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Original Article

PHYTOCHEMICAL SCREENING OF *PSIDIUM GUAJAVA* BARK AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *PSIDIUM GUAJAVA* BARK TANNINS

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ABSTRACT

Objective: Medicinal plants have been used in primary health care over many centuries before the advent of modern medicine. *Psidium guajava* is one of the important medicinal plants having many therapeutic uses. The present study deals with phytochemical investigations of *Psidium guajava* bark and *in vitro* antioxidant activities of *Psidium guajava* bark tannins.

Method: Acetone, Ethanol and Water extracts of *Psidium guajava* bark were assessed for the phytochemical screening. Extraction of tannin from guava bark was assessed using a cold extraction method. Total phenolic content was estimated using Folin-Ciocalteau's method. Tannin was estimated using Folin-Denis method. The antioxidant activity was determined by measuring the scavenging activity of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Nitric oxide radical, super oxide radical activity and Neutrophil-MTT assay. Ascorbic acid and Butylated hydroxytoluene (BHT) were used as standard drugs for the antioxidant studies.

Results: The phytochemical analysis of guava bark revealed the presence of tannins, flavonoids, saponins, glycosides and phenols in all three bark extracts (Acetone, Ethanol and Water). The bark tannin extract possessed strong scavenging activity against DPPH, Nitric oxide radical, super oxide radical and Neutrophil-MTT assays, IC_{50} values were 31.94 µg/ml, 54.37µg/ml, 19.04 µg/ml and 95.41 µg/ml respectively.

Conclusion: From the results, it may be concluded that tannins present in the bark of *Psidium guajava* may be responsible for the antioxidant activity and has promising therapeutic potential, its pharmacological properties which if properly harness can be used in the management of various diseases and can serve as a base for the development of novel potent drug.

Keywords: Medicinal plants, *Psidium guajava*, tannins, antioxidants, neutrophil-MTT assay

INTRODUCTION

The role of free radicals and active oxygen in the pathogenesis of human disease including cancer, aging and atherosclerosis has been recognized [1]. Electron acceptors, such as molecular oxygen, react rapidly with free radicals to become radical themselves, also referred to as reactive oxygen species (ROS). The ROS includes super oxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is also associated with several types of biological damages. Therefore much attention has been focused on the use of antioxidant, especially natural antioxidants to inhibit peroxidation and to protect from damage due to free radicals [2].

Antioxidants which are used nowadays are obtained mainly from two major routes; chemical synthesis and natural living source extraction. According to scientific research, severe toxicity caused by chemical synthetic antioxidants such as genotoxicity, carcinogenicity [3, 4] or hepatotoxicity [5] has been increasingly reported. Hence, the use of synthetic antioxidants is tending to increase and needs replacement with other safer compounds. Meanwhile, natural antioxidants, derived mostly from plants have been reported for high potential in prophylacsis and treatment of many degenerative diseases caused by chain oxidative reactions [6]. An inverse relationship has been reported between consumption of natural antioxidants and mortality from degenerative diseases [7]. Therefore search of non-toxic high potential natural antioxidants is of increasing interest. Tannins are significant plant secondary metabolites subdivided into condensed and hydrolysable compounds in vascular plants. Tannins are antioxidants often characterized by reducing power [8] and scavenging activities [9].

Medicinal plants have played a significant role in ancient traditional systems of medication in many countries. Indigenous herbs are used as remedies against various diseases in the traditional system of medicine or in ethnomedical practices [10]. *Psidium guajava* (guava), one of the most edible plants has long been used as traditional medicine. It has been demonstrated to have several biological activities such as antidiabetic [11], anticough, antibacterial [12] and anti-plasmodic actions [13]. Recently it has been reported to possess high potential for antioxidant activity [14]. The bark of the plant contains tannins (12%-30%) and calcium oxalate crystals, while the seeds contain glycine rich proteins, starch and phenolic and flavonoids compounds [15, 16]. The roots are also rich in tannins [17]. The plant also contains leukocyanidins, sterols and gallic acid in the roots [18]. Recently much attention has directed towards extracts and biologically active compounds isolated from popular plant species. In the present era of drug development and discovery of newer drug molecules, many plant products are evaluated on the basis of their traditional uses [19]. In view of its therapeutic importance, the current study was undertaken to investigate the phytochemical screening and *in vitro* antioxidant potential in *Psidium guajava* bark tannins.

MATERIALS AND METHODS

Collection and authentication of the Plant material

The plant bark was collected from Perinthalmanna, Malappuram, Kerala. The plant material was identified and authenticated by Botanical Survey of India, Coimbatore, Tamilnadu (Voucher No: BSI/SRC/5/23/2010-11/Tech.1785). A voucher specimen has been deposited in the laboratory for future reference.

Preparation of the bark extract

Plant bark was air dried at room temperature for one week to get consistent weight. The dried barks were ground to powder. The guava bark of 2g each was extracted with 40 ml of hexane, benzene, chloroform, dichloromethane, acetone, ethanol, methanol and water (increasing order of polarity) using soxhlet apparatus. All chemicals and reagents used including the solvents were of analytical grade.

Preliminary Phytochemical Screening

Phytochemical analysis was carried out in the acetone, ethanol, and water extracts of the bark of *Psidium guajava* using standard procedures to identify constituents, as described by Harborne (1984), Trease and Evans (1979) [20, 21].

Cold Extraction of tannins from guava bark

Sample (100g) was defatted in soxhlet with diethyl ether and the residue was transferred to a dark bottle. It was then extracted overnight at 1ºC in rotary shaker with 70% acetone (3*300 ml). The residue was again extracted at 1ºC in rotary shaker again with 50% methanol (3*300 ml). The methanolic and acetone extracts were combined and the solvent was distilled off in vacuum using rotary evaporator.

Quantitative Analysis

Estimation of tannins in bark extracts (Folin-Denis Method)

Colorimetric estimation of tannins is based on the measurement of blue color formed by the reduction of phosphotungstomolybdic acid by tannin like compounds in alkaline solution. 1 ml of aliquots sample (10 mg/100 ml) was mixed with 5 ml of Folin-Denis reagent and 20% sodium carbonate and made up to 100 ml, mixed well and absorbance was read at 760 nm after 30 min using spectrophotometer.

Estimation of total phenolic content in bark tannin extract

The total phenol was measured at 765 nm by Folin Ciocalteau reagent [22]. The dilute methanolic extract of bark tannins (0.5 ml of 1:10g ml^{-1}) was mixed with Folin Ciocalteau reagent (5 ml, 1:10 diluted with distilled water) and aqueous sodium carbonate (4 ml, 1M). The mixture was allowed to stand for 15 min and the total phenols were estimated by spectrophotometer at 765 nm.

Estimation of flavonoids content in tannin extract

Aluminum chloride colorimetric technique was used for flavonoids estimation [23]. Extract (0.5 ml of 1:10 g ml^{-1}) in methanol was mixed with 1.5 ml of methanol, 0.1ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. It was left at room temperature for 30 min after which the absorbance of the reaction mixture was measured at 415 nm with a double beam UV/Visible spectrophotometer.

***In Vitro* Antioxidant Assays**

Determination of radical scavenging activity using DPPH assay

DPPH assay was carried out with a standard procedure [24]. 1mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3 ml of extract solution and standard in water at different concentrations. 30 minutes later absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated as,

$$\%\ \text{inhibition} = \frac{A_{conc} - A_{test}}{A_{conc}} \times 100$$

Nitric oxide radical scavenging assay

The method was adopted to determine the nitric oxide radical scavenging activity of tannin extract [24]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which interacts with oxygen to produce nitrite ions determined by the use of Griess reagent. To 1 ml of 10 mM sodium nitroprusside dissolved in 1 ml 5 mM phosphate buffer saline (pH 7.4) was mixed with 0.1 ml of plant extract at various concentrations. The mixture was incubated at 250ºC. After 150 minutes, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent [(1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 minutes with 1 ml of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 10 minutes. The absorbance was measured at 540 nm. The amount of nitric oxide radical was calculated as,

$$\%\ \text{inhibition} = \frac{A_{conc} - A_{test}}{A_{conc}} \times 100$$

Superoxide radical scavenging activity

Super oxide scavenging activities of tannin extract was determined by the standard procedure [25]. 1.25 ml of tetra sodium pyrophosphate (0.052 M, pH 8.3), 0.1 ml phenazene methosulphate (186µM), 0.3 ml NBT (300µM) were added to tannin extract of different concentrations. 0.2 ml of NADH (780 µM) was added and incubated 5 minutes at 30ºC. 0.5 ml acetic acid was added and left for 10 minutes. The absorbance was read at 560 nm. The amount of superoxide radical was calculated as,

$$\%\ \text{inhibition} = \frac{A_{conc} - A_{test}}{A_{conc}} \times 100$$

Neutrophil isolation and superoxide production

Pipetted out 4 ml of anti-coagulant into a 50 ml conical tube and poured 20 ml of whole blood to the side of the tube. Gently inverted the tube several times to mix (for other volumes used 1 ml anti-coagulant for every 5 ml blood). Pipetted out 12 ml (50% of blood volume) of 6% Dextran/ 0.9% NaCl solution into the blood mixture and inverted 18-20 times to ensure adequate mixing. Pipetted out the mixture into 4*15 ml tubes (10ml/tube), Let the four tubes stand at room temperature for 45 min-1hr, or until separation is completed. Return to the settling blood and the yellowish supernatant was pippetted out into a 50 ml tube. Spin at 1500 rpm for 12 minutes at 4ºC low brake. Discard the supernatant and resuspended in 12 ml of the ice-cold distilled H_2O. Sucked up and dispense repeatedly to break the pellet. After 20 seconds 4 ml of 0.6 M potassium chloride was added and mixed several times. The solution was added to 50 ml with Phosphate buffer saline (PBS). It was spinned at 1500 rpm for 6 minutes at 4ºC using a high brake. The supernatant was discarded and resuspended the pellet in 2.5 ml of PBS. The cell suspension was layered over 3 ml of Ficoll-Hypaque (Sigma 1077) in a 15 ml tube. It was spinned at 1500 rpm for 30 minutes at 4ºC using a low brake, resuspended the pellet in 2 ml PBS. The cell concentration was determined using a haemocytometer.

Neutrophil-MTT assay

6 µl (1.25mM) MTT, 150 µl PBS (25mM), 200 µl of neutrophils were added to tannin extract with different concentrations. 0.1 ml NADH (780 mM) was added to tubes and incubated for 20 minutes at 30ºC. 150 µl DMSO was pipetted out to the tubes and the absorbance was read at 570 nm. The neutrophil-MTT assay was determined by,

$$\%\ \text{inhibition} = \frac{A_{conc} - A_{test}}{A_{conc}} \times 100$$

RESULTS AND DISCUSSIONS

The phytochemical screening of acetone, ethanol and water extracts of bark were analyzed (Table 1). Tannins, phenols, saponins and cardiac glycosides were present in all the three solvent extracts. Chemical investigation on the different parts of the plant has

resulted in the isolation of a large number of novel and interesting metabolites [26].

The first extraction with 50% aqueous methanol followed by second extraction with 70% aqueous acetone can also be used for extraction of phenolics. A very long period of phenolic extraction and extraction at high temperature may lead to inactivation of phenolics. The most widely used solvent extraction for phenolic substances is methanol and methanol/ water mixture. Excessive temperature may degrade polyphenolic compounds, that the use of temperature higher than 25ºC is uncommon. This may be a reason why other methods could not extract maximum tannins. The efficiency of the extraction increases along with the number of extraction steps and volume of solvent. Low temperature is advisable to prevent oxidation. Aqueous methanol due to its polarity, extracts polyphenols linked to polar fibrous matrices more effective, while acetone/water mixtures are more useful for extracting polyphenols from protein matrices, since they appear to degrade the polyphenol-protein complexes [27-30]. *Anchomanes difformis* (Blume) Engl leaf extracts revealed that cold extracts contain more tannins, cardiac glycosides and flavonoids while soxhlet extract contains more phenol, alkaloids and steroids [31]. 29% of tannin was quantified by Folin-Denis method against standard tannic acid which was used for determination of extracted tannins by Cold extraction. Tannin like compounds reduces phosphotungstomolybdic acid in alkaline solution to produce a highly colored blue solution [32]. Folin based on the non stoichiometric oxidation of the molecules containing a phenolic hydroxyl group, while Vanillin-HCl is specific for dihydroxyphenols and is particularly sensitive for meta-substituted, di and tri hydroxybenzene containing molecules [33]. For the other available procedures for the determination of tannin, the most widely used is the colorimetric method based on the reduction of phosphomolybdic-phosphotungstic acids by phenols to molybdenum blue in alkaline solutions [34].

Table 1: Preliminary Phytochemical Screening

Phytochemicals	Acetone	Ethanol	Water
Flavonoids	+++	++	+
Alkaloids	-	-	+
Tannins	+++	+++	+++
Proteins	++	-	-
Carbohydrates	-	-	-
Saponins	++	+	+++
Glycosides	++	++	++
Phenols	++	++	++

"+++" Major class group; "++" Moderate quantity group; "+" Very small or trace amount groups**;** "**-**" Absent

The total phenolic compounds in 100 mg of Psidium guajava bark tannin extract was equivalent to tannic acid of 10.75 mg. Total phenolic content determined according to the Folin-Ciocalteau method is not an absolute measurement of the amount of phenolic materials. Different types of phenolic compounds have different antioxidant activities, which is dependent on their structure. The extracts possibly contain different type of phenolic compounds, which have different antioxidant capacities [35]. The contents of total flavonoid that were measured by aluminium chloride colorimetric technique in term of quercetin equivalent was 17.5 mg in 100 mg of tannin extract (Table 2).

Table 2: Estimation of Flavonoid from tannin bark extract

Concentration of Quercitin (µg/ml)	OD at 415 nm
100	0.23
200	0.43
300	0.6
400	0.82

The biological activity of tannins isolated from guava bark was evaluated as their antioxidant capacity to scavenge DPPH free radicals. It was found that as the concentration of tannin increases the capacity to scavange free radical also increases and vice versa. Ascorbic acid was taken as the standard. 400 µg/ml showed 84.44 % of inhibition and IC_{50} of guava tannin was found to be 31.94 µg/ml and that of ascorbic acid 32.61 µg/ml (Table 3).

Table 3: DPPH radical scavenging activity

Concentration (µg/ml)	% Inhibition (tannin extract)	% Inhibition (ascorbic acid)
50	78.26	76.65
100	79.84	77.45
200	80.67	78.98
400	84.44	85.78

Suppression of released nitric oxide may be partially attributed to direct nitric oxide scavenging, as the tannin extracts of *Psidium guajava* decreased the amount of nitrite generated from the decomposition of SNP *in vitro.* The scavenging of NO by the extracts was increased in dose dependent manner (Table 4). At 80 µg/ml, the % inhibition of BHT was 80.29 while that of tannin extract was 68 and IC_{50} of guava tannin and BHT were 54.37 µg/ml and 48.48 µg/ml respectively.

Table 4: Nitric oxide radical scavenging activity

Concentration (µg/ml)	% Inhibition (tannin extract)	% Inhibition (BHT)
20	20.02	40.27
40	38.49	46.27
60	55.17	61.87
80	68	80.29

Superoxide anions indirectly initiated lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals. Some studies reported that the anti-oxidant properties of flavonoids are effective mainly via the scavenging of superoxide anion [36, 37]. Tannin extract was found to possess good scavenging activity on superoxide anions at all the tested concentration. Tannin extract and BHT at concentrations ranging from 20 to 80 µg/ml inhibited the production of superoxide anion radicals by 52.5 to 79% and 60.16 to 79.96% respectively and IC_{50} of guava tannin and BHT were found to be 19.04 µg/ml and 16.62 µg/ml respectively. Tannin extract showed strong superoxide radical scavenging activity. The results are tabulated in Table 5.

Fig.1: Neutrophils isolated from tannin extract of *Psidium guajava*

Table 5: Superoxide radical scavenging activity

Concentration (µg/ml)	% Inhibition (tannin extract)	% Inhibition (BHT)
20	52.5	60.16
40	70.69	73.49
60	74.70	77.12
80	79	79.96

The effect of tannin extracts from bark of *Psidium guajava* on neutrophils cells isolated (Figure 1) were examined by MTT assay.

Neutrophils were induced to release superoxide free radicals by NADH. Dose dependent results were observed, as the concentration of tannin extract increased, super oxide free radicals produced by neutrophils were scavenged accordingly. IC_{50} was found to be 95.41 µg/ml (Table 6).

Table 6: Neutrophil-MTT assay

S.No	Concentration of tannin (µg/ml)	% Inhibition
1	20	15.02
2	40	26.06
3	60	38.00
4	80	48.75
5	100	52.40

CONCLUSION

The results of the present study revealed that the bark extract of *Psidium guajava*, which hold maximum amount of tannins. In the present study we conceded out a systematic record on the relative free radical scavenging activity in bark tannin extract of *Psidium guajava*. Bark extract of *Psidium guajava* showed higher scavenging property, it may be due to the presence of tannins. It was also shown that the bark extract showed significantly higher antioxidant activity in scavenging of DPPH, Superoxide and Nitric oxide free radicals. Neutrophil-MTT assay also revealed that *Psidium guajava* bark tannins have high scavenging activity. So there are many scopes in bark portion and more number of studies can be undertaken like anti-inflammatory, anticancer activities and etc, In future we look forward to check the structural elucidation of the tannins and the potency of bark tannins by means of *in vivo* antioxidant studies.

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