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Original Article

In vitro Antioxidant Activity of Extracts of *Psidium guajava* Leaves

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ABSTRACT

Background: The incidence of diabetes is increasing rapidly in world wide. It produces disturbances in the metabolism of carbohydrate, protein, and lipid. The reports prove that the various oxidative reactions are responsible for the various disorders like diabetes, cancer, aging etc. Evidences recommended that the natural medicines originating from plant source may represent a culturally relevant complementary treatment for diabetes. The present study aims to investigate the antioxidant activity of *P. guajava* leaves in an *in vitro* model of various extracts such as aqueous, ethanol and methanol. **Method:** The scavenging activity of DPPH, reducing power assay, NO, H_2O_2 and SOD is determined. **Results:** The results of the present study, proves all the three extracts which have an antioxidant activity in a dose dependent manner (250, 500, 750, 1000, 1500 µg/ml). The ethanolic extract of *P. guajava* leaves which have high activity when compared to the other two extracts. **Conclusion:** All the three extracts of P. guajava leaves have an antioxidant activity in an *in vitro* model. Among the various extracts, the ethanolic extract has a high activity. Further studies are needed for compound identification and its action in an *in vivo* model.

Key words: *Psidium guajava*, DPPH, Reducing power assay, NO, H_2O_2, SOD.

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INTRODUCTION

Oxidation is an essential process in living organisms to produce energy to biological processes. But uncontrolled oxidation process produces the free radicals and it causes the cell damage and it leads to various diseases such as cancer,[1] atheroscelerosis,[2] aging.[3] etc. In normal conditions our body produces the natural antioxidants and it prevents the cell from oxidative damages, but in pathological conditions our body needs additional supply of antioxidants. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and Butylated Hydroxy Anisole (BHA) are used to prevent the cell damages but it causes the adverse effects due to this, continuous searching for alternative drugs. In now-a-days the researchers are interested to find the drugs from natural sources. In all over the world nearly three-quarters were used plant and plant materials to treat various diseases.[4]

In a Myrtaeceae family, *Psidium guajava* is an important plant. It has grown in all subtropical areas of the world. The leaves of these plant which contains various phytoconstituents such as tannins, flavonoids, terpenoids, phenols etc., in various extracts.[5] In previous study proves that the leaves of *P. guajava* which contains the anti-diabetic activity in an *in vitro* model.[6] The present study aims to investigate the antioxidant activity of *P. guajava* leaves in an *in vitro* model.

MATERIALS AND METHODS

Plant material and extraction

The fresh leaves of *P. guajava* were collected locally and authenticated by the department of Botany, St. Joseph College, Trichy. The shade dried *P. guajava* leaves were powdered mechanically and stored in an air tight container. The extraction was carried out by hot percolation method using Soxhlet apparatus. The various solvents such as aqueous, ethanol and methanol were used. About 100 g of powder was extracted with 600 ml of the various solvents. The extract was concentrated to dryness under controlled temperature 40-50°C. The extract was preserved in refrigerator till further use.

In vitro antioxidant activity - free radical scavenging activity

Scavenging activity of hydrogen peroxide

The ability of the *P. guajava* to scavenge H_2O_2 was determined according to the method.[7] A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). H_2O_2 concentration was determined spectrophotometrically from absorption at 230 nm in a spectrophotometer (SL 159, UV- Visible Spec, Elico, India). Extracts (250, 500, 750, 1000, 1500µg) in distilled water were added to a H_2O_2 solution (0.6 mL, 40 mM). Absorbance of H_2O_2 at 230 nm was determined after ten min against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 of *P. guajava* and standard was calculated using the following equation:

$$\% \text{ Scavenging} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

Reducing Power assay

The reducing power of leaf extracts of *P. guajava* were determined by the method.[8] Substances which have reduction potential react with potassium ferricynaide (Fe^{3+}) to form potassium ferrocynaide (Fe^{2+}), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. Increase in the reduction of ferric to ferrous ion increases the absorbance indicating the reducing ability of ethanolic leaf extract of *P. guajava*.

Varying concentrations of ethanolic leaf extract of *P. guajava* (250, 500, 750, 1000 and 1500 µg) in double distilled water was mixed with 2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide. The mixture was incubated at 50°C for 20 min after which, 1.5 mL of TCA was added and centrifuged at 3000xg for 10 min. From all the tubes, 0.5 mL of supernatant was mixed with 1 mL of distilled water and 0.5 mL of ferric chloride. The absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increasing reducing power. Incubation with water in place of additives was used as the blank.

Scavenging activity of nitric oxide

The method of Garrat (1964).[9] was used to determine the nitric oxide Radical scavenging activity of leaf extracts of *P. guajava*

A volume of 2 ml of 10 mM sodium nitroprusside prepared in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract, at various concentrations (250 - 1500 µg). The mixture was incubated at 25°C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent. The mixture was incubated at room temperature for 30 min, followed by the measurement of absorbance at 540 nm. The amount of nitric oxide radical inhibited by the extract was calculated using the following equation:

$$\% \text{ Scavenging} = \frac{\text{Control CD} - \text{Test OD}}{\text{Control OD}} \times 100$$

Superoxide anion scavenging activity

Superoxide anion scavenging activity of leaf extract of *P. guajava* was determined. The assay was based on the superoxide anion generation from dissolved oxygen by PMS–NADH coupling reaction and reduces NBT. Oxidation of NADH by phenazine methosulphate (PMS) to liberate PMS_{red}, which in turn converts oxidized nitroblue tetrazolium (NBT_{oxi}) to the reduced form NBT_{red}. This forms a violet colour complex indicating the generation of superoxide anion, which was measured spectrophotometrically at 560 nm. A decrease in the formation of colour after addition of the antioxidant was a measure of its superoxide radical scavenging activity.

1 ml of NBT, 1ml of NADH solution and varying volumes of leaf extract of *P. guajava* (250-1500 µg) were mixed well. The reaction was started by the addition of 100 µM of PMS. The reaction mixture was incubated at 30 °C for 15 min. The absorbance was measured at 560 nm in a spectrophotometer. Incubation without leaf extract of *P. guajava* was used as blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging. The % of inhibition was calculated as shown below:

$$\% \text{ Scavenging} = \frac{\text{A Control OD} - \text{A sample}}{\text{A blank}} \times 100$$

DPPH scavenging assay

The ability to scavenging the stable free radical, DPPH was measured as a decrease in absorbance at 517 nm by the method.[10,11]

To a methanolic solution of DPPH (90.25 mM), an equal volume of leaf extract of *P. guajava* (250-1500 µg) was added and made up to 1.0 mL with methanolic DPPH. An equal amount of methanol was added to the control. After 20 min, the absorbance was recorded at 517 nm in a Systronics UV-visible Spectrophotometer. Ascorbic acid was used as standard for comparison. The inhibition of free radicals by DPPH in percentage terms (%) was calculated by using the following equation.

$$\% \text{ Scavenging} = \frac{\text{A Control OD} - \text{A sample}}{\text{A blank}} \times 100$$

Where A control is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound.

RESULT

The present study compares the *in vitro* antioxidant activity of aqueous, ethanolic and methanolic extracts of *P. guajava* leaves with the standard drug by an DPPH, reducing power assay, nitric oxide scavenging activity, H_2O_2 radical scavenging activity and SOD radical scavenging activity. The results are shown in the Table 1 – 5. All the three extracts have an antioxidant activity in an *in vitro* model.

All extracts have inhibition activity against the DPPH in a dose dependent manner. Aqueous extract of *P. guajava* leaves produced 16.21%, 31.29%, 40.91%, 53.67% and 62.39% of inhibition against the DPPH in a dose dependent manner (250 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml and 1500 µg/ml) respectively and its IC_{50} value is 950 µg/ml. Ethanolic extract shows 24.81% of inhibition at 250 µg/ml and 85.26% at 1500 µg/ml and its IC_{50} value is 640 µg/ml. Methanolic extract exhibited 17.09% and 71.49% of inhibition at 250 µg/ml and 1500 µg/ml respectively and its IC_{50} is 800 µg/ml. The standard drug of ascoribic acid, which shows the inhibition of 27.96% and 88.89% of inhibition at 250 µg/ml and 1500 µg/ml respectively, IC_{50} is 575 µg/ml (Table 1). Jamuna *et al*., (2012).[12] to find the similar effect in the methanolic leaf and root extract of *Hypochaeris radicata* in an *in vitro* model.

Table 2 shows the reductive capabilities of *P. guajava* leaves extracts and α-tocopherol. The reducing power of *P. guajava,* has increased the concentration dependently in all the three extracts. The maximum activity of aqueous, ethanolic and methanolic extracts are observed at the maximum dose of 1500 µg/ml at 79.48%, 93.07% and 86.38% respectively. The standard of α-tocopherol inhibitory activity is 94.46%. The IC_{50} value of aqueous, ethanolic and methanolic extract and standard is 375 µg/ml, 225 µg/ml, 275 µg/ml and 220 µg/ml respectively. Based on this result, the ethanolic extract of *P. guajava* which have higher inhibitory activity. The results of the *in vitro* antioxidant properties of methanol and aqueous extracts of *Parkinsonia aculeate* leaves (Sonia Sharma and Adarsh pal vig, 2013).[13] are proved in this study also.

The inhibition against the NO of aqueous, ethanol and methanolic extracts at 1500 µg/ml is 56.84%, 70.71% and 60.63% and their IC_{50} values are 975 µg/ml, 800 µg/ml and 825 µg/ml respectively. The standard drug of ascoribic acid which shows the inhibition of 72.84% of inhibition at 1500 µg/ml, IC_{50} is 750 µg/ml (Table 3). Divya and Vijaya anand (2015).[14] proves the NO inhibition activity of *Terminalia catappa* leaves which favours the antioxidant nature in an *in vitro* model. This may prove in this study also.

The scavenging ability of *P. guajava* extracts on H_2O_2 is shown in Table 4 and it compared with α-tocopherol. The percentage scavenging of H_2O_2 surged with the enhanced concentration of plant extract. The highest Percentage of 59.92%, 89.77%, 73.52%, and 92.49% of scavenging activity is observed at 1500 µg/ml of the aqueous, ethanolic, methanolic and α-tocopherol respectively and the IC_{50} value leaf extract on H_2O_2 radical is 1050 µg/ml, 800 µg/ml, 900 µg/ml and 750 µg/ml. The result of H_2O_2 scavenging activity of this study is like the results of the *in vitro* antioxidant activity of *Cressa cretica.*[15]

The SOD scavenging activity of various extracts of *P. guajava* leaves and butylated hydroxytoluene is shown in Table 5. The higher inhibitory activity (50.19%, 67.68%, 57.80% and 69.72%) is shown at 1500 µg/ml and the lower inhibitory activity (12.61%, 16.92%, 14.39% and 17.75%)

Table 1: DPPH scavenging activity of *P. guajava* leaves extracts.

Test	Concentration (µg/ml)	% nhibition of aqueous Extract	% Inhibition of ethanolic extract	% Inhibition of methanolic extract	% Inhibition of Standard
DPPH	250	16.21	24.81	17.09	27.96
	500	31.29	44.37	33.58	47.10
	750	40.91	59.72	48.91	63.91
	1000	53.67	78.61	63.76	81.32
	1500	62.39	85.26	71.49	88.89
IC_{50}		950	640	800	575

Table 2: Reducing power assay of extracts of *P. guajaval* leaves.

Test	Concentration (µg/ml)	% nhibition of aqueous Extract	% Inhibition of ethanolic extract	% Inhibition of methanolic extract	% Inhibition of Standard
Reducing power assay	250	44.91	57.84	48.36	59.93
	500	52.38	65.17	56.91	67.36
	750	61.82	78.52	68.57	82.98
	1000	70.93	84.82	75.63	88.18
	1500	79.48	93.07	86.38	94.46
IC_{50}		375	225	275	220

Table 3: Nitric Oxide assay of extracts of *P. guajava* leaves.

Test	Concentration (µg/ml)	% nhibition of aqueous Extract	% Inhibition of ethanolic extract	% Inhibition of methanolic extract	% Inhibition of Standard
Nitric Oxide	250	19.26	26.91	22.78	27.18
	500	25.85	36.73	31.63	38.09
	750	39.28	47.90	43.79	50.25
	1000	47.51	59.82	52.05	62.18
	1500	56.84	70.71	60.63	72.84
IC_{50}		975	800	825	750

Table 4: H_2O_2 Assay of *P. guajava* leaf extract.

Test	Concentration (µg/ml)	% nhibition of aqueous Extract	% Inhibition of ethanolic extract	% Inhibition of methanolic extract	% Inhibition of Standard
H_2O_2 Assay	250	16.30	22.58	18.34	24.88
	500	24.95	32.39	27.91	37.27
	750	36.21	47.62	42.64	49.36
	1000	48.37	67.68	57.83	67.76
	1500	59.92	88.77	73.52	92.49
IC_{50}		1050	800	900	750

Table 5: Super Oxide Dismutase assay of extracts of *P. guajava* leaves.

Test	Concentration (µg/ml)	% nhibition of aqueous Extract	% Inhibition of ethanolic extract	% Inhibition of methanolic extract	% Inhibition of Standard
SOD Assay	250	12.61	16.92	14.39	17.75
	500	18.38	24.85	21.73	29.19
	750	28.64	45.64	39.68	39.92
	1000	39.73	55.69	49.74	58.72
	1500	50.19	67.68	57.80	69.72
IC_{50}		1375	900	1000	875

is shown at 250 µg/ml of aqueous, ethanolic, methanolic and standard BHT. The IC_{50} value is 1375 µg/ml, 900 µg/ml, 1000 µg/ml and 875 µg/ml. Vijayakumar *et al*, (2015).[16] proves the increasing concentration of *P. guajava* leaves extract have a high inhibitory activity of SOD, this may true in this study also.

DISCUSSION

The *P. guajava* leaves are popular in an indigenous system of folk medicine. It is mainly used for the treatment of various ailments. Some investigations examined anti-microbial, anti-diarrheic, anti-spasmodic and anti-cancer activity. *P. guajava* leaves contain many major pharmacologically active ingredients, and so many other bioactive compounds. The important active constituents are essential oils, flavonoids, phenolic compounds, triterpenoids and aldehydes. In view of the huge medicinal importance of this plant, the present study is carried out to evaluate the *in vitro* anti-oxidant activity of the *P. guajava* leaves of various extracts.

DPPH is a free radical, it easily damages the cell membrane. DPPH is easily accepting the electrons or hydrogen radical from antioxidant compounds. The DPPH which gains the hydrogen atom from the antioxidant compounds and the colour will be changed. In the present study the indensity of the colour is directly proportional to the inhibitory activity of the antioxidant compound. It shows the inhibitory activity is due to the maximum hydrogen donating ability of *P. guajava* leaves extracts. Based on this result the maximum inhibitory activity is noticed in the ethanolic extract at 1500 µg/ml.

The results obtained in the present study, all the three extracts have an antioxidant activity in a dose dependent manner. The reducing power assay is determined by the electron transfer ability of the plant extracts. The Fe^{3+} ions are converted into Fe^{2+} ions, this ability is minimizing the oxidative damages in the tissues.

In the present study proves that the inhibitory activity NO formation by various extracts of *P. guajava* leaves. The ethanolic extract have a maximum inhibitory activity against the NO formation in the concentration of 1500 µg/ml. NO is a free radical. It changes the structure and functions of the cellular membranes. It is formed from sodium nitroprusside and it react with free radicals to form nitrite. The antioxidant compound which directly reacts with the free radicals and other nitrogen compounds and it prevents the nitric oxide formation. This may prevent the cellular damages.

The present study proves the inhibition of hydroxyl radical production from H_2O_2 in a dose dependent manner. H_2O_2 is easily penetrate to the cell membranes. These molecules are converted into hydroxyl radicals and it damages the cell. Those compounds which donates the electrons to H_2O_2 is called antioxidants. The donating electron reacts with H_2O_2 and it neutralizes, and converted in to water.

SOD is an enzyme involved in the antioxidant defence activity. It produces the H_2O_2 from superoxide anion and thus it prevents the toxic effect. In the present study, the percentage of inhibition is directly proportional to the prevention of cellular damages. The increasing concentration of *P. guajava* leaves extract have a maximum inhibitory activity against the SOD.

CONCLUSION

The present study clearly proves that the extract of *P. guajava* leaves have an antioxidant activity in an *in vitro* model.

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CONFLICT OF INTEREST

The authors declare none.

ABBREVIATIONS USED

P. guajava: Psidium guajava; **BHT:** Butylated hydroxyl toluene; **BHA:** Butylated hydroxyl anisole; **H2O2:** Hydrogen peroxide; **NO:** Nitric Oxide; **PMS:** Phenazine methosulphate; **NBT:** Nitro blue tetrazolium.

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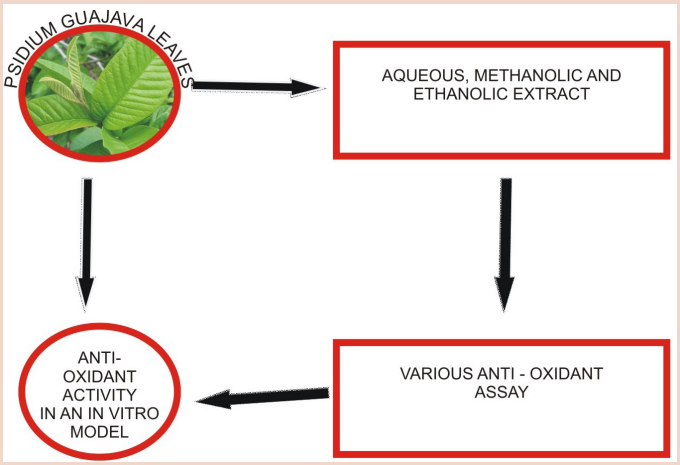
PICTORIAL ABSTRACT



SUMMARY

- The aqueous, methanolic and ethanolic extract of *P. guajava* leaves have an anti-oxidant activity in an *in vitro* model.
- Among these the ethanolic extract of *P. guajava* leaves have a higher activity in an i*n vitro* model.

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