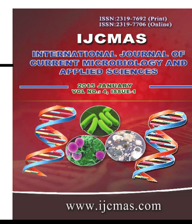

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Original Research Article

Reactive oxygen and nitrogen species scavenging activity of *Psidium guajava* (l) leaf

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A B S T R A C T

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Antioxidant activity, Psidium guajava , Radical scavenging, Reactive oxygen species

Antioxidant activity of aqueous and alcoholic extract of Psidium guajava leaves were carried out for proving its utility in free radical mediated diseases including diabetic, cardiovascular, cancer etc. The aqueous and alcoholic extract were screened for in vitro antioxidant activity by oxygen radical scavenging such as DPPH, nitric oxide scavenging, superoxide scavenging, reducing power and ABTS activity at different concentrations. Throughout the studies leaves extract showed marked antioxidant activity. Among the two extracts, the alcoholic extract of Psidium guajava leaves exhibit significant antioxidant activity than aqueous extract. The antioxidant activity of the leaves extract may be due to the presence of bioflavonoids content in the leaves of Psidium guajava. Overall, the plant extract is a source of natural antioxidants which might be helpful in preventing the progress of various oxidative stress mediated diseases including cancer.

Introduction

Reactive oxygen species (ROS) are produced in the cells by cellular metabolism and other exogenous environmental agents. They are generated by a process known as redox cycling and are catalysed by transition metals, such as Fe^{2+} and Cu^{2+} (Halliwell and Gutteridge, 1999). Overproduction of ROS can damage cellular biomolecules like nucleic acids, proteins, lipids, carbohydrates, proteins and enzymes, resulting in several diseases. Living systems have specific pathways to overcome the adverse affects of various damages.

However, sometimes these repair mechanisms fail to keep pace with such deleterious effects (Halliwell, 1995; Nilsson et al., 2004). Antioxidants scavenge free radicals and are associated with reduced risk of cancer and cardiovascular diseases.

The World Health Organization (WHO) has estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts and their active components (Winston, 1999). Plant

and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities including antioxidant potential (Velavan *et al,* 2007). The majority of the active antioxidant constituents are flavonoids, isoflavones, flavones, anthrocyanins, coumarins, lignans, catechins, and isocatechins. The phenolic compounds in plants act as antioxidants due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators (Javanraedi *et al*., 2003).

With this background and abundant source of unique active components harbored in plants. The chosen medicinal plant namely as *Psidium guajava* L leaves belongs to the Myrtaceae family. Hence, the free radical scavenging activity of *Psidium guajava* leaves were not evaluated. Therefore, the present study were to investigate the free radical scavenging activity of *Psidium guajava* leaves through the free radical scavenging such as DPPH, nitric oxide scavenging, superoxide scavenging, reducing power and ABTS assay.

Materials and Methods

Plant material

Leaves of *Psidium guajava* was selected to screen its biopotentials based on its traditional usage. The fully mature *Psidium guajava* leaves were collected in April 2013 from Thanjavur, Tamil Nadu, India from a single herb. Care was taken to select healthy leaf.

Authentication of plant material

The collected leaves were identified and authenticated by a Botanist Dr. M. JEGADEESAN, Prof. and Head, Department of Environmental and Herbal Sciences, Tamil University, Thanjavur, Tamil Nadu. A Voucher specimen has been deposited at Tamil University Herbarium. The leaves were cut into small pieces and shade dried at room temperature for 15 days.

Preparation of extract

The collected leaves of *Psidium guajava* were washed under running tap water and dust was removed from the leaves . The leaves were dried at room temperature for 15 days and coarsely powdered. The powder (2gm) was extracted with 70% ethanol and 100% aqueous for 48 hours. A semi solid extract was obtained after complete elimination of alcohol and water under reduced pressure. The *Psidium guajava* leaves extract (PGLF) was stored in refrigerator until used. . Doses such as 20, 40, 60, 80 and 100µg/ml were chosen for *in vitro* antioxidant activity. L ascorbic acid as standard was used as a control and prepared by dissolving 10mg of L-ascorbic acid in 100ml of distilled water.

DPPH Radical Scavenging Activity (Spectrophotometric assay)

The free radical scavenging capacity of the extracts of *Psidium guajava* aqueous and ethanolic extracts were determined by using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. Extracts of *Psidium guajava* leaves was mixed with 95% methanol to prepare the stock solution (10mg/100ml). The concentration of extract solution was 10mg/100ml or 100µg/ml. From stock solution 2ml, 4ml, 6ml, 8ml and 10ml of the solution were taken in five test tubes and serially diluted, this was made up to final volume of each test tube to 10ml whose concentration was then 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml respectively. Freshly prepared DPPH

solution (0.004% w/v) was added in each of these test tubes containing extracts and after 10 minutes, the absorbance was taken at 517nm using a spectrophotometer (Systronics UV-Visible Spectrophotometer 119, INDIA). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10mg/100ml or 100μg/ml) of extracts. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank (Soler Evans *et al.,* 1997).

Ferric reducing power assay

This experiment was carried out as described previously (Cuendet *et al.,* 1997). 1ml of the plant extract solution (final concentration 100-500mg/L) was mixed with 2.5ml phosphate buffer (0.2M, pH 6.6) and 2.5ml potassium ferricyanide ($K_3Fe(CN_6)$) (10g/L), then the mixture was incubated at 50°C for 20 minutes. To this 2.5ml of trichloroacetic acid (100g/L) was added, and centrifuged at 3000rpm for 10 minutes.

Finally, 2.5ml of the supernatent solution was mixed with 2.5ml of distilled water and 0.5ml $Fecl_3$ (1g/L) and the absorbance was measured at 700nm in UV-Visible Spectrophotometer (Systronics UV-Visible Spectrophotometer 119, India). Ascorbic acid was used as standard and phosphate buffer as blank solution. The absorbance of the final reaction mixture of two parallel experiments was expressed as mean ± standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

$$\text{\% increase in Reducing power} = \frac{A_{Test}}{A_{Blank}} - 1 \times 100$$

A_{test} is the absorbance of test solution; A_{blank} is absorbance of blank. The antioxidant activity of the leaves extract was expressed as IC_{50} and compared with standard.

Nitric oxide radical scavenging activity

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of NO compete with oxygen leading to reduce production of NO. Nitric oxide was generated from sodium nitroprusside, which at physiological pH liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions (NO_2 -) which diazotize with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink color, which can be measured at 546 nm. Sodium nitroprusside (10 mM, 2 ml) in phosphate buffer saline was incubated with the test compounds in different concentrations at room temperature for 30 minutes. After 30 minutes, 0.5 ml of the incubated solution was added with 1ml of Griess reagent and the absorbance was measured at 546 nm (Polshettiwar *et al.,* 2007).

Superoxide radical scavenging activity (PMS-NADH System)

Superoxide anions were generated using PMS / NADH system. The superoxide anions were subsequently made to reduce nitroblue tetrazolium, which yielded a chromogenic product, which was measured at 560 nm. Phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system was used for the generation of superoxide anion. It was assayed by the

reduction of nitroblue tetrazolium (NBT). About 1ml of nitro blue tetrazolium (156µM), 1ml NADH (468µM) in 100mM phosphate buffer of pH 7.8 and 0.1ml of sample solution of different concentrations were mixed. The reaction was started by adding 100µl PMS (60µM). The reaction mixture was incubated at 25°C for 5 minutes and absorbance of the mixture was measured at 560nm against blank samples. The percentage inhibition was determined by comparing the results of control and test samples (Kumarasamy *et al.,* 2007).

ABTS radical scavenging assay

ABTS (2, 2'-azinobis-3-ethyl benzothiozoline - 6-sulphonic acid) assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process that is associated with a change in absorption. The relatively stable ABTS radical was green and it was quantified spectrophotometrically at 734 nm. ABTS radical cations were produced by the reaction of ABTS and APS. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and the percentage inhibition was calculated.

The stock solutions included were 7 mM ABTS solution and 2.4 mM potassium per sulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowed them to react for 14 hrs at room temperature in dark. The solution was then diluted by mixing 1ml ABTS solution with 60ml methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Leaves extract (1 ml) was allowed to react with 1ml of the ABTS solution and the absorbance was taken at 734 nm after 7 minutes using a spectrophotometer. All determinations were performed in triplicate (n = 3) (Blois *et al.,* 1958).

H_2O_2 scavenging activity

H_2O_2 scavenging ability of aqueous and alcoholic extracts of *Psidium guajava* leaves was determined according to the method of Ali *et al.,* (2009). A solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH 7.4). The aqueous and ethanolic extracts at 30µg/ml concentration in 3.4ml phosphate buffer were added to H_2O_2 solution (0.6ml, 40mM). The absorbance value of the reaction mixture was recorded at 230nm. Blank solution was containing the phosphate buffer without H_2O_2.

Assessment of % inhibition and IC_{50}

Radical scavenging activity of the extract and standard were expressed in terms of % inhibition. It was calculated using the formula $((A_{Control}-A_{Sample})/A_{Control}) \times 100$. Where $A_{Control}$ is the absorbance of the control, and A_{Sample} is the absorbance in the presence of the sample of aqueous and ethanolic extracts. The IC_{50} value is defined as the concentration (in µg/ml) of extracts that produced 50% antioxidant effect. IC_{50} = Concentration of extract / % inhibition X 50.

Results and Discussion

Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol derived radical to stabilise and delocalise the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (Rice-Evans *et al.,* 1997). Preliminary phytochemical analysis indicates that the aqueous and alcoholic extract of *Psidium guajava* leaves

contains flavonoids, saponin, terpenoids, steroids, polyphenols and tannin.

DPPH Assay

DPPH radical scavenging activity of plant extract of PGLE and standard as ascorbic acid are presented in Table 1. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants (Nuutila *et al.,* 2003). Recently, the use of the DPPH$^{\bullet}$ reaction has been widely diffused among food technologists and researchers, for the evaluation of free radical scavenging activity on extracts from plant, food material or on single compounds. In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to the yellow colored 1, 1-diphenyl-1, 2-picryl hydrazine. The molecule of 2, 2-diphenyl-1-picryl hydrazine is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole. The proton transfer reaction of the DPPH$^{\bullet}$ free radical by a scavenger (A-H) causes a decrease in absorbance at 517 nm, which can be followed by a common spectrophotometer set in the visible region.

The effect of antioxidants on DPPH$^{\bullet}$ is thought to be due to their hydrogen donating ability (Sindhu and Abraham, 2006). The half inhibition concentration (IC_{50}) of aqueous, alcoholic extract and ascorbic acid were 66.09, 55.09µgml^{-1}and 48.06 µg ml^{-1} respectively**.** The alcoholic extract exhibited a significant dose dependent inhibition of DPPH activity than aqueous extract. The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity is near to standard as ascorbic acid.

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Miller *et al,* 1993). *Psidium guajava* aqueous and alcoholic extract also moderately inhibited nitric oxide in dose dependent manner (Table 2) with the IC_{50} being 118.56 and 121.89 µg ml^{-1} and ascorbic acid was 53.25µg ml^{-1} respectively**.**

Superoxide anion radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Korycka-Dahl & Richardson, 1978). The superoxide anion radical scavenging activities of the extract from *Psidium guajava* assayed by the PMS-NADH system were shown in Table 3. The superoxide scavenging activity of *Psidium guajava* was increased markedly with the increase of concentrations. The half inhibition concentration (IC_{50}) of aqueous and alcoholic extract of *Psidium guajava* leaves were 164.2, 061.67µg ml^{-1} and ascorbic acid were 33.93µg ml^{-1} respectively**.** These results suggested that alcoholic extract of *Psidium guajava* had notably superior superoxide radical scavenging effects.

Reducing power activity

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of *Psidium guajava* . The reducing capacity of a

compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997; Yildirim *et al,* 2000). Table 4 depicts the reductive effect of *Psidium guajava.* Similar to the antioxidant activity, the reducing power of *Psidium guajava* increased with increasing dosage. All the doses showed significantly higher activities than the control exhibited greater reducing power, indicating that *Psidium guajava* consist of hydrophilic polyphenolic compounds that cause the greater reducing power. The half inhibition concentration (IC50) of aqueous and alcoholic extract of *Psidium guajava* leaves were 200.48, 110.98µg ml-1 and ascorbic acid were 60.63µg ml-1 respectively. These results suggested that alcoholic extract of *Psidium guajava* possess higher reducing power activity than aqueous extract.

ABTS radical scavenging activity

It is known that proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm, which decreases with the scavenging of proton radicals (Mathew and Abraham, 2006; Adedapo*et al*., 2008) . The 2,2'-azinobis-3-ethylbenzothiazoline6-sulfonic acid (ABTS) activity of the formulation were comparable to standard ascorbic acid. This implies that the plant extract may be useful for treating radical-related pathological damage, especially at higher concentration (Olayinka *et al*., 2010). Since the IC50 value of ascorbic acid (Table 5), which is known to be a potent antioxidant is 49.56µg/ml, which is significantly low, implies that a very less amount of this antioxidant would give a remarkably high effect in fighting oxidative damage. However, aqueous and alcoholic extract of *Psidium guajava* leaves shown IC50 values were 59.21, 61.28µg/ml, which necessarily indicates that rather a higher dose is required to achieve a desirable effect.

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can nactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects (Miller *et al*., 1993). It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. As shown in Table 6, aqueous and alcoholic extract of *Psidium guajava* leaves extract demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner with an IC50 of 76.28, 81.43µg /ml and ascorbic acid was 47.37 µg/ml (Table 6).

In conclusion, the results of the present study showed that the aqueous and alcoholic extract of *Psidium guajava* leaves possesses antioxidant activity. Among the two extracts, the alcoholic extract of *Psidium guajava* leaves exhibit significant antioxidant activity than aqueous extract. The potential antioxidant activity of *Psidium guajava* leaves due to the presence of flavonoids and polyohenols. Thus, it can be concluded that *Psidium guajava* leaves can be used as an accessible source of natural antioxidants with consequent health benefits.

Table 1 *In vitro* Free Radical scavenging effect of *Psidium guajava leaves* by DPPH method

Concentration (µg/ml)	Aqueous extract	Alcoholic extract	Ascorbic acid	
			Concentrations	%inhibition
20	36.82±0.24	41.94±1.41	10µg/ml	6.32±1.80
40	54.45±1.05	61.90±1.61	20µg/ml	12.78±1.85
60	68.66±1.12	70.74±0.40	30µg/ml	25.05±0.42
80	72.00±0.80	78.52.1.11	40µg/ml	28.73±0.57
100	75.65±0.66	90.75±1.34	50µg/ml	52.01±0.79
IC_{50} µg/ml	66.09	55.09	…………..	48.06

Percentage Scavenging (Mean ± SD) of Triplicates

Table 2 *In vitro* Free Radical scavenging effect of *Psidium guajava leaves* by nitric oxide scavenging assay

Concentration (µg/ml)	Aqueous extract	Alcoholic extract	Ascorbic acid	
			Concentrations	%inhibition
20	31.59±1.32	19.32±0.40	10µg/ml	27.09 ±0.78
40	33.02±0.32	25.43±0.34	20µg/ml	31.74 ±0.38
60	34.47±0.46	31.86±0.80	30µg/ml	33.75 ±1.12
80	38.49±0.44	39.10±0.42	40µg/ml	40.5 ±0.90
100	42.17±1.40	41.02±0.26	50µg/ml	46.94 ±0.38
IC_{50} µg/ml	118.56	121.89	…………..	53.25

Percentage Scavenging (Mean ±SD) of Triplicates

Table 3 *In vitro* Free Radical scavenging effect of *Psidium guajava leaves* by superoxide radical scavenging assay method

Concentration (µg/ml)	Aqueous extract	Alcoholic extract	Ascorbic acid	
			Concentrations	%inhibition
20	17.95±0.80	35.75±0.56	10µg/ml	66.2.44±0.40
40	20.27±0.33	53.92±0.60	20µg/ml	66.55±0.50
60	25.94±0.49	64.57±1.16	30µg/ml	67.86±0.39
80	29.11±0.37	67.17±0.15	40µg/ml	70.94±0.09
100	30.45±1.24	81.07±012	50µg/ml	73.67±1.45
IC_{50} µg/ml	164.2	061.67	…………..	33.93

Percentage Scavenging (Mean ± SD) of Triplicates

Table 4 *In vitro* Free Radical scavenging effect of *Psidium guajava leaves* by reducing power assay

Concentration (µg/ml)	Aqueous extract	Alcoholic extract	Ascorbic acid	
			Concentrations	%inhibition
20	7.49±1.04	3.88±1.75	10µg/ml	3.9±0.24
40	18.67±0.8	20.16±2.14	20µg/ml	12.17±0.83
60	22.79±4.24	25.92±1.54	30µg/ml	16.56±0.44
80	23.49±3.26	32.00±1.47	40µg/ml	20.63±0.83
100	24.94±3.27	45.05±1.35	50µg/ml	41.23±0.84
IC_{50} µg/ml	200.48	110.98	…………..	60.63

Percentage Scavenging (Mean ± SD) of Triplicates

Table 5 *In vitro* Free Radical scavenging effect of *Psidium guajava leaves* by ABTS radical scavenging assay method

Concentration (µg/ml)	Aqueous extract	Alcoholic extract	Ascorbic acid	
			Concentrations	%inhibition
20	42.13±0.62	51.31±0.09	10µg/ml	4.51±0.46
40	57.23±0.74	60.74±0.72	20µg/ml	10.55±0.29
60	64.17±0.38	68.00±0.79	30µg/ml	39.25±0.69
80	75.59±0.51	78.10±0.17	40µg/ml	43.93±0.77
100	84.44±0.37	81.59±0.08	50µg/ml	50.44±0.84
IC_{50} µg/ml	59.21	61.28	…………..	49.56

Percentage Scavenging (Mean ±SD) of Triplicates

Table 6 *In vitro* Free Radical scavenging effect of *Psidium guajava leaves* by H_2O_2 method

Concentration (µg/ml)	Aqueous extract	Alcoholic extract	Ascorbic acid	
			Concentrations	%inhibition
20	36.95±0.35	14.85±0.47	10µg/ml	20.30±0.15
40	43.40±0.48	26.36±1.04	20µg/ml	25.68±0.39
60	58.61±0.10	36.21±0.11	30µg/ml	42.46±0.29
80	58.67±4.2	42.03±0.79	40µg/ml	44.88±0.18
100	65.54±0.84	61.40±1.49	50µg/ml	52.77±0.20
IC_{50} µg/ml	76.28	81.43	…………..	47.37

Percentage Scavenging (Mean ± SD) of Triplicates

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