Broad Spectrum Antimicrobial Activity of *Psidium guajava* Linn. Leaf

[1*]Egharevba, Henry Omoregie; [1]Iliya, Ibrahim; [2]Ibekwe Nneka; [3]Abdullahi, Makailu Sabo; [4]Okwute, Simon Koma; [1]Okogun, Joseph Ibumeh

[1]Department of Medicinal Plant Research and Traditional Medicine
National Institute for Pharmaceutical Research &Development (NIPRD), Idu Industrial Layout, Idu, P.M.B. 21 Garki, Abuja, Nigeria.
[2]Department of Medicinal Chemistry and Quality Control, NIPRD
[3]National Research Institute for Chemical Technology, Zaria, Nigeria.
[4]Department of Chemistry, University of Abuja, Nigeria
*Corresponding author: Phone number: +234 8036453033; E-mail: omoregieegharevba@yahoo.com

ABSTRACT: *Psidium guajava* Linn. leaf was extracted successively with hexane, ethylacetate and methanol. Another crude extract of aqueous methanol was also carried out. The extracts were tested *in vitro* for activity against standard strains microbes and clinical isolates. The zones of inhibition, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined. The *in vitro* antimicrobial screening revealed that the extract exhibited varying activity against different microbes with zones of inhibition ranging from 14-35mm, MIC ranging from 1.25 - 10mg/ml, and MBC/MFC of 2.5-20mg/ml for the sensitive organisms at the tested concentrations. The highest activity was an MIC of 1.25 mg/ml and MBC of 2.5mg/ml. None of the extract exhibited activity against fungi isolates except the standard strains of *Candida albicans.* The activities observed could be due to the presence of some of the secondary metabolites like, Tannins, saponins, terpenes and flavonoids which were detected and have previously been reported in the plant. [Nature and Science 2010;8(12):43-50] (ISSN: 1545-0740).

Key words: *Psidium guajava*, extracts, phytoconstituents, antimicrobial, MIC, MBC, MFC

INTRODUCTION

Increase development of resistance to current antibiotics has strengthened scientific research for discovery of new drug. However new leads/hits in drug discover are being developed from natural sources due to the growing scientific link between the folkloric medicinal use of some of these natural products, especially of plant origin, to biological activity. Hence plants continue to provide a good source for new drugs (Kunle and Egharevba 2009; Kunle *et al* 2003; Begum *et al* 2002).

Psidium guajava Linn (Myrtaceae) is one of such plants in folk medicine that has been used for the management of various disease conditions and is believed to active. Various parts of the plant has been used in traditional medicine to manage conditions like malaria, gastroenteritis, vomiting, diarrhoea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, and a number of other conditions (Jaiarj *et al* 1999; Abdelrahim *et al* 2002; Lutterodt 1989). Thus its used in traditional medicine is well established against enteric human bacteria. The morphology of the plant has been described severally in literature (Burkill 1997; Irvine 1961; Anas *et al* 2008) However, although some antibacterial screening has been done selectively by different researcher in the field, none has actually screen the leaves of the plant against a broad spectrum of microbes. This study is aimed at determine where the activity against a broad spectrum of human disease causing microbes, which include eight standard strains and twenty-two clinical isolate from the northern part of Nigeria lies in the various extracts of the leave.

MATERIALS AND METHODS

All the solvents and reagent used in the study were of Analar grade and, unless otherwise stated, were sourced from Zayo-Sigma, Abuja, Nigeria.

Collection and Extraction of Plant Material

The plant collected in January 2010 from Chaza Suleja, Nigeria and identified by the Taxonomist in the Department of Medicinal Plant Research and Traditional Medicine of the National Institute for Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria. A voucher specimen was deposited at the herbarium of the department. The leaves were rinsed with clean water and air-dried for three weeks, and then pulverized using a mechanical grinder. Pulverized leaf was kept separately in an air-tight cellophane bag until used.

Phytochemical screening

The presence of some basic secondary metabolites in the pulverized plant material were determined using standard methods (Sofowora 2008; Evans 2002). Proximate analysis was also carried out to determine the moisture content, total ash value, acid insoluble ash value, alcohol and water extractive values.

Preparation of extracts

Exactly 400g each of the pulverized plant was macerated successively in Hexane, ethylacetate and 98% methanol for 48hrs each. The mixtures were then filtered under vacuum and the filtrates concentrated using a rotatory evaporator. The methanol concentrate was evapourated to dryness in a water bath. For aqueous methanolic extract, 70% methanol in water was used as extraction solvent and the concentrate was freeze-dried using a table-top freeze-dryer. The extracts were stored in an airtight sample bottles and kept in a desiccator until used.

Preparation of Extract Stock Concentration for Antimicrobial screening

A test stock concentration of 10mg/ml for aqueous methanol, methanol and ethylacetate extracts were prepared by dissolving 0.1g of each extract in 10mls of distilled water in separate test tubes. For the hexane extract a concentration of 20mg/ml was prepared by dispersing 0.2g in 10mls of distilled water. The positive control drugs were sparfloxacin (0.2mg/ml), erythromycin (0.5mg/ml) and flouconazole (0.5mg/ml), all of sigma chemicals UK obtained from Zayo-Sigma Abuja Nigeria.

Antimicrobial Screening

Organism Source

The organisms used include standard strains, *Staphylococcus aureus NCTC 6571, Bacilluc subtilis NCTC 8236, Eschericia coli NCTC 10418, Pseudomonas aeruginosa NCTC 6750, Salmonella typhimurium ATCC 9184, Klebsiella pneumonia ATCC 10031* and *Staphylococcuc aureus ATCC 13704,* obtained from the department of Pharmaceutical Microbiology, Ahmadu Bello University (ABU) Zaria, Nigeria, and clinical isolates, *Staphylococcus aureus, Methicilin Resistant Staphylococcus aureus, Streptococcus pyogenes, Streptococcus faecalis, Corynebacterium ulcerans Listeria monocytogenes, Bacillus subtilis, Bacillus cereus, Escherichia coli, Klebsiella pneumonia, Klebsialla ozaenae, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas flourescense, Salmonella typhimurium, Shigella dysenteriae, Aspergillus fumigates, candida albicans, Microsporum gypseum and Trichophyton rubrum,* obtained from the department of medical Microbiology Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, Nigeria. All the organisms were checked for purity and maintained at 4^oC in slants of nutrient agar and sabouraud dextrose agar (SDA) for bacteria and fungi respectively. Well diffusion method described by Hugo and Russel (1992) was used to determine the antimicrobial activities (zone of inhibition) of the extracts against the organisms.

Preparation of the Inoculum

A loopful of the test organism was taken from their respective agar slants and sub-cultured into test-tubes containing nutrient broth for bacteria and sabouraud dextrose liquid for fungi. The test-tubes were incubated for 24hrs at 37^oC for bacteria and for 48hrs at 30^oC for the fungi. The obtained microorganisms in the broth were standardized using normal saline to obtain a population density of 10^8cfu/ml for the bacteria. For the fungi, fungal spores were harvested after 7 days old SDA slant culture was washed with 10ml normal saline in 2% Tween 80 with the aid of glass beads to help in dispersing the spores. The spores suspension were standardized to 10^5cfu/ml.

Preparation of Media

The medium was prepared according to manufacturer's instruction (Oxoids Limited Basingstoke, Hampshire, England). 40g of Blood Agar (52g of SDA) were weighed into a conical flask 1000ml of distilled water was added and capped with a cotton wool. The media were boiled to dissolution and then sterilized at 121^oC for 15mins. The media were allowed to cool to 45^oC and 20ml of the sterilized medium was poured into sterile petri-dishes and allowed to cool and solidify. The plates were labeled with the test microorganism (each plate with a test microbe). The microbes were spread evenly over the surface of the medium with the aid of a glass spreader. The plates were dried at 37^oC for 30mins and divided into two sets to be used for well diffusion method and disc diffusion method respectively.

Zone of Inhibition - Well Diffusion Method

A standard cork borer of 5mm in diameter was used to cut well at the center of each inoculated plate and the agar removed from the well. 0.1ml of the text solution (extract) was then introduced into the well created at the center for each plate. The bacteria plates were incubated at 37^oC for 24hrs while the fungal plates were incubated at 30^oC for 1-7days, and observed for the zone of inhibition of growth. The zones were measured with a transparent ruler and the result recorded in millimeters. The screening was done in triplicates. Sterilized distilled water was used as negative control.

Minimum Inhibitory Concentration - Broth Dilution Method

MIC of the extracts were also carried out using broth dilution method as described in Ibekwe *et al*, 2001. The nutrient broth and sabouraud dextrose liquid were prepared according to the manufacturer's instruction (10ml of each broth was dispensed into separate test-tube and was sterilized at 121^{o}C for 15mins and then allowed to cool. Two-fold serial dilution of the extract in the broth were made from the stock concentration of the extract to obtain 10, 5, 2.5, 1.25, 0.625mg/ml for aqueous methanol, methanol and ethylacetate extracts, and 20, 10, 5, 2.5, and 1.25mg/ml for the hexane extracts. 0.1ml of the standardized inoculums of the microbes were then inoculated into the different concentrations of the extracts in the broth. The test tubes of the broth were then incubated at 37^{o}C for 24hrs and 30^{o}C for 1-7days for bacteria and fungi respectively and observed for turbidity of growth. The lowest concentration which showed no turbidity in the test tube was recorded as the MIC.

Minimum Bactericidal/Fungicidal Concentration - Broth Dilution Method

Blood and sabouraud media were prepared, sterilized at 121^{o}C for 15mins and was poured into sterile petri-dishes and left to cool and solidify. The contents of the MIC in the serial dilution were then sub-cultured onto the media and incubated at 37^{o}C for 24hrs and 30^{o}C for 1-7days for bacteria and fungi respectively, and observed for colony growth. The MBC/MFC was the plate with the lowest concentration of extract and without colony growth.

Determination of activity index

The activity index of the crude plant extract was calculated as

$$\text{Activity index (A.I.)} = \frac{\text{Mean of zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

Determination of proportion index

The proportion index was calculated as

$$\text{Proportion index (P.I.)} = \frac{\text{Number of positive results obtained for extract}}{\text{Total number of tests carried out for each extract}}$$

RESULTS

The results of phytochemical screening and proximate analysis are shown in tables 1 and 2, while that of microbial screening are as shown in table 3 and 4below.

Table 1: Phytochemical Analysis

METABOLITE	RESULT
Carbohydrate	+
Tannins	+
Glycosides	+
Saponins	+
Terpenes	+
Sterols	+
Flavonoids	+
Resins	+
Balsams	+
Alkaloids	-

+ = present, - = not detected

Table 2: Proximate Analysis

Parameter	Values (%)
Moisture content	3.67
Water-soluble extractive value	3.54
Alcohol-soluble extractive value	21.34
Total ash value	3.30
Acid-insoluble ash value	0.25

Table 3: Zone of Inhibition

S/N	TEST ORGANISM	STRAINS	ZONE OF INHIBITION (mm)							ACTIVITY INDEX			
			P. gujava Leaf Extract				Control drugs						
			Am	m	e	h	Sp	Er	Fl	Am	m	e	h
1	*Staphylococcus aureus*	NCTC 6571	20	30	24	24	29	22	0	0.91	1.36	1.09	1.09
2	*Bacillus subtilis*	NCTC 8236	19	32	20	0	20	22	0	0.86	1.45	0.91	0.00
3	*Escherichia coli*	NCTC 10418	20	30	0	0	22	24	0	0.83	1.25	0.00	0.00
4	*Pseudomonas aeruginosa*	NCTC 6750	20	29	22	20	24	0	0	0.83	1.21	0.92	0.83
5	*Salmonella typhimurium*	ATCC 9184	22	30	20	0	25	27	0	0.81	1.11	0.74	0.00
6	*Klebsiella pneumoniae*	ATCC 10031	20	32	22	22	25	29	0	0.69	1.10	0.76	0.76
7	*Staphylococcus aureus*	ATCC 13704	21	35	19	24	20	27	0	0.78	1.30	0.70	0.89
8	*Candida albicans*	ATCC 10231	20	30	0	20	0	0	22	0.91	1.36	0.00	0.91
9	*Staphylococcus aureus*	Isolate	24	32	29	18	20	21	0	1.14	1.52	1.38	0.86
10	*Methicilin Resistant Staph. aureua*	Isolate	22	30	27	17	0	27	0	0.81	1.11	1.00	0.63
11	*Streptococcus pyogenes*	Isolate	20	29	0	0	20	26	0	0.77	1.12	0.00	0.00
12	*Streptococcus faecalis*	Isolate	24	27	17	0	24	29	0	0.83	0.93	0.59	0.00
13	*Corynebacterium ulcerans*	Isolate	25	30	30	15	25	30	0	0.83	1.00	1.00	0.50
14	*Listeria monocytogenes*	Isolate	25	30	27	0	25	24	0	1.04	1.25	1.13	0.00
15	*Bacillus subtilis*	Isolate	0	32	25	17	20	25	0	0.00	1.28	1.00	0.68
16	*Bacillus cereus*	Isolate	20	34	27	17	24	26	0	0.77	1.31	1.04	0.65
17	*Escherichia coli*	Isolate	0	0	30	0	27	20	0	0.00	0.00	1.50	0.00
18	*Klebsiella pneumoniae*	Isolate	22	31	27	0	26	19	0	1.16	1.63	1.42	0.00
19	*Klebsiella ozaenae*	Isolate	20	30	30	14	24	18	0	1.11	1.67	1.67	0.78
20	*Proteus mirabilis*	Isolate	0	0	25	0	22	20	0	0.00	0.00	1.25	0.00
21	*Proteus vulgaris*	Isolate	0	27	25	0	0	24	0	0.00	1.13	1.04	0.00
22	*Pseudomonas aeruginosa*	Isolate	19	29	24	0	19	22	0	0.86	1.32	1.09	0.00
23	*Pseudomonas flourescenses*	Isolate	0	30	0	15	0	24	0	0.00	1.25	0.00	0.63
24	*Salmonella typhimurium*	Isolate	20	29	27		20	22	0	0.91	1.32	1.23	0.00
25	*Shigella dysenteriae*	Isolate	20	30	25	14	20	20	0	1.00	1.50	1.25	0.70
26	*Aspergillus flavus*	Isolate	0	0	0	0	0	0	27	0.00	0.00	0.00	0.00
27	*Aspergillus fumigatus*	Isolate	0	0	0	0	0	0	23	0.00	0.00	0.00	0.00
28	*Candida albicans*	Isolate	0	15	15	0	0	0	24	0.00	0.63	0.63	0.00
29	*Microsporum gypseum*	Isolate	0	14	16	0	0	0	20	0.00	0.70	0.80	0.00
30	*Trichophyton rubrum*	Isolate	0	17	15	0	0	0	24	0.00	0.71	0.63	0.00

[1]Am = 70% aqueous methanol extract of *P. guajava* leaf*;* m= *methanol* extract of *P. guajava* leaf*; e= ethylacetate* extract of *P. guajava* leaf*; h= hexane* extract of *P. guajava* leaf*;* Sp= Sparfloxacin; Er = Erythromycin; Fl = Flouconazole.

[2]A.I. used Erythromycin primarily, unless where there was no activity, in which case sparfloxacin or flouconazole were used.

[3]**Proportion Index: Am=0.67; m=0.87; e=0.80; h=0.43**

Table 4: MIC and MBC/MFC

S/N	TEST ORGANISM	STRAINS	*P.guajava* Leaf Extracts							
			MIC				MBC//MFC			
			Am	m	e	h	Am	m	e	h
1	*Staphylococcus aureus*	NCTC 6571	2.5	1.25	2.5	5	10	2.5	5	20
2	*Bacillus subtilis*	NCTC 8236	5	1.25	2.5	-	10	2.5	10	-
3	*Escherichia coli*	NCTC 10418	2.5	1.25	-	-	10	2.5	-	-
4	*Pseudomonas aeruginosa*	NCTC 6750	2.5	2.5	2.5	5	10	5	10	20
5	*Salmonella typhimurium*	ATCC 9184	2.5	1.25	2.5	-	10	2.5	10	-
6	*Klebsiella pneumoniae*	ATCC 10031	2.5	1.25	2.5	5	10	2.5	10	20
7	*Staphylococcus aureus*	ATCC 13704	2.5	1.25	5	5	10	2.5	10	20
8	*Candida albicans*	ATCC 10231	2.5	1.25	-	5	10	2.5	-	20
9	*Staphylococcus aureus*	Isolate	2.5	1.25	2.5	10	10	2.5	5	20
10	*Methicilin Resistant Staph. aureua*	Isolate	2.5	1.25	2.5	10	10	2.5	5	20
11	*Streptococcus pyogenes*	Isolate	2.5	2.5	-	-	10	5	-	-
12	*Streptococcus faecalis*	Isolate	2.5	2.5	5	-	10	5	10	-
13	*Corynebacterium ulcerans*	Isolate	2.5	1.25	1.25	10	5	5	2.5	20
14	*Listeria monocytogenes*	Isolate	2.5	2.5	-	-	5	2.5	-	-
15	*Bacillus subtilis*	Isolate	-	2.5	2.5	10	-	2.5	5	20
16	*Bacillus cereus*	Isolate	2.5	2.5	2.5	10	10	2.5	5	20
17	*Escherichia coli*	Isolate	-	-	1.25	-	-	-	2.5	-
18	*Klebsiella pneumoniae*	Isolate	2.5	1.25	2.5	-	10	2.5	5	-
19	*Klebsiella ozaenae*	Isolate	2.5	1.25	1.25	10	10	2.5	2.5	20
20	*Proteus mirabilis*	Isolate	-	-	2.5	-	-	-	5	-
21	*Proteus vulgaris*	Isolate	-	2.5	2.5	-	-	5	5	-
22	*Pseudomonas aeruginosa*	Isolate	5	2.5	2.5	-	10	5	10	-
23	*Pseudomonas flourescenses*	Isolate	-	1.25	-	10	-	2.5	-	20
24	*Salmonella typhimurium*	Isolate	2.5	2.5	2.5	-	10	5	5	-
25	*Shigella dysenteriae*	Isolate	2.5	1.25	2.5	10	10	2.5	5	20
26	*Aspergillus flavus*	Isolate	-	-	-	-	-	-	-	-
27	*Aspergillus fumigatus*	Isolate	-	-	-	-	-	-	-	-
28	*Candida albicans*	Isolate	-	5	5	-	-	10	10	-
29	*Microsporum gypseum*	Isolate	-	5	5	-	-	10	10	-
30	*Trichophyton rubrum*	Isolate	-	5	5	-	-	10	10	-

[1]Am = 70% aqueous methanol extract of *P. guajava* leaf*;* m= *methanol* extract of *P. guajava* leaf*; e= ethylacetate* extract of *P. guajava* leaf*; h= hexane* extract of *P. guajava* leaf

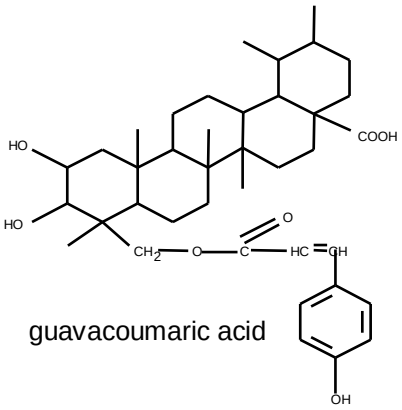
DISCUSSION

The leaf extracts of *P. guajava* showed a broad spectrum antibacterial actvitiy (gram +ve and –ve). The activity was strong against most enteric bacteria both standard strains and clinical isolates. However the ethylacetate extract was not active against typed strains but active against isolate of E. coli. This was also what was obtained in the work of Goncalves *et al*., 2008, who worked on seabob isolates and typed strains.. The methanol and ethylacetate extracts also activity against standard strains *C. albicans* ATCC 1023 and some of the clinical isolates of fungi although there was no activity *Aspergillus flavus* and *fumigates.* The methanol and ethylacetate extract exhibited broader spectrum activity although the methanol extract was generally more active and had lower MIC against most of the organisms, which also support previous works (Goncalves et al., 2008, Rabe and Van Staden, 1997). The lower activity of the hexane extract was previously insinuated in the low activity of its essential oil by Gonclaves et al. However essential oil components like 1,8-cineol, p-cimene etc has been isolated from the *Psidium* genus.The results obtained is also comparable to that reported by Anal et al on *Staphylococcus aureus*, but at variance with the results obtained for fungi in the work of Hema *et al*. The plant has also been shown to be active against acne causing bacteria like *Propionibacterium acnes* and accompanying *staphylococci spp.* (Qa'dan *et al*., 2005).

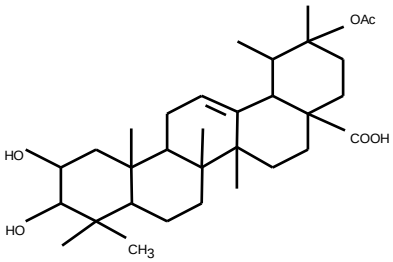
The activity exhibited may be as a result of the phytochemicals contained. Although alkaloid was not detected, other components like tannins, saponins, flavonoids and terpenes have been severally reported to exhibit antibacterial and antioxidants activities (Kunle and Egharevba 2009, Ayoola *et al* 2008). Flavonoids are known to possess good antioxidant properties and guava has been reported to have comparatively high antioxidant activity against 2,2-Diphenyl-1-picryl hydrazyl (DPPH) radicals and antioxidants are known to exhibit some level of antimalaria activity due to their ability to minimize the oxidative damage induced by the parasites on the red cells (Ayoola *et al* 2008). Flavonoids like morin-3-O-lyxoside, morin-3-O-arabinoside, quercetin-3-O-arabinoside, guaijavarin and quercetin has been isolated and found to exhibit good antibacterial activity against some enteric bacteria (Arima and Danno, 2002; Rattanachaikunsopon and Phumkhachorn, 2010), and may be a useful food preservative. The absence of alkaloid from the leaves has also been reported by Ayoola *et al*. Some triterpenoid reported to have been isolated include guavanoic acid and guavacoumaric acid (Begum *et al*, 2002).

SOME COMPOUNDS FROM PSIDIUM GENUS.

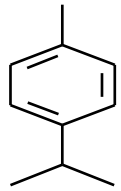
1,8-cineol, p-cimene (Gonclaves et al 2008); quercetin, morin-3-O-arabinoside, quercetin-3-O-arabinoside morin-3-O-lyxoside (Rattanachaikunsopon and Phumkhachorn, 2010); 3β-p-E-Coumaroyloxy-2agr-methoxyurs-12-en-28-oic acid, guajanoic acid, β-sitosterol, oleanolic acid, ursolic acid (Begun et al 2004); ursolic acid, 2-α-hydroxyursolic acid, 2-α-hydroxyoleanolic acid, morin-3-O-α-L-arabopyranoside, quercetin, hyperin, myricetin-3-O-β-D-glucoside, quercetin-3-O-β-D-glucuronopyranoside, 1-O-galloyl-beta-D-glucose (Fu *et al*., 2009); morin-3-O-α-L-lyxopyranoside, guaijavarin (Arima and Danno, 2002); 20β-acetoxy-2α,3β-dihydroxyurs-12-en-28-oic acid (guavanoic acid), and 2α,3β-dihydroxy-24-p-z-coumaroyloxyurs-12-en-28-oic acid (guavacoumaric acid), 2α-hydroxyursolic acid, jacoumaric acid, isoneriucoumaric acid, asiatic acid, ilelatifol D and β-sitosterol-3-O-β- -glucopyranoside (Begum et al 2002); Viridiflorol, epi-[α]-muurolol, α-cadinol, epi-[α]-cadinol, caryophyllene oxide, β-caryophyllene, vitamin C, vitamin A, pectin, alanine, α-humulene, α-hydroxyursolic acid, α-linolenic acid, alpha-selinene, amritoside, araban, arabinose, arabopyranosides, arjunolic acid, aromadendrene, ascorbic acid, ascorbigen, asiatic acid, aspartic acid, avicularin, benzaldehyde, butanal, carotenoids, caryophyllene, catechol-tannins, crataegolic acid, D-galactose, D-galacturonic acid, ellagic acid, ethyl octanoate, essential oils, flavonoids, gallic acid, glutamic acid, goreishic acid, guafine, guavacoumaric acid, guaijavarin, guajiverine, guajivolic acid, guajavolide, guavenoic acid, guajavanoic acid, histidine, hyperin, ilelatifol D, isoneriucoumaric acid, isoquercetin, jacoumaric acid, lectins, leucocyanidins, limonene, linoleic acid, linolenic acid, lysine, mecocyanin, myricetin, myristic acid, nerolidiol, obtusinin, octanol, oleanolic acid, oleic acid, oxalic acid, palmitic acid, palmitoleic acid, pectin, polyphenols, psidiolic acid, quercetin, quercitrin, serine, sesquiguavene, tannins, terpenes, and ursolic acid (Tropical Plant Database, 2010).



guavacoumaric acid



guavanoic acid



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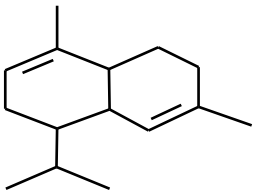

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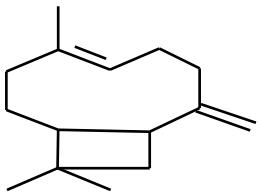
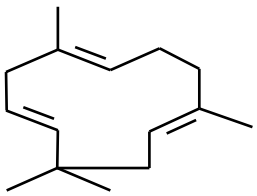


Caryophyllene



Humulene

CONCLUSION

Psidium guajava is highly exploited traditionally for ethnomedicinal purposes and this study corroborates its wide traditional application for the management of gastrointestinal problem and respiratory infections. The study also shows that the plant may be a good as an antibacterial preparation but may not be very useful as antifungi. The plant may be a good source of antioxidant drug for use in the management of malaria and other ailments especially infectious diseases.

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