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STUDY OF GC-MS AND HPLC CHARACTERIZED METABOLIC COMPOUNDS IN GUAVA (*Psidium guajava* L.) LEAVES

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Psidium guajava leaves are rich source of nutrients, antioxidants, phytoconstituents and biological active compounds. The study was designed to elucidate secondary metabolites like alkaloids, saponins, flavonoids, tannins and glycosides in extracts of guava leaves through Gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) by qualitative as well as quantitative procedures. These metabolites were further tested for their antimicrobial potential against two-gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two-gram negative (*Escherichia coli* and *Pasteurella multocida*) bacteria and three pathogenic fungal strains (*Asprrgillus niger*, *Fusarium solani* and *Aspergillus flavus*). GC-MS analysis revealed the presence of major constituents like Ca- Carrophyllene (22.70%), α cubebene (11.2%) and alpha-Humulene (5.91%). The ethyl-acetate, methanol, *n*-hexane and chloroform extracts were tested for antibacterial and antifungal activities against above mentioned microbes. Among all the tested solvent extracts, Chloroform and ethyl acetate extracts of *P. guajava* demonstrated more sensitivity towards the growth of *B. subtilis* and *P. multocida* with MIC of 230±3.02, 316.±6.2 and 237±5.09 and 288±1.55 µg/ml, respectively. Methanolic extracts showed higher MIC against *S. aureus* (233±5.51 µg/ml) and *E. coli* (192±2.05 µg/ml), respectively. The findings of this current study would provide the way to use guava as a potential therapeutic agent to combat antimicrobial and antifungal resistance.

Keywords: *Psidium guava*, secondary metabolites, phyto-medicines, antibacterial activities, antifungal resistance

INTRODUCTION

Many of the fruits and vegetables have the potential to act as functional foods and thus impart physiological and therapeutic benefits. Literature reports reveal that phytochemicals, especially, phenolics, present in fruits and vegetables are the major bioactive compounds that are associated with human health benefits. A wide array of such bioactive compounds distributed in fruits and vegetables can have complementary role in regulating different mechanisms such as stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, and antioxidant, antibacterial and antiviral effects (Thirumurugan, 2010). There has been established a strong relationship between the phenolics and the multiple biological activities of fruits and vegetables.

The guava (*Psidium guajava*) is a potential medicinal plant belongs to family *Myrtaceae* and native to South America, but now vastly cultivated in tropical and subtropical regions of various countries of the world (Biswas *et al*., 2013; Mehmood *et al*., 2013, 2014, 2016; Kareem *et al*., 2018). There are two important varieties of guava including red guava and white guava are being used in folk medicine from ancient time to treat a number of ailments. Leaves, fruits, roots and barks of guava have been used traditionally to treat diarrhea and stomachache diseases in various countries. Despite all other parts, the guava leaves are considered an important source of essential oils, rich in resin, tannins, cineol, flavonoids, triterpenes, mineral salts, malic acid, cellulose, eugenol, fat and chlorophyll. Leaves are used to treat gastrointestinal, respiratory disorders, obesity, hypertension and diabetes mellitus (Bernhoft *et al*., 2010). Leaf extracts have been used as anti-inflammatory, analgesic, hepatoprotective, and also possess antioxidant and antimicrobial properties. The therapeutic potential of guava is due to the presence of phenolics, quercetin glycosides, vitamin C, acetic acid, protocatechuic acid, citric acid, glutamic acid, malonic acid, cis-aconitic acid, trans-aconitic acid, epicatechin, asparagine and xanthine (Raghunandan *et al*., 2011). In addition, betulinic acid and lupeol are known to manage cardiovascular disease and atherosclerosis (Mukhtar and Ahmad, 2000; Thirumurugan, 2010).

It is widely accepted that the intake of phenolics through our diet in the form of fruits and vegetables is strongly linked with protection against cardiovascular heart diseases, certain cancer, and chronic health disorders (Abdelrahim *et al*., 2002). Some phytochemicals of fruits and vegetables have been reported to have strong antioxidant potential and exhibit multiple biological functions to modify activation of some metabolic process and detoxification/disposition of toxins and

carcinogens thus leading towards decreased incidence of several health-related problems. Such therapeutic and medicinal properties of fruits and vegetables are mainly attributed to their contents of bioactive compounds and antioxidants (Biswas *et al*., 2013).

Guava is also famous for its antimicrobial, anti-inflammatory, antitumor, anti-hyperglycemic, antiallergic and antimutagenic activities (Abdelrahim *et al*., 2002). It has been used to treat acne lesions, wounds, cough and dental diseases (Jaiarj *et al*., 1999). Flavonoids present in the leaves of guava which make it famous antibacterial agent. In addition, it obtained from leaves include morin-3-*O*-arabinoside, quercetin and morin-3-*O*-lyxoside. However, quercetin-3-*O*-arabinoside constituted strong antibacterial action (Lutterodt, 1992). Natural phyto-medicines provide the foundation for the development of modern drugs to treat enormous infectious and metabolic disorders (Richard *et al*., 2013). The current study was aimed to determine the concentrations of various secondary metabolites present in guava leaves, and to evaluate their potential for phytochemicals and antimicrobial activities against various pathogenic bacteria and fungi.

MATERIAL AND METHODS

***Preparation of extracts*:** *Psidium guajava* leaves were collected from the Botanical garden, University of Agriculture, Faisalabad, Pakistan. Extracts were made by mixing 20g powder of washed, dried and ground leaves separately in 200ml of ethyl acetate, n-hexane, ethanol, chloroform, acetone, methanol and water. Samples were placed in arbitrary shaker at 120 rpm for 24 h at 25°C. The supernatants were filtered and stored at 4°C in 100ml sterilized bottles for further use (Bauer *et al*., 1966).

***Oil extraction from leaves*:** The oil extraction from leaves was performed by hydro distillation method by taking 100g leaves for 4 hours in Clevenger apparatus (Nostro *et al*., 2000).

***GC-MS analysis*:** The GC–MS analysis of essential oils was carried out in GS-MS instrument (Model; company name, origin). Identification of various components in the leaf was done by comparing their mass spectra with GC–MS mass spectral library. Their retention indices were compared with authentic compounds, and with values cited in the literature (Tachakittirungrod *et al*., 2007).

***Antimicrobial assay*:** Two gram positive (*Staphylococcus aureus* and *Bacillus subtilis)* and two gram negative bacteria *(Escherichia coli* and *Pasteurella multocida)* as well as three fungal strains (*Aspergillus niger, Fusarium solani* and *Aspergillus flavus*) were selected to investigate the inhibitory potentials of guava leaf extracts. The microorganisms were obtained from Department of Microbiology, University of Agriculture, Faisalabad. The bacteria were freshly cultured on nutrient agar plates followed by incubation at 37°C for 24 hrs to pick up fresh colonies with sterilized inoculating loop, and then, vortexed in sterile physiological saline prior to inhibition tests.

The inoculum of bacterial strains (*S. aureus, B. subtilis, E. coli* and *P. multocida*) were prepared in 2.8% nutrient broth. About 1µl/1ml of suspension of each bacterial strain was transferred to their respective labeled tubes followed by pouring in respective petri plates. The antibacterial assays of leaves extracts were performed on 2.8% sterilized nutrient agar plates using disc diffusion method. The leaves extract (1µl/1ml of suspension) were applied on 6mm paper wicks followed by placing the wicks on the medium in each petri plate. The samples were incubated at 37°C for 24 hrs. The antibacterial activity was estimated in terms of zone of inhabitation and was measured by zone reader (Brahmachari *et al*., 2013). The results were noted as mean of three independent replicates for each extract. Control petri plates were prepared and preceded in the same way except that extract was not applied to these plates.

The antifungal activity was estimated by using sterilized growth medium (30ml) mixed with 100µl of chloramphenicol solution (Nostro *et al*., 2000). The inoculum of (200µl/30ml of selective medium) various fungal strains *A. niger, F. solani* and *A. flavus* were transferred to their respective labeled suspension followed by pouring in respective petri plates. Petri plates were incubated for 48hrs at 28°C. Small filter paper discs containing 100µl of extract were placed on medium in each petri plate followed by incubation at 28°C. The antifungal activity was estimated in terms zone of inhabitation around disk and it was measured by zone reader. Minimum concentration of inhibition of extracts was determined according to modified resazurin microtitre-plate assay (Vieira *et al*., 2001).

***Phytochemical profiling*:** Phytochemical screening of leaf extracts was done to detect the presence of Alkaloids, Flavonoids, Steroids, Saponins and Tannins as per established protocols (Biswas *et al*., 2013).

***Estimation of phenolic contents*:** Total phenolic contents were estimated using standard protocols. Methanolic leaf extract was prepared by dissolving 100mg extract in 25ml methanol following by filtering through 0.2µl filter before HPLC (Rheodyne, USA). The 4µl of each methanolic extract was used for HPLC analysis at 30°C with 1.3ml/min flow rate in a mixture of acetonitrile and methanol as mobile phase. Standard curves using calibrated phenolic standards were used for the identification and concentration of phenolic compounds and expressed as parts per million (ppm) for each leaf extract.

RESULTS AND DISCUSSION

***GC-MS analysis*:** Table 1 and peaks found in Figure 1 demonstrates the concentrations of various chemical compounds found in the leaves of *P. guajava.* 33 components in the guava leaf were identified through GC-MS analysis.

Table 1. Concentrations of various chemical compounds found in the leaves of *P. guajava*.

Sr.	RT	%Area	Name of Compound
1	28.33	22.707	Caryophyllene oxide
2	16.94	15.653	Caryophyllene
3	14.12	8.019	Alpha-Cubebene
4	21.01	6.206	Calamenene, cis
5	18.27	5.901	Alpha-Humulene
6	16.16	5.535	Alpha -Bulnesene
7	29.48	4.651	Humulene epoxide II
8	19.47	3.143	Neryl acetate
9	17.36	2.844	ϒ -Bisabolene, (Z)
10	25.29	2.841	Alpha-Farnesene
11	28.51	2.442	ϒ-Bisabolene, (E)
12	18.07	2.209	Δ-Cadinene
13	17.16	2.088	ϒ-Cadinene
14	26.20	1.780	Longifolene
15	15.25	1.263	Alpha-Bergamotene
16	29.24	1.079	ϒ -Gurjunene
17	19.89	1.057	Geranyl acetate
18	17.66	1.030	Alpha-Cadinene
19	11.52	0.927	Sabinen
20	18.95	0.915	Ar-Curcumene
21	30.26	0.851	Pentacosane
22	22.70	0.821	Naphthalene, 1,2-dihydro-1,1,6-trimethyl-
23	26.34	0.821	Alpha-Farnesene
24	26.89	0.816	Cadina-1(2),4-diene, cis
25	18.50	0.770	Selina-3,7(11)-diene
26	11.81	0.733	D-Limonene
27	30.43	0.644	Cadalene
28	18.72	0.587	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-
29	17.91	0.471	Citronellyl formate
30	13.80	0.405	Δ -Elemene
31	12.65	0.371	Eucalyptol
32	27.87	0.294	Farnesol, (2Z,6E)
33	26.77	0.115	1,3,3-Trimethyl-2-hydroxymethyl-3,3-dimethyl-4-(3-methylbut-2-enyl)-cyclohexene

Among these, carrophyllene oxide (22.70%), carrophyllene (15.6%), alpha cubebene (8.02%) and alpha-humulene (5.91%) exhibited highest concentrations in the leaf extracts. While quantitatively, the major components found in a study conducted in Egypt were 2- hex-3-enyl acetate (11%) and the corresponding alcohol (7.5%), pentan-2-one (9.15%), cinnamyl alcohol (10.2%), 3-phenylpropyl acetate (5 %) and the corresponding alcohol (3.5%) (Vernin *et al*., 1991).

The results indicated that methanol extract of guava leaf possessed highest inhibitory potential against all tested bacterial strains (*S. aureus*, *B. subtilis*, *E. coli*, *P. multocida*) and fungal strains (*A. niger*, *F. solani*, *A. flavus*) (Table 2). The *P. multocida* and all the tested fungal strains were resistant to *n*-hexane. However, it possessed lowest antimicrobial activity against *S. aureus*, *B. subtili* and *E. coli* among all the tested organic extracts of guava. While in a study from India the methanolic extract exhibited antibacterial activity against *E. coli* with minimum inhibitory concentration, 0.78 µg/ml, minimum bactericidal concentration of 50 µg/ml, and appreciable antifungal activity with minimum inhibitory concentration of 12.5 µg/ml (Dhiman *et al*., 2011). The effect of guava leaf extracts was greater on *S. aureus* than on *E. coli* and *Salmonella* spp. Based on statistical analyses, the antimicrobial action of the methanol extract for concentrations 484.30 µg/disc and 1,453.10 µg/disc (Goncalves *et al*., 2008). Similar significant results were also found in study conducted in Nigeria (Geidam *et al*., 2007).

Based on the inhibitory potential, the organic extracts were graded as Methanol > Ethyl acetate > Chloroform > *n*-hexane (Lee *et al*., 1998). The gram positive bacteria were susceptible

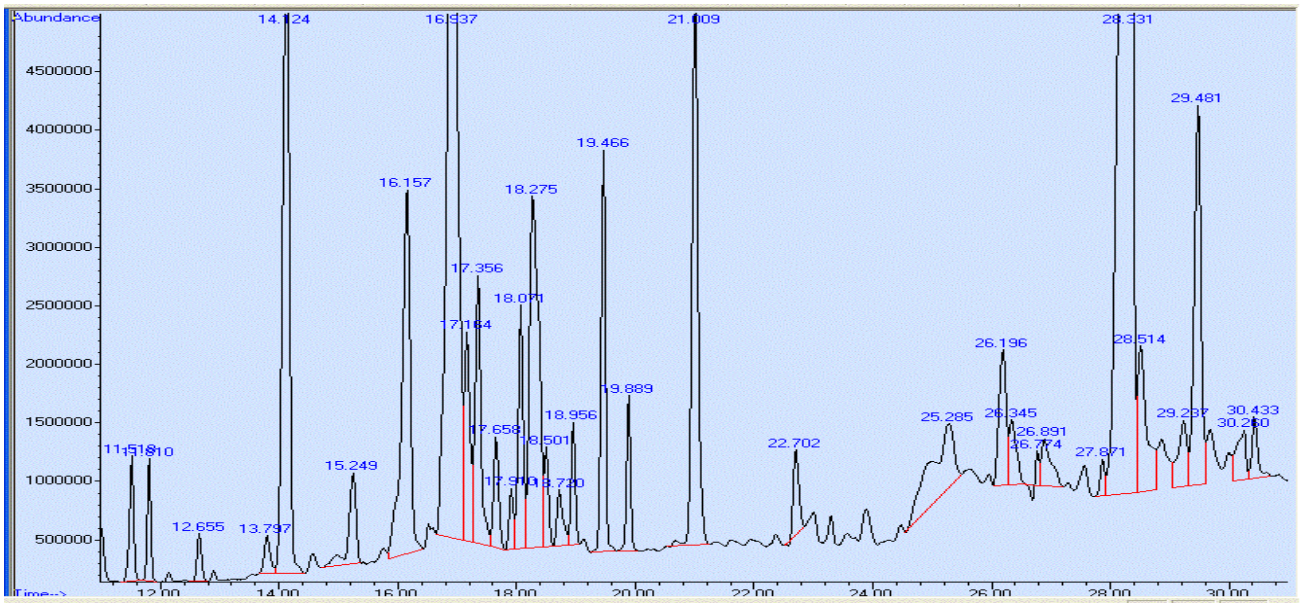

Figure 1. GC-MS chromatogram of guava leaves showing concentrations of various compounds.

Table 2. Antibacterial and antifungal activities of *Psidium guajava* tested against microorganisms by disc diffusion method.

Solvents used	Zone of inhibition of bacteria (mm)				Zone of inhibition fungal strains (mm)		
	B. subtilis	***S. aureus***	***E. coli***	***P. multocida***	***A. niger***	***F. solani***	***A. flavus***
n-hexane	11.33±0.57*	12.33±1.15	11.5±1.00	-	-	-	-
Chloroform	18.66±1.52	16.33±3.05	13.0±1.73	15.10±1.00	18.66±0.57	19.00±2.00	22.66±1.52
Ethyl acetate	21.00±1.00	22.33±1.15	19.0±2.00	20.33±1.15	23.00±2.00	20.33±2.30	21.20±2.00
Methanol	24.33±1.52	25.66±3.51	21.0±2.00	23.20±2.00	24.33±0.57	21.66±0.57	26.33±2.50
Chloramphenical	25.00±1.00	25.66±0.57	25.0±1.00	25.62±0.57	25.33±1.52	25.00±1.00	24.66±1.15

*Mean±Standard Error of Deviation

Table 3. Antibacterial activities in terms of minimum inhibitory concentration (MIC) of *P. guajava*.

Solvents	Minimum Inhibitory Concentration (ml)			
	B. subtilis	***S. aureus***	***E. coli***	***P. multocida***
n-Hexane	112.0±5.21*	167.0±6.12	110.0±1.10	-
Chloroform	230.0±3.02	98.0±4.05	161.0±1.23	316.0±6.20
Ethyl acetate	237.0±5.09	119.0±5.05	108.0±2.00	288.0±1.55
Methanol	115.0±4.22	233.0±5.55	192.0±2.05	167.0±2.10
Chloramphenicol	87.0±4.02	55.0±3.27	89.0±5.03	77.0±4.27

*Mean ± Standard Error of Mean.

Table 4. Phytochemical analysis in the leaves of *P. guajava*.

	Alkaloids	Flavonoids	Tannins	Steroids	Saponins	Glycosides	Glycosides
Qualitative analysis	++	++	++	+	+	++	+
Quantitative analysis	7%	9%	9%	6%	4.5%	8%	4%

to all organic extracts may be due to the mesh like layer of peptidoglycans through which extract penetrate to inside the cell (Biswas *et al*., 2013) while in gram negative bacteria thin lipopolysaccharide barrier is present which restricted the penetration of extract plant based antimicrobial agents.

The leaf extracts of guava were found highly effective at high concentrations against all the tested bacterial strains. However, n-Hexane showed more potential towards *S. aureus* (147±4.27) and *B. subtilis* (112±5.21). Chloroform and ethyl acetate extracts of *P. guajava* demonstrated more sensitivity towards the growth of *B. subtilis* and *P. multocida* with MIC of 230±3.02, 316.±6.2, 237±5.09 and 288±1.55 µg/ml, respectively. Methanolic extracts showed higher MIC against *S. aureus* (233±5.51) and *E. coli* (192±2.05) µg/ml, respectively (Table 3).

The phytochemical analysis of guava leaves extracts depicted various concentrations of phytochemicals (Table 4). HPLC analysis was completed by using Shim Pak CLC-ODS(C-18) column at 280 nm by using UV-Visible Detector (Table 5).

Table 5. Phenolic contents in the leaves of *Psidium gujava*.

Phenolic compound	Retention time (Min.)	Area (%)	Concentration in ppm
Quercetin	3.133	1.8	1.565
Vanillic acid	13.07	8.5	1.274
Syringic acid	16.87	4.6	2.147
m-Coumeric acid	20.31	9.5	3.689
Cinamic acid	24.86	18.5	2.345

The results of current study revealed that guava leaves extracts possessed variant number of secondary metabolites which are responsible for antimicrobial and antidiarrheal activities. Phytochemical contents are nonnutritive chemicals in the plant that exhibited disease preventive characteristics.

***Conclusions*:** It is inferred from the above said discussion that *Psidium guajava* leaves extracts possessed substantial antimicrobial and antifungal activity due to presence of good profile of phytoconstituents. The potent antimicrobial potential of *Psidium guajava* leaves makes it a good candidate to be used in natural therapies and medicine. In addition, HPLC analysis of methanolic extract of *Psidium guajava* leaves shows that plants have potential to combat oxidant due to the presence of polyphenols as an antioxidant. The ultimate aim of this work is to bring up *Psidium guajava* as a potent medicinal plant by highlighting its phytochemical composition along with Polyphenols and good profile of GC-MS based analyzed valuable compounds. The utilization of indigenous plant as potential source of antimicrobial agents as well as a food is highly needed. Further studies can be carried out towards isolation of individual bioactive components as well as to assess the potential of this medicinal herb for multiple biological activities.

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