Chemical Constituents and Antioxidant Activity of Fresh Leaves of *Psidium guajava* Cultivated in Pakistan

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Summary: The *in vitro* antioxidant activities of the methanol extract of fresh leaves of *Psidium guajava* cultivated in Pakistan and its different fractions were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. The methanol extract, main ethyl acetate fraction and its polar sub fraction showed high free radical scavenging activity with EC_{50} 11.72, 11.72 and 46.8 µg/mL respectively. The first two values are comparable with that of reference compound ascorbic acid (EC_{50} 9.4 µg/mL). The known antioxidants gallic acid (**1**), methyl ferulate (**2**) and methyl *p-E*-coumarate (**3**) were isolated from the ethyl acetate insoluble fraction. Their structures were identified by mass, ^{1}H- and ^{13}C-NMR spectroscopy. Compounds **2** and **3** are reported for the first time from the genus *Psidium*.

Keywords: *Psidium guajava*, DPPH scavenging activity, reactive oxygen species, gallic acid, methyl ferulate, methyl *p*-E-coumarate.

Introduction

Psidium guajava Linn. (Myrtaceae) commonly known as guava, is one of the most gregarious fruit trees. It is a native of tropical America and has long been naturalized in southeast Asia. It is also very common as an adventive in pastures and wayside thickets throughout the tropics and subtropics. Different parts of the plant are used in the indigenous system of medicine for the treatment of various human ailments. The fruit is tonic, cooling, laxative and anthelmintic. It is also good in colic, diarrhea, dysentery and bleeding gums. Guava leaves are used for the treatment of diarrhea, for the relief of cough, pulmonary disorders, vomiting and ulcers and as an astringent for bowels. A paste of the leaves is also applied for rheumatism and to cuts and wounds. They are also used to cure cerebral affections, nephritis, cochexia, rheumatism, epilepsy, cholera and toothache. The young leaves and shoots are used as tonic in the diseases of the digestive functions and prescribed as febrifuge and antispasmodic. A decoction of different parts of the plant is also recommended as a gargle for sore throats, laryngitis and swelling of the gums [1-5]. The pharmacological studies carried out on *P. guajava* [6] demonstrated that it possesses spasmolytic [7], antidiarrheal [8], antimicrobial [9], hypoglycemic [10] and antioxidant properties [11-13]. A vast number of flavonoids, terpenoids, tannins and other phenolics have been reported from different parts of the plant.

Reactive oxygen species (ROS) are produced in metabolic and physiological processes and can cause damage to cell components. ROS are removed from the system *via* enzymatic and non-enzymatic antioxidative mechanism. Under certain conditions, the increase in oxidants and decrease in antioxidants can not be prevented and the oxidative disorders develop. ROS are involved in a number of degenerative diseases such as atherosclerosis, cancer, diabetes mellitus, AIDS, cirrhosis, aging, immunosuppression, inflammation, ischemic heart disease, hair loss and neurodegenerative disorders such as Alzheimer's and Parkinson disease [14-20].

Synthetic antioxidants such as BHT (butylated hydroxytoluene) or BHA (butylated hydroxyanisole) are not only expensive but they also have inherent cytotoxicity reflecting adverse effect on human health [21] therefore in recent years, the utilization of natural antioxidants from plant materials for therapeutic usage is of great interest and mandatory [22]. Recently, the antioxidant activity of aqueous extract of dried fruit and oven dried guava leaves [11], different solvent extracts of the dried leaves [12, 13] has been reported. These studies indicated that *Psidium guajava* extracts and fractions

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have the potential to be developed as new antioxidant agents to prevent or to cure the oxidative disorders.

In the present studies antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay has been investigated for the first time in the methanol extract (PG) of fresh and uncrushed leaves of the plant cultivated in Pakistan. Different fractions (PG-EA, PG-PES, PG-ES, PG-EA-S, PG-EAIS and PG-BS) obtained from PG were also tested for DPPH free radical scavenging assay at 500 µg/mL concentration.

Results and Discussions

PG and PG-EA were found to be most active as they showed 87% and 86% inhibition of DPPH free radical, respectively with EC_{50} values of 11.72 µg/mL while PG-EA-IS, PG-BS, PG-PE-S and PG-EA-S fractions showed 74%, 66%, 53% and 42% inhibition of the free radical, respectively (Table-1). Gallic acid (**1**) [23], methyl ferulate (**2**) [24-26] and methyl p-*E*-coumarate (**3**) [24, 25, 27] have been isolated from polar sub-fraction (PG-EA-IS) of PG-EA. These compounds are known for their radical scavenging properties [28, 29]. Furthermore **2** and **3** are reported for the first time from the genus *Psidium*. Strong free radical scavenging properties in PG and PG-EA may be due to the presence of these and other phenolic constituents and their synergic effect (Fig. 1).

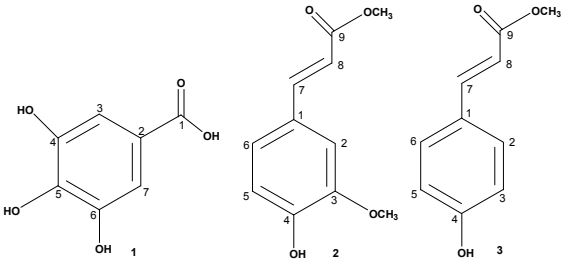

Fig. 1: Structures of Isolated Compounds (**1-3**).

Table-1: *In vitro* DPPH free radical scavenging activity of the methanol extract (**PG**) of *P. guajava* and its fractions at 500 µg/mL concentration.

S.No.	Samples	% Inhibition	EC_{50} (µg/mL)
1	PG	87	11.72
2	PG-BS	66	250
3	PG-EA	86	11.72
4	PG-PE-S	55	500
5	PG-E-S	53	500
6	PG-EA-S	42	>500
7	PG-EA-IS	74	46.8
8	Ascorbic acid (positive control)	87*	9.4

* % inhibition at 200 µg/mL concentration

Experimental

Plant Material

The leaves of *Psidium guajava* were collected from Karachi region in October, 2010. The plant was identified by Prof. Dr. Muhammad Qaiser, Department of Botany, University of Karachi, Karachi, Pakistan and a voucher specimen [KUH-GH No. 53976] has been deposited in the Herbarium of the same department.

Extraction and Fractionation

Fresh and uncrushed leaves (2.0 kg) of *Psidium guajava* were repeatedly extracted (3 times) with MeOH at room temperature. The concentrated syrupy residue (PG) obtained on removal of the solvent under reduced pressure was partitioned between EtOAc and water. The aqueous phase was extracted with *n*-BuOH and the organic layer after washing with water, drying over Na_2SO_4 and upon removal of solvent under vacuum gave a residue (PG-BS). The EtOAc layer was washed, dried over Na_2SO_4, treated with charcoal and filtered. The charcoal bed was further washed with MeOH-C_6H_6 (1:1). The EtOAc filtrate and MeOH-C_6H_6 washings were combined on the basis of TLC and freed of the solvent under reduced pressure to obtain fraction PG-EA which was divided into petroleum ether soluble (PG-PE-S) and petroleum ether insoluble (PG-PE-IS) fractions. The PG-PE-IS fraction was further divided into ether soluble (PG-ES) and ether insoluble (PG-EIS) fractions. The PG-EIS fraction was further divided into ethyl acetate soluble (PG-EA-S) and ethyl acetate insoluble portions (PG-EA-IS). The PG-EA-IS afforded various fractions (Fr1 to Fr9) upon VLC. Fr1 was subjected to CC (column chromatography) to afford three pure compounds (**1-3**) persevering for antioxidant activities.

Characterization of Gallic acid (**1**)

Off white needles ($CHCl_3$-MeOH, 1:1), m.p. 251-252 ^oC (lit. m.p. 250-251^oC); IR (KBr) ν_{max} cm^{-1}: 3450-2635 (O-H, COOH), 1700 (C=O), 1615, 1542, 1450 (aromatic C=C) and 1252 (C-O); HREIMS *m/z* 170.0224 $[M]^+$(calcd. for $C_7H_6O_5$, 170.0215); ^{1}H-NMR (acetone-d_6, 400 MHz): δ 7.14 (2H, s, H-3 and H-7); ^{13}C-NMR (acetone-d_6, 100 MHz): δ 167.4 (C-1), δ 144.9 (C-4 and C-6), δ 137.8 (C-5), δ 120.8 (C-2), δ 109.1 (C-3 and C-7).

Characterization of Methyl ferulate (**2**)

Colorless crystals ($CHCl_3$-MeOH, 1:1), m.p. 64-65 °C (lit. m.p. 65 °C); IR (KBr) ν_{max} cm^{-1}: 3448.6 (O-H), 2970.4, 2931.5 (C-H), 1720.2 (α, β unsaturated C=O), 1633.4 (C=C), 1608.1, 1572.5, 1452.0 (aromatic C=C) and 1144.6 (C-O); HREIMS *m/z* 208.0742 $[M]^+$, (calcd. for $C_{11}H_{12}O_4$, 208.0735); ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.60 (1H, d, *J* = 15.0 Hz, H-7), δ 7.06 (1H, dd, *J* = 8.0 and 2.0 Hz, H-6), δ 7.01 (1H, d, *J* = 2.0 Hz, H-2), δ 6.92 (1H, d, *J* = 8.0 Hz, H-5), δ 6.26 (1H, d, *J* = 15.0 Hz, H-8), δ 5.84 (1H, br.s, OH), δ 3.90 (3H, s, Ar OCH_3), δ 3.72 (3H, s, $COOCH_3$); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 51.4 (OCH_3), δ 56.2 (Ar OCH_3), δ 111.4 (C -2), δ 115.5 (C -8), δ116.0 (C-5), δ 123.8 (C -6), δ 127.4 (C -l), δ 145.7 (C -7), δ 148.6 (C -3), δ 150.0 (C -4), δ 167.8 (C -9).

Characterization of Methyl p-E-coumarate (**3**)

Light yellow needles ($CHCl_3$-MeOH, 1:1), m.p. 138-139 °C (lit. m.p. 137-139 °C); IR (KBr) ν_{max} cm^{-1}: 3452.2 (O-H), 2972.2, 2930.4 (C-H), 1725.4 (α, β unsaturated C=O), 1630.2 (C=C), 1606.8, 1574.8, 1450.8 (aromatic C=C) and 1142.4 (C-O); HREIMS *m/z* 178.0634 $[M]^+$, (calcd. for $C_{10}H_{10}O_3$, 178.0629); ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.60 (1H, d, *J* = 16.0 Hz, H-7), δ 7.42 (2H, d, *J* = 8.6 Hz, H-2 and H-6), δ 6.84 (2H, d, *J* = 8.6 Hz, H-3 and H-5), δ 6.25 (1H, d, *J* = 16.0 Hz, H-8), δ 3.78 (3H s, OCH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 51.5 (OCH_3), δ 115.2 (C-8), δ 116.5 (C-3,5), δ 126.9 (C-l), δ 130.9 (C-2,6), δ 145.3 (C-7), δ 160.5 (C-4), δ 168.0 (C-9).

Determination of Antioxidant Activity

Antioxidant activity of main guava methanol extract and its various fractions was determined by the method reported by Lee and co-workers [30]. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) solution was prepared in ethanol (333 μM solution) and the sample solutions were prepared in dimethylsulfoxide (DMSO). Reaction mixtures containing 10 μL of test samples and 90 μL of DPPH solution were added in 96- well microtiter plates (final concentration of test sample was 500 μg/mL for fractions and 200 μg/mL for pure compound and final concentration of DPPH in the well was 300 μM). Plates were incubated at 37 °C for 30 minutes. Absorbance was measured at 515 nm using spectrophotometer. Percent inhibition by sample treatment was determined by comparison with a DMSO treated control group and calculated using the formula given below.

DPPH scavenging effect (%) = $[(A_0-A_1) / A_0) \times 100]$.

where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the sample.

Ascorbic acid was used as standard control. The EC_{50} value calculated denotes the concentration (in μg/mL) of sample required to scavenge 50% of DPPH.

Conclusions

A significant antioxidant activity in the methanol extract of fresh leaves of *P. guajav*a and ethyl acetate fraction of the extract provides a scientific basis for the use of this plant in traditional medicine. The antioxidant mechanism may be attributed to the free radical-scavenging ability of the constituents present in *P. guajava* which include flavonoids [31], ascorbic acid [32], ferulic acid and gallic acid [28, 29] etc. These studies reveal that synergism exists among the compounds present in the crude extract (PG) and ethyl acetate fraction (PG-EA). The known antioxidants (**1-3**) were isolated from the plant. Compound **2** and **3** are reported for the first time from the genus *Psidium*.

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