Madras Agric.J., 2023; https://doi.org/10.29321/MAJ.10.200805

A Comparative Study on the Antioxidant and Antidiabetic Activities off Ethanolic Leaf Extracts of *Psidium Guajava* L. Varieties

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Abstract

Antioxidants play an important role in protecting the cell from damage caused by oxidative stress (OS). Plants have potential phytochemicals, which possess antioxidant properties. The present study was designed to investigate the screening of phytochemcials, antioxidant, and antidiabetic properties of ethanolic leaf extracts of *Psidium guajava* varieties (Lucknow 49 and TRY G1). The leaf extracts were screened for various phytochemicals (alkaloids, flavonoids, saponin, phenols and glycosides) and antioxidant activities. Antidiabetic actives were evaluated by *in vitro* analysis using standard protocols. The antioxidant activities were determined by DPPH (1,1-diphenyl-2-picrylhydrazine) radical scavenging assay, superoxide radical scavenging assay and nitric oxide radical scavenging assay methods. The phytochemicals screening of the extracts revealed the presence of alkaloids, flavonoids, saponin, phenols, tannins, and glycosides. Among the varieties, Lucknow 49 had higher level of α-amylase inhibitor activity of 62.46 and α -glucosidase inhibitor activity of 98.40 at 100 µg/mL than TRY G1. Both the varieties of the extracts showed antioxidant activities in dose dependent manner, and values were compared with standard ascorbic acid. Lucknow 49 showed the

maximum DPPH (60.68%), superoxide radical (65.67%) and nitric oxide radical(61.36%) scavenging activities than TRY G1 variety. The IC_{50} values of the variety Lucknow 49 were also confirmed antioxidant potentials. The results indicate that both the varieties of *Psidium guajava* had significant antioxidant and antidiabetic potentials, hence the plant leaves could serve as effective antidiabetic agents and potent free radical scavengers, which may be used for pharmaceutical applications. However, further exploration is necessary for effective usage in both traditional and modern system of medicines.

Keywords: *Phytochemicals; Antidiabetic; Antioxidant; Psidium guajava; Oxidative stress*.

1.0 Introduction

A free radical is an unstable molecule that has an unpaired electron. This unstable radical has the tendency to become stable through electron pairing with biological macromolecules such as proteins, lipids and nucleic acids (Rahman *et al.,* 2015). Radical-caused cell damage can become more extensive due to weakened cellular antioxidant defense systems. All biological systems have inherent antioxidant defense mechanisms that remove damaged molecules, but these mechanisms can be incompetent. Therefore dietary intake of antioxidants is very important to protect cells from damage caused by free radicals (Sherki *et al.*, 2002). Oxidative stress is one of the causes of free radicals and reactive oxygen species (ROS). These can be formed under normal physiological conditions, and when not eliminated appropriately, they can become harmful (Pattanayak *et al*., 2011).

Oxidative damage can lead to breakdown or hardening of lipids due to lipid peroxidation, which results in cell death or makes it nonfeasible for the cell to get nutrients or to receive and send signals (Gaikwad *et al*., 2011). Antioxidants prevent and stabilize the damage caused by free radicals by supplying electrons. Antioxidants also turn free radicals into waste by-products, and then it is eliminated from the body. Consumption of fruits and vegetables lower the risk of several diseases caused by free radicals (Hamid *et al.,* 2002). Diabetes mellitus is one of the major health issues worldwide, it is a metabolic disorder of multiple etiologies distinguished by an improper metabolism of glucose with a disorder of carbohydrate, fat, and protein metabolism as a result of defects in insulin secretion and/or insulin action (Tafesse *et al.,* 2017). According to

International Diabetes Federation (IDF) report, elevated blood glucose is the third uppermost risk factor for premature mortality, following high blood pressure and tobacco use globally (Barcelo and Rajpathak, 2001).

Despite several antidiabetic agents that have been introduced in the market from natural and synthetic sources, diabetes continues as a significant medical problem worldwide. The currently available modern drugs used to treat diabetes are often associated with boundaries such as inadequate efficacy, high cost, and various side effects (Alme *et al.,* 2020). Medicinal plants with antidiabetic activity can be used as an alternative medicine in managing diabetes, especially in developing countries, due to their cost effectiveness, accessibility, and lower side effects. Medicinal plants of more than 1200 species are used throughout the world in traditional medicine for curing diabetes (Piero *et al.,* 2015). In the recent decade, the role of antioxidants has been increasingly recognized as a critical influence on the biochemistry of living beings (Umesha *et al.,* 2013). Hence, this study was conducted for the comparison of antioxidant and antidiabetic activities of ethanolic leaf extracts of Lucknow 49 and TRG G1 guava varieties.

2.0 Materials and methods

2.1 Collection of plant materials

The leaves of *Psidium guajava*, namely Lucknow 49 and TRY G1 were collected from the orchard of Horticultural College and Research Institute for Women, TNAU, Trichy. The leaf samples were washed separately with fresh water and then shade dried at room temperature separately. The dried leaves were finely powdered, sieved, and then stored in sterile airtight containers at 4 ^{o}C under refrigeration for further use.

2.2 Chemicals

Alpha glucosidase, porcine pancreas alpha amylase, *p*-nitrophenyl-α-D-glucopyranose (*p*-NPG), 3,5- dinitrosalicylic acid (DNS), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), nitro blue tetrazolium (NBT), rutin and acarbose were purchased from Sigma Chemical Co., USA. All other chemicals used including the solvents, were of analytical grade.

2.3 Extraction of plant materials

Hundred grams of powdered leaf samples of Lucknow 49 and TRY G1 were taken in a conical flask separately. The samples were extracted with 250 mL of three different solvents viz., hexane, ethanol and water. The contents of the flasks were kept in mechanical shaker over night. This suspension was filtered and the residue was resuspended with an equal volume of solvents for 48 hours extraction and then filtered again. The two filtrates were pooled, and the solvents (hexane and ethanol) were evaporated. The dried residue was used for further analysis.

2.4 Preliminary phytochemical screening

The phytochemical screening of hexane, ethanol, and water extracts of leaves of Lucknow 49 and TRY G1 was carried out with standard procedures for determining the presence of phytochemicals (Peach and Tracey, 1955).

***2.5 In vitro* antidiabetic activity**

A. *α*-amylase inhibitor assay

A starch solution (1% w/v) was prepared by stirring 1g starch in 100 mL of 20 mM of phosphate buffer (pH 6.9) containing 6.7 mM of sodium chloride. The enzyme solution was prepared by adding 27.5 mg of porcine pancreatic amylase α- amylase in 100 mL of 20 mM of phosphate buffer (PBS, pH 6.9) containing 6.7mM of sodium chloride. To 100 µLof (20, 40, 60 80, 100 µg/mL) plant extracts, 200 µLporcine pancreatic amylase was added and the mixture was incubated at 37 ºc for 20 min. To the reaction mixture 100 µL(1%) starch solution was added and incubated at 37 ºC for 10 min. The reaction was stopped by the addition of 200 µL DNSA (1g of 3,5 di nitro salicylic acid, 30g of sodium potassium tartarate and 20 mL of 2N sodium hydroxide and made up to a final volume of 100 mL with distilled water) and kept it in a boiling water bath for 5 minutes. The reaction mixture was diluted with 2.2 mL of water and taken the absorbance at 540 nm. For each concentration, blank tubes were prepared by replacing the enzyme solution with 200 µLin distilled water. Control, representing 100% enzyme activity, was prepared in a similar manner, without extract. The experiments were repeated thrice using the same protocol (Ali *et al.*, 2006).

B. *α* -glucosidase inhibitior assay

The inhibition of *α* -glucosidase activity was determined using the modified published method (Kim *et al.*, 2011). One mg of α- glucosidase was dissolved in 100 mL of phosphate buffer (pH 6.8). To 100 µLof (20, 40, 60, 80, 100 µg/mL) plant extracts, 200 µL-glucosidase were added and the mixture was incubated at 37 ºC for 20 min. To the reaction mixture 100 µL3mM -nitrophenyl –D glucopyranoside (*p*-NPG) was added and incubated at 37 ºC for 10 min. Then to the reaction mixture was added 2 mL Na_2CO_3 (0.1M) to terminate the reaction and the *α* -glucosidase activity was measured at 405 nm on UV-VIS spectrophotometer by measuring the quantity of *p* –nitrophenol released from *p*-NPG. Acarbose was used as positive control of α-amylase and *α* -glucosidase inhibitors. The concentration of the extract required to inhibit 50% of *α* -amylase and *α* –glucosidase activities under the assay conditions was defined as the IC_{50} value.

2.6 *In vitro* antioxidant activities

A. DPPH radical scavenging assay

The free radical scavenging activity of the plant extracts and ascorbic acid was determined using the stable radical DPPH (1,1-diphenyl-2- picrylhydrazyl) (Bolis, 1958). The extract at different concentrations (100–500 µg/mL) was placed in test tubes and 3.9 mL of freshly prepared DPPH solution (25 mg L) in methanol was added in each test tube and mixed. 30 min later, the absorbance was measured at 517 nm in UV-visible spectrophotometer. The capacity to scavenge the DPPH radical was calculated by using the formula as follows

$$\text{DPPH scavenged (\%)} = (Ac - At)/Ac \times 100$$

Where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was calculated with the help of concentration in mg of dry material per mL (mg / mL) that inhibits the formation of DPPH radicals by 50%. Each value was determined using the regression equation.

B. Nitric oxide radical (NO.) scavenging assay

Nitric oxide generated from sodium nitro prusside (SNP) was measured according to the method of Marcocci *et al.* (1994). The total volume of reaction mixture 5.0 mL containing SNP (5 mM) in phosphate-buffered saline (pH 7.3), with or without the plant extract at different concentrations (100–500 µg/mL) was incubated at 25 °C for 180 min in the visible polychromatic light source (25W tungsten lamp). The nitric oxide radical thus generated interacted with oxygen to produce the nitrite ion (NO.) which was assayed at 30 min intervals by mixing 1.0 mL of incubation mixture with an equal amount of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the coloured mixture (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured in 546 nm using spectrophotometer. The nitrite generated in the presence or absence of the plant extract was estimated using a standard curve based on sodium nitrite solutions of known concentrations. Each experiment was conducted with at least three duplicates and the data presented as an average of three independent determinations.

C. Superoxide radical ($O_2^{\cdot -}$) scavenging assay

This assay was carried out on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) as described by Martinez *et al.* (2001) with slight changes in procedure of Awah *et al.,* 2010. Briefly, each 3.0 mL reaction mixture contained 0.05 M PBS (pH 7.8), 13 mM methionine, 2 M riboflavin, 100 MEDTA, NBT (75 M) and 1.0 mL of test sample solutions. The tubes were kept in fluorescent light 725 lumens, 34 W and absorbance was read at 560 nm after 20 min. The entire reaction was covered in aluminium foil. The percentage inhibition of superoxide anion was estimated using the following equation:

$$\% \text{ Inhibition} = A_o - A_S / A_0 \times 100$$

Where, *A*0 is absorbance of the control, and *A*s is absorbance of the tested sample. The IC_{50} represented the concentration of the extract that inhibited 50% of radical.

2.7 Statistical analysis

The data are presented as the mean ± standard deviation (SD) of three independent triplicate experiments. Statistical analysis was performed by using statistical package SPSS version 21.0 software. The differences in treated and untreated groups were assessed through one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests.

3.0 Results and Discussion

3.1 Preliminary Phytochemical Screening

The phytochemical investigation of three different solvents (hexane, ethanol and water) extracts of leaves of *Psidium guajava* varieties (Lucknow 49 and TRY G1) were carried out with standard procedures for determining the presence or absence of phytochemicals. Qualitative phytochemicals analysis results are represented in the Table 1.

Preliminary phytochemical screening of the leaf extracts (hexane, ethanol and water) of both the varieties (Lucknow 49 and TRY G1) showed positive results for alkaloids, flavonoids, saponin, phenols, tannins and glycosides. Among the extracts, ethanolic extract of both the varieties were showed the maximum presence of phytochemicals when compared to hexane and water. So the ethanolic extract of *Psidium guajava* was used for further investigations.

3.2 Antidiabetic activity

A. α–amylase inhibitor activity

In the present study, the *in vitro* *α*–amylase inhibitor activity of the ethanolic leaf extracts of Lucknow 49 and TRY G1 were examined, and the results are presented in table 2. From the table it was evident that, increasing the sample concentration range from 20 to 100 µg/mL, inhibition percentage also increased in dose-dependent manner. Among the varieties of extracts at concentrations of (20-100 µg/mL) Lucknow 49 was observed the higher *α*–amylase inhibitory activities of 62.46 followed by TRY G1, which showed 28.36 at 100 µg/mL. Standard drug of acarbose showed the values of 72.77 at 100 µg/mL.

α–glucosidase inhibitor activity

α–glucosidase inhibitor activity of ethanolic extracts of Lucknow 49 and TRY G1 were assayed and the results are represented in Table 3. The results revealed that extracts of all the three varieties had significant inhibitory activity. The percentage inhibition of all the varieties at 20- 100 µg/ mL concentrations showed dose dependent manner. Among the varieties, Lucknow 49 was observed the higher α– glucosidase inhibitor activity of 32.78 at 20 µg/mL and 98.40 at 100 µg/mL concentrations. The lowest α –glucosidase inhibitor activity was observed in TRY G1, which showed minimum activity of 5.80 at 20 µg/mL and maximum of 52.43 at 100 µg/mL. Acarbose was a standard drug for α - glucosidase inhibitor activity, which showed values of 84.94 at 100 µg/mL.

The plant extract might be acted as starch blockers and prevents the absorption of starch in to the body. Blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose. The secondary metabolites such as phenols, flavonoids, saponins, alkaloids, tannins and terpenoids are present in the plant extract could be responsible as being effective inhibitors of α-amylase and α-glucosidase.

***3.3 In vitro* antioxidant activity**

A. DPPH radical scavenging activity

The evaluation of anti-radical scavenging properties of the ethanolic leaf extracts (Lucknow 49 and TRY G1) of *Psidium Guajava* were executed by DPPH radical scavenging assay. The percentage inhibition of DPPH radicals by the plant extracts was determined, and the results are represented in the Table 4.

From the table, it was found that the extracts of both varieties at different concentrations (100, 200, 300, 400, and 500 µg/mL) showed their activities in dose-dependent manner. The scavenging effect of the extracts was compared with standard ascorbic acid. In both the varieties Lucknow 49 showed the maximum DPPH radical scavenging activities from 13.50 to 60.68 at 100 to 500 µg/mL respectively. The standard ascorbic acid showed inhibition of 73.21 at 50µg/mL and the IC_{50} values were found 403.90 and 478.35 respectively.

B. Superoxide radical scavenging activity

The superoxide radical scavenging activity of the ethanolic leaf extracts of selected varieties of *Psidium guajava* was analysed and the results are depicted in the Table 5. The plant extracts of the two varieties had significant scavenging activities on superoxide radicals. The highest percentage of inhibition (65.67 %) was observed in Lucknow 49 at 500 µg/mL concentration. Standard ascorbic acid showed the value of 62.37 at 50µg/mL.

C. Nitric oxide radical scavenging activity

The nitric oxide scavenging activity of the ethanolic leaf extracts of selected varieties of *Psidium Guajava* were analyzed and the results are given in Table 6. The nitric oxide scavenging activity was maximum in Lucknow 49 (61.36) and TRY G1 (48.32) at 500 µg/mL concentration. This was compared with the standard ascorbic acid, which possess 73.56 at 50 µg/mL.

Conclusion

Results of the study clearly showed that both the varieties of *Psidium guajava* L. had alkaloids, flavonoids, saponin, phenols, tannins and glycosides. The extract had a significant amount of α-amylase and α- glucosidase inhibitory activities that may be due to bioactive compounds present in the leaves of Lucknow 49 and TRY G1. Lucknow 49 had strong DPPH, superoxide radical and nitric oxide radical scavenging activities than that of TRY G1. Hence, the ethanolic leaf extracts of Lucknow 49 and TRY G1 plant could be used as functional food and in the pharmaceutical preparations.

Acknowledgement

We are thankful to Honourable Vice-chancellor of Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu and The Dean, Horticultural college and Research Institute for Women, Tiruchirappalli for providing all necessary facilities.

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Table 1. Phtochemical screening of leaf extracts of three varieties of *Psidium guajava*

S. No	Tests	Lucknow 49			TRY G1		
		hexane	Ethanol	Water	Hexane	ethanol	Water
1	Alkaloids	-	+	+	-	+	+
2	Flavonoids	-	++	+	-	++	+
3	Saponins	-	-	-	-	+	-
4	Phenols	+	++	+	+	+	+
5	Steroids	-	-	-	-	-	-
6	Glycosides	-	++	+	-	++	+
7	Tannins	+	+	+	+	+	+
8	Triterpnes	-	-	-	-	-	-
9	Resins	-	+	+	-	+	+

(+) presence (-) absence

Table 2. *α*–amylase inhibitor activity of selected varieties of *Psidium guajava* leaf extracts and standard acarbose

Sample concentration (µg/mL)	% inhibition		
	Lucknow 49	TRY G1	Std. *acarbose*
20	10.73±0.71	2.37±0.17	10.63±0.55
40	20.37±1.36	7.31±0.50	36.31±1.91
60	38.67±1.19	21.24±0.61	45.69±1.38
80	42.41±1.45	27.66±0.73	54.37±2.00
100	62.46±1.05	28.36±1.55	72.77±1.54

Table 3. *α*- glucosidase inhibitor activity of selected varieties of *Psidium guajava* leaf extracts and standard acarbose

Sample concentration (µg/mL)	% inhibition		
	Lucknow 49	TRY G1	Std. *acarbose*
20	32.78±1.86	5.80±0.27	22.76±0.67
40	55.51±1.76	28.59±1.72	64.47±0.74
60	70.51±1.88	32.65±1.90	70.88±0.41
80	84.66±1.23	43.25±1.03	73.24±0.25
100	98.40±1.94	52.43±1.10	84.94±0.45

Table 4. DPPH radical scavenging activity of selected varieties of *Psidium guajava* leaf extracts and standard ascorbic acid

Sample concentration (µg/mL)	% inhibition	
	Lucknow 49	TRY G1
100	13.50±0.36	10.47±0.35
200	18.55±0.56	17.56±0.41
300	28.73±0.57	34.57±1.29
400	47.23±1.05	44.00±1.56
500	60.68±0.87	53.48±1.17
IC_{50}Value	403.90	478.35
Std. *Ascorbic acid* at (50µg/mL)	77.21±1.28	

Table 5. Super radical scavenging activity of selected varieties of *Psidium guajava* leaf extracts

Sample concentration (µg/mL)	% inhibition	
	Lucknow 49	TRY G1
100	11.70±0.49	10.76±0.16
200	28.73±0.58	19.67±0.11
300	3.51±1.06	27.62±1.79
400	51.63±1.64	41.58±1.56

500	65.67±2.05	58.31±1.48
IC_{50}Value	390.03	482.68
Std. *Ascorbic acid* at (50µg/mL)	62.37±0.96	

Table 6. Nitric oxide radical scavenging activity of selected varieties of *Psidium guajava* leaf extracts

Sample concentration (µg/mL)	**% inhibition**	
	Lucknow 49	**TRY G1**
100	7.74±0.417	6.84±0.52
200	12.49±1.46	22.65±0.94
300	30.45±1.21	27.44±0.96
400	41.45±0.87	34.51±1.68
500	61.36±1.01	48.32±1.59
IC_{50}Value	483.72	489.21
Std. ***Ascorbic acid*** **at (50µg/mL)**	73.56±1.56	