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Antioxidant power of phytochemicals from *Psidium guajava* leaf

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Abstract: Dried ground leaves of *Psidium guajava L*. (guava) were extracted by water and aqueous ethyl alcohol 50% (1:10) ratio, and the total phenolic content in the extracts was determined spectrophotometrically according to Folin-Ciocalteu's phenol method and calculated as gallic acid equivalent (GAE). Remarkably high total phenolic content 575.3 ±15.5 and 511.6±6.2 mg of GAE/g of dried weight material (for ethanol guava leaf extracts and water guava leaf extracts, respectively) were obtained. The antioxidant activity of lyophilized extracts was determined at ambient temperature by means of a 2,2-diphenyl-1-picrylhydryzyl ($DPPH^{\cdot}$) colorimetry with detection scheme at 515 nm. The activity was evaluated by the decrease in absorbance as the result of $DPPH^{\cdot}$ color change from purple to yellow. The higher the sample concentration used, the stronger was the free radical-scavenging effect. The results obtained showed that ascorbic acid was a substantially more powerful antioxidant than the extracts from guava leaf. On the other hand, the commercial guava leaf extracts and ethanol guava leaf extracts showed almost the same antioxidant power whereas water guava leaf extracts showed lower antioxidant activity. The parameter EC_{50} and the time needed to reach the steady state to EC_{50} concentration ($T_{EC_{50}}$) affected the antiradical capacity of the sample. The antioxidant efficiency (AE) has been shown to be a more adequate parameter for selecting antioxidants than the widely used EC_{50}. This study revealed that guava leaf extracts comprise effective potential source of natural antioxidants.

Key words: Antioxidant capacity, Radical-scavenging activity, $DPPH^{\cdot}$ free-radical, Colorimetry, Guava, Polyphenol
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INTRODUCTION

There is a considerable epidemiological evidence indicating association between diets rich in fresh fruits and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer. Free-radicals are generated continuously in the body due to metabolism and disease (Yeum *et al*., 2003). In order to protect themselves against free radicals, organisms are endowed with endogenous (catalase, superoxide dismutase, glutathione peroxidase/reductase) and exogenous (C and E vitamins, β-carotene, uric acid) defences; yet these defence systems are not sufficient in critical situations (oxidative stress, contamination, UV exposure, etc.) where the production of free radicals significantly increases (Mondon *et al*., 1999).

It is generally assumed that the active dietary constituents contributing to these protective effects are the antioxidants (vitamins, carotenoids, polyphenols, sterols). *Psidium guajava Linn*, belonging to the family of *Myrtaceae*, has been used as health tea. Its leaf contains copious amounts of phenolic phytochemicals which inhibit peroxidation reaction in the living body, and therefore can be expected to prevent various chronic diseases such as diabetes,

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cancer, heart-disease (Kimura *et al*., 1985). Furthermore, decreasing of free-radicals has antioxidizing effect in the body, meaning these guava leaf polyphenols can prevent arterial sclerosis, thrombosis, cataract and inhibit senescence of the body and skin (Okuda *et al*., 1982). Many people habitually take medicinal decoction of guava leaf for long for treatment of diarrhoea (Watt and Branchwizk, 1969), and therefore, the safety of guava leaves have empirically been confirmed (Hamada and Kitanaka, 1999). People in China use guava leaf as anti-inflammatory and haemostatic agent (Liu, 1988). It was reported that the leaves of *P. guajava Linn* contain an essential oil rich in cineol, tannins and triterpenes. In addition, three flavonoids (quercetin, avicularin, and guaijaverin) have been isolated from the leaves (Khadem and Mohammed, 1959). The antioxidant activity of phenolic compounds is determined by their molecular structure and, more specifically, by the position and degree of hydroxylation of the ring structure. The antioxidative activity is conventionally used to indicate the ability of antioxidant to scavenge some radicals. Phenolic compounds are typical active oxygen scavengers in foods and have been evaluated by several methods. One among tests proposed for assessment of antioxidative activity (AOA) is $DPPH^{\cdot}$ free-radical colorimetry (Brand-Williams *et al*., 1995), whose color changes from purple to yellow in the presence of antioxidants. The kinetics of decolorization reactions directly relate to the types of antioxidants and to their different concentrations. The more rapidly the absorbance decreases, the more potent is the antioxidant activity of the antioxidants in terms of hydrogen donating ability (Yen and Duh, 1994). The rapid reduction of $DPPH^{\cdot}$ radical by antioxidants allows the evaluation of antioxidant power of different antioxidants.

This work aimed at assessing the total antioxidant activity of guava leaf extracts for comparison with the antioxidant activity of synthetic antioxidant such as ascorbic acid, using a $DPPH^{\cdot}$ free-radical since guava leaf extracts have been found to contain a wide range of phenolic compounds which may act as antioxidants.

EXPERIMENTAL DETAILS

Standard and reagents

The standard guava leaf extract was purchased from Bizen Chemical Co., Ltd (Japan).

Ascorbic acid and methanol (99.95 % purity) were of analytical grade, and obtained from the Chemical Depot of Southern Yangtze University (Wuxi, China), while ($DPPH^{\cdot}$) 2,2-diphenyl-1-picrylhydrazyl was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Other reagents used were of high purity.

Preparation of guava leaf extracts

Dried guava leaves were ground and extracted in our laboratory using deionized water and aqueous ethyl alcohol 50 % (1:10) ratio, since they have shown efficacy with practically no toxicity toward many food ingredients and are marketable throughout the world. The extracts were filtered through Whatman #2 filter paper (Whatman International Limited, Kent, England) using a chilled Büchner funnel and concentrated to about 10% of the original volume by a rotary evaporator at 40 °C. The concentrate was then lyophilized. The amount of total phenolic content in the guava leaf extracts was determined according to the procedure of Singleton and Rossi (1965) modified by Hoff and Singleton (1977), using Folin-Ciocalteu's phenol reagent to develop a pigment whose absorbance was determined at 765 nm. The results were expressed as gallic acid equivalent (GAE).

Free radical-scavenging method

The antioxidant activity of guava leaf extracts was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, $DPPH^{\cdot}$ (Brand-Williams *et al*., 1995). A methanolic solution (0.1 ml) of sample of various concentrations (0.1–0.5 mg/ml) was placed in a cuvette, and 4 ml of (6×10^{-5}) mol/L methanolic solution of $DPPH^{\cdot}$ was added. The mixture was shaken vigorously and then, absorbance measurements commenced immediately. The decrease in absorbance at 515 nm was determined continuously with data

capturing at 60 seconds intervals with the UV-Vis spectrophotometer Model Leng Guang 722 (Shanghai Analytical Instruments Factory), until the reaction reached a plateau. Methanol was used to zero the spectrophotometer. The absorbance of the DPPH· radical without antioxidant (i.e. the control) was measured daily. Special care was taken to minimize the loss of free radical activity of the DPPH· radical stock solution as recommended by Blois (1958). All determinations were performed in triplicate. The calibration curve was determined using two blanks and the values were averaged. The DPPH· concentration in the reaction medium was calculated from the following equation as determined by the linear regression:

$$A_{515\ \mathrm{nm}} = 0.0044\ [\mathrm{DPPH}^{\cdot}]_T - 0.0017$$

where $[\mathrm{DPPH}^{\cdot}]_T$ was expressed as mg/liter; with $r = 0.999$. The percent inhibition of the DPPH· by the samples was calculated according to the formula of Yen and Duh (1994) as follows:

$$\%\ \mathrm{Inhibition} = [(A_{C(0)} - A_{C(t)}) / A_{C(0)}] \times 100$$

where $A_{C(0)}$ was the absorbance of the control at t = 0 min and $A_{C(t)}$ was the absorbance of the antioxidant at t, which varied with the different concentrations.

Statistical analysis

Total polyphenolic content was presented as mean value plus or minus standard deviation. Statistical analysis between experimental results was based on student's t-test. Significant difference was statistically considered at the level of $P < 0.005$.

RESULTS AND DISCUSSION

Antioxidant activity of different extracts from guava leaf according to the DPPH· radical scavenging method

The results (Figs.1–2) showed that the decrease in absorbance of the DPPH· radical was due to its reduction by different antioxidants. Absorbance decreases as a result of a color change from purple to yellow as the radical was scavenged by antioxidant through donation of hydrogen to form the stable DPPH-H. The data showed that DPPH· Solution was bleached with all the samples tested. However, differences could be observed through different antioxidants used and their concentrations.

Table 1 shows that ascorbic acid is superior inhibitor of DPPH· compared to guava leaf extracts. From Table 1, ascorbic acid showed high percentage inhibition while standard guava leaf extracts, ethanol guava leaf extracts and water guava leaf extracts respectively, showed decreasing inhibition

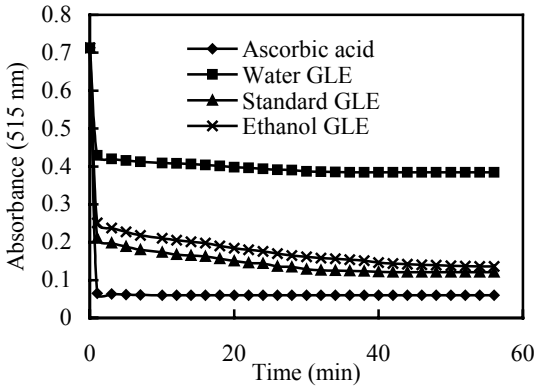

Fig.1 Time course of absorbance reduction of 4 ml of 6×10^{-5} mol/L 2,2-diphenyl-1-picrylhydryzyl (DPPH·) in methanol and each tested sample (ascorbic acid, water GLE, ethanol GLE, standard GLE) at concentration: 0.25 mg/ml, by the decolorization reaction of DPPH· radical from purple to yellow. GLE: guava leaf extracts

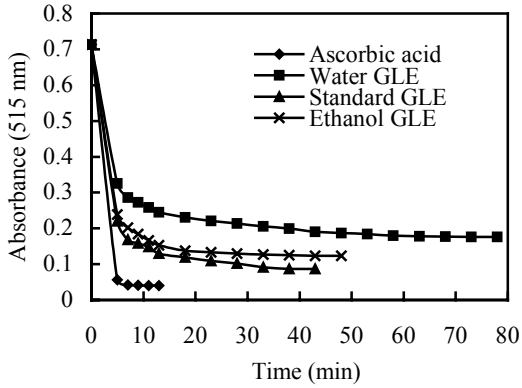

Fig.2 Time course of absorbance reduction of 4 ml of 6 $\times10^{-5}$ mol/L 2,2-diphenyl-1-picrylhydryzyl (DPPH·) in methanol and each tested sample (ascorbic acid, water GLE, ethanol GLE, standard GLE) at concentration: 0.5 mg/ml, by the decolorization reaction of DPPH· radical from purple to yellow. GLE: guava leaf extracts

Table 1 Comparison of antioxidant activity of guava leaf extracts with ascorbic acid expressed as % inhibition

Concentration (mg/ml)	Ascorbic acid	STD (GLE)	Ethanol (GLE)	Water (GLE)
0.1	35.76	31.69	27.62	18.23
0.25	91.58	83.02	75.87	46.14
0.4	92.42	85.69	80.92	47.12
0.5	94.38	87.79	82.74	75.13

GLE: guava leaf extracts, STD: standard

effect for all concentrations. For all the antioxidants, with higher the concentrations, the bleaching ability of the DPPH· solution was nearly complete. Ascorbic acid was substantially more active whatever the concentrations used (Table 1) in comparison with different extracts from guava leaf. With high concentration of ascorbic acid, it was possible to observe nearly a 100% free radical scavenging effect. Ethanol GLE and standard GLE had nearly the same effect for the similar concentrations used. As for ascorbic acid, ethanol GLE, standard GLE and water GLE, a clear gradient activity was seen from the first to the last concentration used (Table 1). Ascorbic acid, known to act as antioxidant (Larson, 1988) and different extracts from guava leaf showed good free radical-scavenging activity depending on the concentration used. The higher the concentration used the higher the free radical-scavenging effect.

Total phenolic content values of standard GLE, ethanol GLE and water GLE were 598.25±4, 575.3 ±15.5 and 511.6±6.2 mg of gallic acid equivalent (GAE)/g of dry weight material, respectively. The average of total phenolic content of standard, ethanol and water GLE were significantly different at $P<0.005$. Ascorbic acid, a potential antioxidant, was used as positive control of antioxidant activity compared with the extracts of guava leaf found to contain significant amounts of natural phenolics that are antioxidants. Guava leaf extracts (GLE) analysis revealed they contain different phenolic compounds such as 1–tannic acid; 2–procatechuic acid; 3–caffeic acid; 4–ferulic acid; 5–rutin trihydrate; 6–quercetin dihydrate (the HPLC profile was not reported). Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids (Hopia *et al*., 1999). Due to the diversity and complexity of the natural mixtures of phenolic compounds in the different extracts of guava leaf, it is rather difficult to characterize every compound and assess or compare their antioxidant activities (Zheng and Wang, 2001). The present study showed that the amount of ethanol guava leaf extracts to total phenolic content was higher than that of water guava leaf extracts. Moreover, it can be seen from Table 1 that ethanol GLE exhibited higher antioxidant activity (expressed in terms of percentage inhibition) than water guava leaf extracts for all the concentrations tested. Antioxidant activity of the extracts may also be attributable to unidentified substances or to synergistic interactions.

Fraction of the residual DPPH· solution plotted versus time

The absorbance of the solution decreases depending on the intrinsic antioxidant activity (AOA) of the antioxidant as well as on the speed of the reaction between DPPH· and the same antioxidant. In case of rapid kinetic behaviour, practically all samples at high concentrations reacted within a very short time, and a steady state was reached almost immediately. On the other hand, slow kinetic behaviour (lowest concentration for each sample) implied longer periods before the steady state was reached. Moreover, ascorbic acid showed rapid kinetic behaviour, and is a strong reducer of a DPPH· radical, i.e. it has high antioxidant activity (AOA).

Clearly, the reaction kinetics depended on the concentration of the different samples. For example, samples containing lower concentrations reacted slowly (steady state was not reached within a short time) with DPPH·, while at the highest concentration level of each sample, the steady state was apparently reached after 8 minutes for ascorbic acid, after 12 minutes for standard GLE, after 20 minutes for ethanol GLE and after 24 minutes for water GLE.

Data on the time course of absorbance (reaction kinetics) (Figs.1–2) enabled assessment of the extent of AOA for each sample. To do so, it was necessary to convert data on the reaction kinetics

into new plots displaying the fraction of residual $DPPH^{\cdot}$ in solution as a function of time. The fraction of residual $DPPH^{\cdot}$ ($DPPH^{\cdot}_{res}$) was calculated from:

$$\%DPPH^{\cdot}_{res} = [DPPH^{\cdot}]_t / [DPPH^{\cdot}]_{t=0}$$

where $[DPPH^{\cdot}]_t$ and $[DPPH^{\cdot}]_{t=0}$ are concentration of $DPPH^{\cdot}$ at $t = 0$ and $t = t$, respectively.

$[DPPH^{\cdot}]_t$ was determined according to the linear regression equation below:

$$A_{515\ nm} = 0.0044\ [DPPH^{\cdot}]_T - 0.0017$$

Figs.3–6 are time dependent plots of residual $DPPH^{\cdot}$ for various concentrations of ascorbic acid and different types of guava leaf extracts. From the different figures, the decrease in absorbance was function to the concentration. The higher the concentration, the greater was the decrease in absorbance. Using data from preceding figures, new plots can be constructed to display fractions of $DPPH^{\cdot}$ left in the solution at steady state condition as a function of sample concentration (mg antioxidant/g $DPPH^{\cdot}$) as shown in Fig.7.

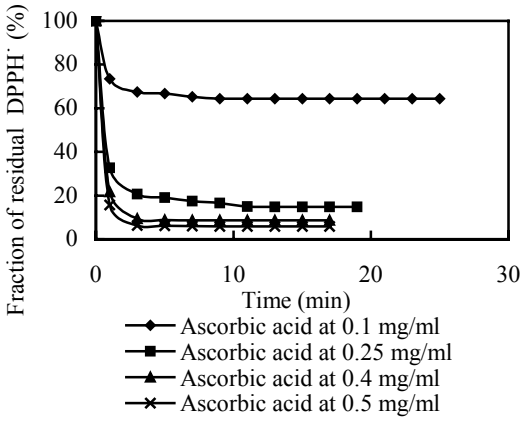

Fig.3 Fraction of the residual $DPPH^{\cdot}$ plotted versus time for concentrations of ascorbic acid varying from 0.1 to 0.5 mg/ml at the steady state. The high the sample concentration the decrease was the residual $DPPH^{\cdot}$

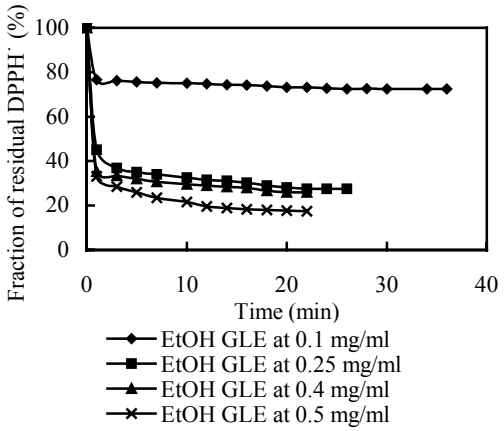

Fig.4 Fraction of residual $DPPH^{\cdot}$ plotted versus time for concentration of ethanol guava leaf extracts (EtOH GLE) varying from 0.1 to 0.5 mg/ml to reach the steady state. The high the sample concentration the decrease was the residual $DPPH^{\cdot}$

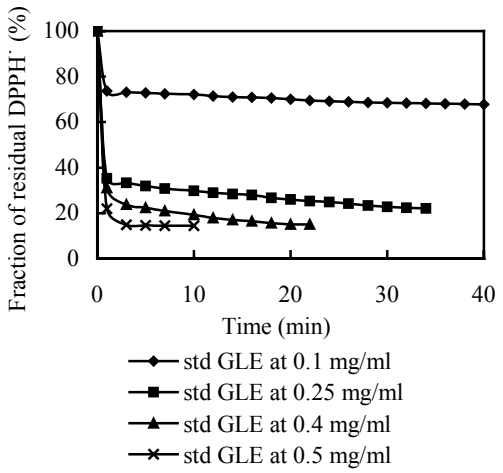

Fig.5 Fraction of residual $DPPH^{\cdot}$ plotted versus time for concentration of standard guava leaf extracts (std GLE) varying from 0.1 to 0.5 mg/ml to reach the steady state. The high the sample concentration the decrease was the residual $DPPH^{\cdot}$

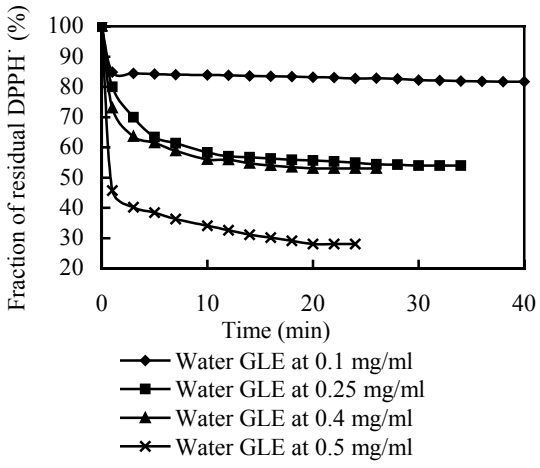

Fig.6 Fraction of residual $DPPH^{\cdot}$ plotted versus time for concentration of water guava leaf extracts (Water GLE) varying from 0.1 to 0.5 mg/ml to reach the steady state. The high the sample concentration the decrease was the residual $DPPH^{\cdot}$

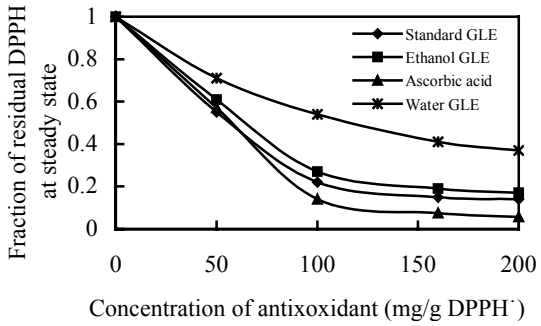

Fig.7 Fraction of the residual DPPH· left at the steady state plotted, versus the ratio (mg of antioxidant/g of DPPH·). From the graph one obtains 54 mg ethanol GLE, 130 mg water GLE, 53 mg standard GLE, and 50 mg ascorbic acid as efficient concentration EC_{50}

At this stage it is useful to introduce a concept of efficient concentration EC_{50} (i.e. Radical-scavenging activity) defined as the amount of antioxidant concentration needed to reduce the initial DPPH· concentration, $C_{DPPH^{\cdot}\,(t=0)}$ by a factor of two (at steady state). The parameter EC_{50} is a direct quantitative measure for antioxidative activity (AOA); highly effective (high antioxidative activity) antioxidant is characterized by low EC_{50} value, and vice versa.

Then the parameter EC_{50}, which reflects the depletion of 50% radical-scavenging activity, was calculated for each type of sample. The EC_{50} values of the different samples taken into account were obtained from the plot of the absorbances against the concentrations and are summarized in Table 2.

Table 2 shows that the lower the EC_{50}, the shorter was the reaction time and the higher the antiradical efficiency. Inspection of Fig.7 showed that EC_{50} per g DPPH·=54 mg ethanol GLE, 130 mg water GLE, 53 mg standard GLE and 50 mg ascorbic acid. Our results showed that ethanol GLE contained higher radical-scavenging activity than water GLE due to the higher amount of phenolic content, which agreed well with the report of Yamagushi *et al*.(1998). The parameter, antiradical efficiency (AE) which is more discriminatory than EC_{50} according to Sanchez-Moreno *et al*.(1997) was used to define the antioxidant capacity of guava leaf extracts. Considering that both EC_{50} and the time needed to reach the steady state to EC_{50} concentration ($T_{EC_{50}}$), which was calculated graphically, affect the antiradical capacity, it was reliable to assess that parameter as $AE=1/EC_{50}T_{EC_{50}}$.

The concentration of antioxidant needed to decrease by 50% the initial substrate concentration (EC_{50}) is a parameter widely used to measure the antioxidant power (Robak and Gryglewski, 1988; Yoshida *et al*., 1989; Vinson *et al*., 1995). The lower the EC_{50}, the higher was the antioxidant power. Table 2 shows that ascorbic acid was a substantially more powerful antioxidant than the extracts from guava leaf. On the other hand, the standard GLE and ethanol GLE showed almost the same antioxidant power whereas water GLE showed lower antioxidant activity (Table 2). The ranges of time at the steady state was determined for each type of antioxidant and varied with concentrations. The higher the concentration, the shorter was the time needed to reach the steady state. Many attempts to explain the structure-activity relationship of some polyphenols have been reported by Hagerman *et al*.(1998). It is known that monophenols are less

Table 2 50% Radical scavenging activity concentration of antioxidants and their antiradical efficiencies

Antioxidant	EC_{50} (mg antioxidant/g DPPH·)[a]	Ranges of time at the steady state (min) for antioxidant concentrations	$T_{EC_{50}}$ (min)[a]	AE($\times10^{-3}$)
Water GLE	130±1.0	24–40	45±4	0.31
Standard GLE	54±3.2	20–30	36±1	0.52
Ethanol GLE	53±2.0	10–38	42±6	0.42
Ascorbic acid	50±0.5	7–14	25±2	0.80

GLE: guava leaf extracts; $T_{EC_{50}}$ Time needed to reach the steady state to EC_{50} concentration; AE: Antiradical efficiency

[a]: Each value is the mean ± standard deviation of a triplicate

efficient than polyphenols. The accessibility of the radical center of $DPPH^{\cdot}$ to each antioxidant could also influence the antioxidant power (Yoshida *et al*., 1989).

There is little information on the kinetic behaviour of the antioxidant compounds in the oxidation process (Sanchez-Moreno *et al*., 1997). Halliwell (1990) reported that the antioxidant power results first from the capacity to prevent the autoxidation of free radical-mediated oxidation of the substrate in low concentration and second, that the resulting radical after scavenging must be stable. In our model we consider that "short time" should be added to the first condition resulting "low concentration and short time" because the reaction time is also important to define antioxidant capacity. In case of the high concentration (>0.5 g/ml), the results after scavenging (not shown) were not stable due to interference of food pigments.

Table 2 shows the significance of antiradical efficiency (AE) in comparison with EC_{50} ascorbic acid, ethanol GLE and standard GLE which were nearly equal, being superior to that of water GLE. However, the AE of ascorbic acid was 1.5-fold that for standard GLE and 1.9-fold that for ethanol GLE whereas standard GLE was 1.2-fold that for ethanol GLE. The classification order of AE for the tested antioxidants was: ascorbic acid > standard GLE > ethanol GLE > water GLE. This fact showed that AE is a more adequate parameter for selecting antioxidants than the widely used EC_{50}.

Guava leaf extracts showed potential antioxidant activity and can be used to extend the shelf life of foodstuffs, to reduce wastage and nutritional losses by inhibiting and delaying oxidation. However, antioxidant cannot improve the quality of an already oxidized food product (Coppen, 1983). Increased intake of guava leaf extracts is therefore good for our health.

CONCLUSIONS

Ascorbic acid, known to act as antioxidant and different extracts from guava leaf showed good free radical-scavenging activity depending on the concentration used. The higher the concentration used the higher the free radical-scavenging effect. The parameter, antiradical efficiency (AE) has been found to be a more adequate parameter for selecting antioxidants than the widely used EC_{50}. Therefore, supplementing a balanced diet with guava leaf extracts may provide health-promoting effects. It will also be important to study the synergistic effect of different phenolic compounds contained in guava leaf extracts.

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