.26	0.5
Grape seed extract 1	0.82	0.38	0.54
Grape seed extract 2[a]	0.73	0.85	0.69
Grape skin extract 1	5.79	3.24	n.d[b]
Grape skin extract 2	6.7	8.46	n.d
Grape skin extract 3	6.75	3.0	n.d
(+) Catechin	2.52	3.43	2.70
Calcium ascorbate	0.65	n.d	n.d

[a] Sample dissolved in a small amount of base first and either made to pH 8 or pH 2.5.
[b] n.d. Not determined.

3. Results and discussion

Both NBT and WST-1 methods clearly demonstrated that *Pinus* bark extracts 1 and 2 and both grape seed extracts acted as better superoxide radical-scavengers than vitamin C, calcium ascorbate, trolox and all three grape skin extracts (Table 1 and 2).

Results from the NBT analysis revealed that Bark 1 and Bark 2 are 13 and 14 times more potent antioxidants, respectively, in basic conditions, than vitamin C (Table 1). Bark 1 exhibits approximately 3- and 1.5-fold more scavenging of superoxide radicals than grape seed extracts 1 and 2, respectively. Bark 1, when it is dissolved in acidic and distilled water, appeared to possess two-fold greater antioxidant activity compared to bark 2 extract and both grape seed extracts (Table 1). Even though the grape skin extracts had between 20–35% of the total phenolic content of other flavonoid extracts (Table 3), NBT assay results showed only 5–20% of the antioxidant activity for these extracts compared with the other flavonoid extracts (Tables 1 and 2).

Table 2
Antioxidant activity (50% inhibition levels for each sample in μg/ml) for different samples dissolved in distilled water and basic solutions by WST-1 assay

Samples	Distilled water	Basic (pH 8)
Vitamin C	1.93	1.95
Bark 1	0.79	1.24
Bark 2	0.98	1.21
Grape seed extract 1	0.67	0.77
Grape seed extract 2	0.69	0.60
Grape skin extract 1	6.91	n.d[a]
Grape skin extract 2	8.83	n.d
Grape skin extract 3	7.34	n.d
(+) Catechin	0.59	0.50
Calcium ascorbate	2.05	n.d
Trolox[b]	8.98	n.d

[a] n.d. Not determined.
[b] Trolox was dissolved in ethanol as it is not soluble in water.

Catechin showed much less antioxidant activity than the plant-derived mixtures in the NBT assay.

Both grape seed extracts possess more antioxidant activity than both bark extracts in the WST-1 method. The results of WST-1 assay showed that Bark1 is a better antioxidant than Bark 2 in distilled water solutions; however, the antioxidant activities were similar in basic solutions. Grape skin extracts showed approximately 10% of the antioxidant activity of other flavonoid extracts in the WST-1 assay. Trolox, a vitamin E analogue, had the weakest superoxide radical-scavenging activity among the samples studied. Bark 1 and 2 extracts were 11 and 9 times more active than trolox, while the grape seed extracts were 13 times more active. In the WST-1 assay catechin was the best antioxidant.

The paired difference test was used to observe whether there was any significant difference at a 95% confidence interval between the same substances dissolved, either in distilled water or basic solution, and analyzed by NBT and WST-1 assays. Neither of these test showed a significant difference with the results for the distilled water solutions, $t = 0.11$ ($t_{\text{critical}} = 1.81$) or for the basic solutions, $t = 0.18$ ($t_{\text{critical}} = 2.02$). However, the results show there are small differences, although they are not statistically significant, between the NBT and WST-1 IC_{50} results. In particular, Bark 1, dissolved in distilled water, has a much lower IC_{50} value in the NBT assay than in the WST-1 assay, whilst the converse of this is true for catechin dissolved under the same conditions.

The major difference between WST-1 and the NBT assay systems used, other than the dyes, is that the WST-1 method involved catalase. Superoxide radicals can react with water to form hydrogen peroxide. Hydrogen peroxide could be scavenged by flavonoids. Catalase catalyzes the breakdown of hydrogen peroxide to water and oxygen. Hence, it is expected that the amount of radical-scavenging material needed in the WST-1 assay would be less than that required for the NBT assay to reach the IC_{50}. This was not the case for many of the extracts analysed, especially in basic solutions. Since some flavonoids can bind to proteins, it is possible the plant extracts which had increased IC_{50} in the WST-1 method, may have bound to the catalase.

Table 3
Total phenol analysis by Folin–Ciocalteu method[a]

Samples	Total phenols (μg/ml)
Bark 1	9.64±0.66
Bark 2	8.92±0.68
Grape Seed 1	10.1±0.74
Grape Seed 2	8.51±0.65
Grape Skin 1	3.39±0.18
Grape Skin 2	2.71±0.27
Grape Skin 3	2.89±0.11
Catechin	37.1±0.92

[a] Values are expressed as mean±standard deviation of three determinations.

It is thought that flavonoids may inhibit the superoxide-producing enzyme xanthine oxidase, which is used in both the NBT and WST assays, by binding to it (Lu & Foo, 2000). Therefore, urate formation was measured, to test whether xanthine oxidase was inhibited. There were no differences in the rates of urate formation between samples containing plant extracts and the control. The results of this experiment suggest that the polyphenolics in the plant extracts examined did not inhibit the xanthine oxidase.

Free radical-scavenging activity, possessed by the flavonoids in these plant extracts, is dependent on the concentration and type of radical-scavenging phenolics present in each extract. Total phenolic content was measured for each extract using the Folin–Ciocalteau method and these results are listed in Table 3. Total phenols versus reciprocal values of IC_{50} of flavonoid extracts (Table 1) in pH 8 solutions gave a better correlation ($R^2 = 0.7829$) than extracts dissolved in distilled water ($R^2 = 0.6012$). A poor correlation ($R^2 = 0.1242$) was found for extracts in pH 2.5 solutions. This result indicates that samples dissolved in a basic medium are more suitable for flavonoid extracts than those dissolved in distilled water or acidic media for the antioxidant assays. As the flavonoids examined were slightly acidic, they were most soluble in base or distilled water, but were difficult to dissolve in acidic solutions. Differences in antioxidant activity observed for the samples at different pHs may be due to differences in solubility. In most cases, it appeared that the antioxidant activities of flavonoid mixtures dissolved in acid were similar to or slightly lower than those for samples dissolved in distilled water. Paired difference tests, performed at the 95% confidence interval, showed that there was no statistical differences between the three

solutions: distilled water and base $t = 0.12$ ($t_{critical} = 1.86$), distilled water and acid $t = 0.18$ ($t_{critical} = 2.02$) or acid and base $t = 0.18$ ($t_{critical} = 2.02$). Bioavailability studies have shown that flavonoids are absorbed in the small intestine where the pH is 8 (Griffiths, 1982; Hollman et al., 1996; Hollman & Katan, 1998). In acidic solution, procyanidin polymers can be broken down (Wong, 1973). Therefore it is possible that depolymerisation may occur in the stomach.

The relationship between free radical-scavenging activity and the concentration of flavonoids present in the extract is complicated. Research has shown that the anti-radical activity of procyanidins (the primary anti-radical flavonoids present in grape seed and pine bark extracts) is strong for high concentrations (>1000 mg/l). When concentrations of procyanidins are lower (200–1000 mg/l), the activity is weaker. However, when the concentrations are below 200 mg/l, the anti-radical activity increases (Saint-Cricq de Gaulejac, Provost, & Vivas, 1999). The concentrations of the crude extracts examined were between 100 and 1000 mg/l for the grape seed and *Pinus* bark extracts while the concentrations for the grape skin extract were between 1000 and 10,000 mg/l. Since these crude extracts contain mixture of compounds, the actual concentrations of the procyanidins are lower than the value stated. Both bark and grape seed extracts have shown IC_{50} values between 350 and 960 mg/l.

Antioxidant activity is also dependent on the structure of the free radical-scavenging compounds, the substituents present on the rings of the flavanoids and the degree of polymerization. Although there is some debate as to whether the degree of polymerization increases the antioxidant capacity, it appears that epicatechin and epicatechin polymers are better antioxidant than catechin and catechin polymers and the B procyanidins are better antioxidants than the A procyanidins (Hagerman et al., 1998; Ricardo da Silva et al., 1991; Saint-Cricq de Gaulejac et al., 1999). The structural criteria for the most successful free radical-scavengers are either (1) a 3-hydroxy group on an unsaturated C ring, (2) 2,3-double bond with the 3-OH group and 4-one in the C ring, or (3) an *ortho*-OH substitution pattern in the B ring where the OH groups are not glycated (Rice-Evans, & Miller, 1996; Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995; Rice-Evans, Miller, & Paganga, 1996; Van Acker et al., 1996).

The major polyphenolic components in all three types of plant extracts examined are the catechin and epicatechin procyanidins which meet the first and third structural criteria for being a good antioxidant. *Pinus radiata* extract (Bark1) contains quercetin, which is a well known free radical-scavenger and meets the second and third structural criteria. Both pine bark extracts also contain taxifolin, which has some antioxidant activity as it meets the third structural criterion. Grape seed, grape skin and pine bark extracts contain phenolic acids that can contribute some antioxidant activity. Grape skins contain a variety of other flavonoids, which contribute to the antioxidant activity as they fulfil the first and third structural criteria.

4. Conclusion

Solubility is an important physical factor in sample preparation for anti-oxidant activity measurement. Type of solvent and pH affect the solubility and lead to different results. This might be the reason for the differences in anti-oxidant activities between the samples analyzed. Although not statistically significant, the basic solvent in the NBT assay was identified as a better solvent than distilled water or acid in the flavonoid extracts examined for maximum solubility and antioxidant activity. The basic pH examined is similar to that of blood or in the intestine, where the compounds are bioavailable.

Pinus bark extracts 1 and 2 and grape seed extracts 1 and 2 are more effective superoxide radical-scavengers than the antioxidant vitamin C and trolox. Although, grape skin extracts are weak antioxidants, they are slightly more efficient free radical-scavengers than trolox.

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