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Screening of *in vitro* free radical scavenging activities of the fruit extracts of *Psidium guajava* L.

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The objective of the current investigation was to evaluate the *in vitro* free radical scavenging properties of fruit extracts of two varieties of *Psidium guajava* L. (PG) i.e., Lalit (LA) and *Allahabad safeda* (AS). The ethanolic fruit extracts of LA and AS of PG were obtained from the soxhlet extraction procedure and the same was used for *in vitro* antioxidant assays. Total phenols, flavonoid contents and free radical scavenging assays viz., ABTS, DPPH, nitric oxide and superoxide were analysed at 100, 200, 300, 400, 500, 600 and 700 µg/mL concentrations. Phytochemical investigation exhibited higher phenols, flavonoid contents in fruit extracts of LA compared to AS. The fruit extracts of PG showed remarkable ABTS, DPPH, nitric oxide and superoxide free radical scavenging activity in a dose dependent manner. The fruit extracts of LA exhibited higher free radical scavenging activity in contrast to AS. The present investigation revealed, a high *in vitro* antioxidant property of LA due to its higher phenolic content. Hence, the fruit extract of *Psidium guajava* L. can be used in therapeutics for the treatment of pathologic conditions involving free radicals.

Keywords: Antioxidants, Free radicals, Fruit extract, Phenolic and flavonoid contents, *Psidium guajava* L.

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Natural products are plausible drugs for humans or livestock species and such obtained consequences can intervene in synthesis of effective drugs[1]. Reactive oxygen species (ROS) are generated as by-products of biological reactions and they forge homeostatic inconsistent in the body leading to oxidative stress[2-4]. Additionally, ROS may be initiated in the cell as a sequel of environmental stress. Increased ROS production may embark on vast array of degenerative disorders such as aging, immunodeficiencies, neurologic disorders, inflammation, arteriosclerosis, cardiovascular disease and distinct types of cancers[5-7]. ROS are unceasingly produced during normal physiological events and eliminated by antioxidant defense mechanisms[8]. There is an equilibrium between generation of ROS and their elimination by the antioxidant system in organisms. Under pathologic conditions, ROS are overproduced which leads to oxidative stress. An imbalance between antioxidant defense mechanisms and ROS can catalyse to oxidative modification in the cellular membrane or intracellular molecules[9]. The health enhancing effect of plants is due to their antioxidant properties and antioxidants could provide protection to living organisms from the damage led due to elevated levels of ROS and lipid peroxidation[10]. Numerous medicinal plants have been identified for their antioxidant activities and it has been evidenced that, crude or isolated compounds from them are efficacious antioxidants[11-14].

Fruits have long been contemplated as a rich source of exogenous antioxidants. In recent times, antioxidant potential of fruit has received a greater emphasis in maintaining the levels of oxidative stress. The current study, involves the screening of antioxidant property of an important Indian fruit, *Psidium guajava* L. (PG). PG is rich in Vitamin C, hence it plays a salient role in antidiabetic activity[15]. The fruit of PG contains iron, anti-viral compound, anti-inflammatory compound, saponin, oleanolic acid, arabopyranoside[16]. PG fruit has copious amount of antioxidants, polyphenols and ascorbic acid. Studies have been piloted on assessing the antioxidant property of different part of PG viz., stem, leaf and root[17].

Natural antioxidants plays an indispensable role in retarding the advancement of many degenerative disorders and capable of scavenging free radicals and hence considered as an effective therapeutic drugs

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because of their negligible side effects[18]. Therefore, there is a keen interest in exogenous antioxidants from natural sources. Very few literatures are available on the antioxidant property of two fruit extracts of PG. Hence our study was designed to know the antioxidant prospect of two varieties of PG, viz., Lalit (LA) and *Allahabad safeda* (AS) under *in vitro* condition.

Methodology

Plant material

The two fruits varieties of PG viz*.,* Lalit (LA) and Allahabad safeda (AS) were collected from Regional Horticultural Research and Extension Centre, UHS campus Bengaluru{longitude of 77°56′ E and latitude of 13° 9′ N and 942.37} and were authenticated by Dr Rama Rao V, Research officer (S-2) Botany with an authentication number (Acc. No: RRCBI-18272).

Preparation of fruit extract

Fresh fruits of *Psidium gujava* L. were cleansed with tap water and rinsed with distilled water. Later, chopped and dessicated for about 45-50 days and pulverized using a mechanical blender and subjected to soxhlet extraction. Soxhlet apparatus was thoroughly dried and approximately 70 g of coarse fruit powder was wrapped in a filter paper. The fruit powder wrapped in a filter paper is known as timble. The timble was placed in the soxhlet tube which was fixed to a round bottom flask containing suitable solvent for extraction. The other end of the soxhlet tube was fixed to the reflux condenser. The whole apparatus was placed in the heating mantle. Extraction was done at a suitable temperature depending on the solvent. It was refluxed for 24 h. The end point of extraction was determined by observing the siphon tube which circulates the solvent through the timble. The solvent in the siphon tube becomes colourless when the extraction is complete. The extraction procedure was repeated successively with few various solvents of increasing polarity viz*.,* petroleum ether, benzene, chloroform and ethanol. After extraction, the extract was dried in a desiccator. The ethanolic filtrate was selected for the current investigation.

Materials and Methods

Quantification of phytochemical constituents

Total phenolic contents

The total phenolic contents in the fruit extracts were determined by using modified Folin–Ciocalteu's method[19]. Calibration curve were prepared in different aliquots of gallic acid, and mixed with 0.5 mL of Folin Ciocalteu's reagent, 2 mL of 20% sodium carbonate, vortexed and incubated for 35-40 min at room temperature and absorbance was measured at 760 nm by using UV-VIS 1800 spectrophotometer (Thermo Fisher Scientific). Results were expressed as milligram gallic acid equivalent/gram extract.

Total flavonoid content

The flavonoid contents in the fruit extracts were determined spectrophotometrically as per the standard protocol[20] with slight modifications. 0.5 mL of aluminium chloride solution (1.2%) was added to 0.5 mL of fruit extracts and incubated for 1 h at room temperature and the absorbance was measured at 420 nm. Results were calculated as rutin (mg/g extract).

Antioxidant scavenging activity

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) scavenging activity

The ABTS activity of the fruit extracts was evaluated by following the standard method[21]. Prior to this assay, ABTS solution was prepared by allowing, 7 mM of ABTS in distilled water with 2.4 mM potassium persulfate and stored in a shady place for 14-16 h at room temperature. 1 mL of ABTS solution was added to both the extracts and incubated for 30 min at room temperature. The ability of radical scavenging property was determined at the absorbance of 734 nm.

DPPH free radical scavenging activity

The DPPH scavenging activity was measured with slight modifications as per the protocol[22]. 1 mL of both the extracts were added into 3 mL of DPPH solution (Hi Media Laboratories Pvt. Ltd., Mumbai) and incubated in darkness for 30 min in room temperature. The absorbance was measured at 517 nm by using UV-VIS 1800 spectrophotometer (Thermo Fisher Scientific - USA) and DPPH radical activity was calculated using the formula,

$$\text{DPPH scavenging activity \%} = \frac{(A_1 - A_2)}{A_1} \times 100$$

Where, A_1= absorbance of the control,

A_2 = absorbance of the fruit extract/standard.

Nitric oxide scavenging assay

Nitric oxide scavenging assay was evaluated with slight modifications as per the protocol[23]. To various aliquots of sample, 0.5 mL of sodium nitroprusside

solution was added (10 mM) and incubated at room temperature (25-27°C) for 180 min. Then for all the samples, equal volume of freshly prepared Griess reagent (equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) was added and absorbance was read at 546 nm.

Super oxide radical scavenging activity

The reaction mixture (Potassium phosphate buffer – 250 mM, Methionine – 13 mM, Riboflavin – 2 µM, EDTA – 0.1 mM, Nitrobluetetrazolium – 75 µM) was prepared. For, different concentrations of samples, 3 mL of reaction mixture was added and absorbance was read at 560 nm for 2 min of time intervals and ascorbic acid was used as the standard.

% inhibition of superoxide radical = OD (blank) - OD (extract) x 100

Analysis

Results were calculated by Mean ± SE of each parameters and mean values of different groups were compared using one way ANOVA followed by Duncan's multiple range test and considered significant ($p<0.05$).

Results

Quantitative phenols and flavonoid content

PG fruit extract of LA exhibited higher phenols and flavonoid content in contrast to AS. The quantified phenolic content of fruit extracts of LA and AS was found to be 40.10±0.70 and 36.43±0.47 mg/gallicacid equivalent/gm extract, respectively. Similarly, the quantified flavonoid content of fruit extracts of LA and AS was found to be and 39.11±0.95, 36.87±1.241 mg rutin equivalents (mg/g), respectively.

ABTS scavenging capacity

The PG fruit extracts of LA and AS exhibited concentration in dose dependent (100 to 600 µg/mL) manner with the IC50 values of 790.11 µg/mL (40.46% inhibition) and 790.69 µg/mL (37.5% inhibition) respectively. In comparison, the ABTS radical scavenging activities of gallic acid ascorbic acid were greater than that of LA and AS extracts with the IC50 value of 411.47 µg/mL (65% inhibition) and 409.94 µg/mL (65.8% inhibition) respectively. The ABTS radical scavenging capacity of LA extract was higher than AS (Table 1 & 2).

DPPH free radical scavenging capacity

The PG fruit extracts of LA and AS exhibited concentration dependent (100 to 500 µg/mL) scavenging property of DPPH with IC50 values of 277.11 µg/mL (72.7 % inhibition) and 325.14 µg/mL (68.16 % inhibition) respectively. However, gallic acid and ascorbic acid showed higher DPPH radical scavenging property with IC50 values of 272.60 µg/mL (74.33% inhibition) and 266.85 µg/mL (75.53% inhibition), respectively. The fruit extract of LA showed maximum DPPH radical scavenging property in contrast to AS (Table 1 & 3).

Nitric oxide radical scavenging capacity

PG fruit extracts of LA and AS exhibited concentration dependent (100 to 600 µg/mL) activity with a IC50 value of 435.98 µg/mL (67.86% inhibition) and 505.59 µg/mL (61.23% inhibition), respectively. The radical scavenging capacity of gallic acid was higher than the extracts with IC50 value of 399.38 µg/mL (73.66% inhibition). The extract of LA showed higher percent inhibition compared to that of AS (Table 1 & 4).

Superoxide radical scavenging activity

Results exhibited decreased in percentage of inhibition in super oxide radical activity of PG fruit extracts of LA and AS in dose dependent manner (100 to 700 µg/mL) with a IC50 values of

Table 1 — The percentage inhibition with IC50 value of DPPH, ABTS, nitric oxide and super oxide free radical generation by fruit extracts of LA, AS, gallic acid and ascorbic acid.

	LA		AS		Gallic acid		Ascorbic acid	
	% inhibition	IC50 µg/mL	% inhibition	IC50 µg/mL	% inhibition	IC50 µg/mL	% inhibition	IC50 µg/mL
DPPH (500 µg/mL)	72.7%	277.11	68.16%	325.14	74.33333%	272.60	75.53333%	266.85
ABTS(600 µg/mL)	40.46667	735.11	37.5	790.67	65.53333	411.47	65.8	409.94
Nitric oxide (700 µg/mL)	67.86667	435.98	61.23333	505.59	73.66667	399.38	-	
Super Oxide (100 - 700 µg/mL)		45.38		71.38	-			71.37

Table 2 — % inhibition of ABTS radical generation of fruit extracts of LA, AS, gallic acid and ascorbic acid.

Concentration (µg/mL)	LA	AS	Gallic acid	Ascorbic Acid
100	13.43 ± 2.062[a]	13.53 ± 0.23[a]	18.46 ± 1.03[a]	19.06 ± 0.52[a]
200	22.63 ± 1.70[b]	22.96 ± 0.42[b]	27.66 ± 1.47[b]	28.83 ± 0.75[b]
300	31.16 ± 0.88[c]	33.93 ± 0.54[c]	38.33 ± 1.36[c]	38.60 ± 1.44[c]
400	40.66 ± 1.16[d]	41.10 ± 0.20[d]	50.73 ± 1.14[d]	51.60 ± 1.13[d]
500	52.83 ± 1.50[e]	48 ± 0.55 [e]	60.50 ± 1.09[e]	61.13 ± 0.94[e]
600	56.36 ± 0.73[e]	50.96 ± 0.55[f]	65.26 ± 1.39[f]	65.65 ± 0.35[f]
ANOVA F Value (df =4, 20)	11.21 p<0.001	123.47 p<0.001	51.62 p<0.001	51.62 p<0.001

Note: Mean values with different superscripts are significantly (p<0.05) different as judged by Duncan's multiple test, df = degree of freedom, LA- Lalit, AS-*Allahabad safeda*.

Table 3 — % inhibition of DPPH radical generation of fruit extracts of LA, AS, gallic acid and ascorbic acid.

Concentration (µg/mL)	LA	AS	Gallic acid	Ascorbic Acid
100	24.80 ±0.60[a]	22.90 ± 0.55[a]	27.40 ± 0.37[a]	30.40 ± 0.46[a]
200	40.76 ± 0.33[b]	34.73 ± 2.36[b]	40.56 ± 0.37[b]	39.43 ± 0.82[b]
300	57.13 ± 0.20[c]	46.16 ± 2.06[c]	54.83 ± 0.49[c]	54.60 ± 2.79[c]
400	68.76 ± 0.43[d]	63.13 ± 1.58[d]	69.66 ± 0.33[d]	70.06 ± 0.56[d]
500	72.70 ± 1.08[e]	68.16 ± 1.59[d]	74.33 ± 1.02[e]	75.53 ± 1.11[e]
ANOVA F Value (df=4, 20)	11.21 p<0.001	123.47 p<0.001	51.62 p<0.001	51.62 p<0.001

Note: Mean values with different superscripts are significantly (p<0.05) different as judged by Duncan's multiple test, df = degree of freedom, LA- Lalit, AS-*Allahabad safeda*.

Table 4 — % inhibition of nitric oxide radical generation of fruit extracts of LA, AS and gallic acid.

Concentration (µg/mL)	AS	LA	Gallic acid
100	18.33 ± 0.66[a]	4.50 ± 0.66[a]	10.50 ± 0.75[a]
200	27.86 ± 0.57[b]	18.60 ± 0.75[b]	24.90 ± 0.64[b]
300	35.23 ± 1.33[c]	34.66 ± 0.52[c]	41.10 ± 0.86[c]
400	45.43 ± 0.67[d]	49.96 ± 0.29[d]	57.93 ± 1.33[d]
500	57.76 ± 0.71[e]	61.60 ± 2.94[e]	69.43 ± 0.92[e]
600	60.40 ± 0.34[f]	67 ± 0.98[f]	72.90 ± 0.55[f]
700	61.23 ± 0.29[f]	67.86 ± 0.93[f]	73.66 ± 0.28[f]
ANOVA F Value (df=4, 20)	11.21 p<0.001	123.47 p<0.001	51.62 p<0.001

Note: Mean values with different superscripts are significantly (p<0.05) different as judged by Duncan's multiple test, df = degree of freedom, LA- Lalit, AS-*Allahabad safeda*.

45.38 µg/mL and 71.38 µg/mL, respectively. However, free radical scavenging activity of ascorbic acid was similar to the extract of AS with IC50 value of 71.37 µg/mL. The extract of AS showed higher percentage inhibition compared to that of LA (Table 5).

Discussion

Medicinal herbs and their products have been the mainstay of conventional remedies worldwide. Presence of phytochemicals in the plant products are generally harmless and have the capacity to intercept with long term disorders. Redox imbalance on the body occurs due to detrimental damage done by free radical which can be balanced by many artificial emerging drugs but they have been linked with diverse side effects and the only alternate way is to consume natural products[25-27]. Numerous medicinal herbs were reported to have antioxidant activity and possess different phenolic compounds and flavonoids[28]. These compounds are gaining extensive

Table 5 — % inhibition of super oxide radical generation of fruit extracts of LA, AS and ascorbic acid.

Concentration (µg/mL)	LA	AS	Ascorbic acid
100	80.50 ± 0.32^{a}	89.06 ± 0.57^{a}	89.73 ± 1.10^{a}
200	74.13 ± 0.93^{b}	84.50 ± 0.72^{b}	85.06 ± 0.86^{b}
300	66.76 ± 0.38^{c}	75.56 ± 0.61^{c}	74.36 ± 2.20^{c}
400	45.96 ± 0.38^{d}	65.66 ± 1.03^{d}	66.23 ± 1.38^{d}
500	29.50 ± 0.45^{e}	53.43 ± 1.03^{e}	52.33 ± 1.14^{e}
600	26.80 ± 0.46^{f}	50.83 ± 0.99^{f}	51.56 ± 0.82^{e}
700	25.53 ± 0.46^{f}	49.56 ± 0.56^{f}	51.03 ± 0.43^{e}
ANOVA F Value	11.21	123.47	51.62
(df=4, 20)	$p<0.001$	$p<0.001$	$p<0.001$

Note: Mean values with different superscripts are significantly ($p<0.05$) different as judged by Duncan's multiple test, df = degree of freedom, LA- Lalit, AS-*Allahabad safeda*.

attention in past few years because of their physiological role viz*.,* free radical quenching, anti-diabetic, anti-carcinogenic and anti-inflammatory effects[29]. In the current study, a substantial level of phenols in fruit extracts of LA and AS is correlated with their free radical scavenging property. The results of our current investigation is consistent with earlier reports and it also revealed the relationship between total phenolic contents and anti-oxidant capacity of extracts[30,31].

Our results revealed that, PG fruits possess remarkable amount of flavonoids and phenols. Phenols and flavonoids are the chief plant constituents because of their scavenging ability due to their OH groups. The phenolic compounds may contribute directly to antioxidant potential action[32-34]. The phenolic compounds play a role of powerful protecting agents against lethal effects of oxidative stress[35-37]. Research revealed that fruits are high in phenolic contents with abundant antioxidant properties, which can vary depending upon the fruits[38].

DPPH radical assay is a subtle way to estimate the *in vitro* antioxidant capacity of natural products which have been obtained from the plant source. DPPH is one of the free radical which is stable at room temperature and reduced in the presence of an antioxidant molecule, it shows its characteristic absorption at 517 nm whereas its degree of discolouration indicates the scavenging potential of antioxidant in the extract. DPPH is capable enough to identify active ingredients and incorporate the antioxidant activity of numerous samples with various different concentrations at less time span[39]. Lower the IC50 value depicts higher the DPPH activity. LA extract exhibited high radical property compared to AS extract. Likewise, the determination of ABTS radical scavenging capacity revealed, LA extract is more potent in contrast to AS extract. The pulp and peel fractions of PG showed remarkable DPPH and FRAP activities. Our current investigation is in line with the above reports[40].

Nitric Oxide (NO) is a potent pleiotropic mediator which is generated through histiocytes, sensory neurons etc. NO is a diffusible free radical, plays a vital role in diverse biological system such as antidiabetic, antimutagenic activities and pathogenesis of inflammatory systems. The incessant levels of nitric oxide is noxious, which leads to degenerative disorders linked with numerous inflammatory conditions including Type 1 diabetes, autoimmune disorders and ulcerative colitis[41,42]. Noxious act of NO elevates when it combines with superoxide radical, forming a highly reactive peroxynitrite anion (ONOO)[43]. In our current investigation, LA effectively scavenged the nitric oxide radicals compared to AS and exhibited concentration dependent scavenging activity.

Superoxide possesses few detrimental actions which inflicts upon the cells in the body and subsequently leads to numerous ailments. Thus, a proposal has been entrenched to gauge the comparative interceptive capability of the antioxidant extracts to scavenge the superoxide radical[44]. In our current study, fruit extract of AS manifested greater superoxide radical scavenging activity compared to LA extract. Inhibition of generation of superoxide radicals in *in vitro* reaction mixture is the probable mechanism of scavenging the superoxide ions. The radical scavenging capability of LA extract is possibly dependent on the number and the location of OH groups in the phenolic compounds present in the extract[45].

Fruit extracts of PG, possess potent antioxidant properties and therefore consumption of this can avert the diseases in which free radicals are involved. However, further investigations on antioxidant activity and related mechanisms have to be investigated under *in vivo* system. In addition, investigations related to isolation and purification of antioxidants in this plant need to be conducted in future.

Conclusion

Our current investigation revealed the potent antioxidant property of ethanolic fruit extract of LA and AS. The PG fruit extract of LA showed considerable free radical scavenging ability in contrast to AS. Due to the free radical potential of PG extracts, it can be inferred as phenolic compounds are accountable for potent antioxidant activities. Hence, the fruit extract of *Psidium guajava* L. can be used in therapeutics for the treatment of pathologic conditions involving free radicals.

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Conflict of interest

The authors proclaim no conflict of interest.

Authorship Statement

The experimental design of this study and guidance was given by Dr D Usha Anandhi and practical aspects, and data analysis of the study was carried out by B H Sowmya.

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