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ANTIBACTERIAL, CYTOTOXICITY AND GC-MS ANALYSIS OF *Psidium Guajava* EXTRACTS

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ABSTRACT
Leaves of Psidium guajava was extracted with water, ethanol and chloroform using percolation technique. The extracts were subjected to thin layer chromatography, bioactivity and toxicity studies as well as GC-MS analysis. The TLC chromatogram revealed 3, 4 and 6 bands respectively for the water, ethanolic and chloroform extracts. Contact bio-autographic assay conducted on the extracts against the tested bacteria showed that, out of the thirteen (13) fractions tested only one (1) showed activity on the test bacteria (Rf3).The fraction was active against all the tested bacteria with strong zones of inhibition against S. Paratyphi B, moderate against S. Paratyphi A and weak against S. Typhi. S. typhi showed slight sensitivity, S. paratyphi moderately sensitive, while S. paratyphi B showed strong sensitivity. Result of the brine shrimp lethality assay showed that all the extracts were toxic in the order of chloroform extracts* (31.112µg/mL) *more toxic, and then ethanolic extracts (*39.903µg/mL) *and lastly water extracts* (49.534µg/mL)*. The GC-MS analysis was conducted on the most active fraction (Rf3).In the total ion chromatograms (TIC), constituents including fatty acids, heterocyclic compounds and esters among others were found. The major constituents successfully matched and identified were Oleic acid (Peak 6, 35.56% peak area), Palmitic acid (Peak 2, 20.26% peak area)and were all reported to be active principles.
Key words: bacteria, Bioactivity, GC-MS analysis, *Psidium guajava*, thin layer chromatography, toxicity.

INTRODUCTION

The Genus *Psidium* belongs to the Family Myrtaceae, which is considered to have originated in tropical South America. Guava crops are grown in tropical and subtropical areas of the world like Asia, Egypt, Hawaii, Florida, Palestine, and others. The Genus *Psidium* comprises approximately 150 species of small trees and shrubs in which only 20 species produce edible fruits and the rest are wild with inferior quality of fruits (Scopus*et al.,* 2011). *Psidium guajava,* a common plant with edible fruit has enjoyed many medicinal applications as antibacterial, anti-inflammatory, anti-malaria, haemostatic, antispasmodic, as a tonic, anti-diarrheal, anti-diabetic, anti–rheumatism in traditional medicine (Olajide*et al.,* 1999).A number of works have been carried out on *P. guajava* and many compounds of medicinal importance have been isolated (Abdelrahim *et al.,* 2002).According to World Health Organization more than 80% of the world populations still rely on herbal medicines as primary sources of health care. Millions of Africans of all ages rely on herbal medicine for primary health care (Begum *et al.,* 2002).

GC-MS is one of the best techniques to identify the bioactive constituents of long chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids, amino and nitro compounds etc. (Ramasamy, 2012). A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties (Senthamarai and Basker, 2012).

Brine- shrimp lethality test provides a quick, inexpensive and desirable alternative to complexities encountered with animal models. And also predicts possible anti-cancer activity because a positive correlation exists between this test and the 9KB (human nasopharyngeal carcinoma) cytotoxicity (McLaughlin*et al*., 1991).

The aim of this study was to determine the toxicity status of the *Psidium guajava*extracts, phytochemicals profile in the plant leaves using GC-MS and also ascertain the most bioactive fraction(s) using bio-autography study.

MATERIALS AND METHODS

Sample Collection

The plant material(leaves) was collected from the staff quarters of Bayero University, Kano, oldcampuson November, 2015. The sample was identified by a botanist, in the Department of Plant Biology, Bayero University, Kano; voucher specimenwas deposited in the department's herbarium. The sample voucher number is BUKHAN 0113 (Aliyu, 2006).

Extractionof Plant Material

The leaves after collection were air-dried at room temperature for 3 weeks. Dry leaves were then pound in a porcelain mortar to a fine powder for ease of extraction of active compounds. The dried powdered plant leaves (50g) was extracted with ethanol (250ml), chloroform (250ml) and water(500ml) for one week. The extracts were filtered using Whatman No. 1 filter paper. The resulting filtrates were allowed to evaporate in a water bath at 100^{o}C. The filtrate wass carefully labeled and stored in the refrigerator for further use (Fatope *et al.,* 1993).

Test Organisms and Biochemical Tests

Salmonella Typhi, *Salmonella* ParatyphiA, and *Salmonella* ParatyphiB,were collected from Department of Microbiology, Aminu Kano Teaching Hospital, Kano, Nigeria. The test bacterial identity was characterized by observing their cultural growth characteristics and various biochemical tests as described by Cheesbrough, (2006).

Standardization of Inoculum

Using sterile inoculation wire loop, 2-4 colonies from an overnight culture of the test organism was transferred into a tube of saline until the turbidity of the suspension matched the turbidity of the 0.5 McFarland Standard as described by the National Committee for Clinical Laboratory Standard (NCCLS, 2008).

Preparative Thin Layer Chromatography

Preparative thin layer chromatography was conducted on both the ethanolic, chloroform and water extracts in order to find the best solvent system that can fractionate the extracts in the main analytical TLC. Glass slides were coated with silica gel (stationary phase). The slides were dried and activated by heating in an oven for thirty minutes at 110^{o}C. The thickness of the adsorbent was made to around 0.5 to 2.0mm thickness according to (Joseph and Bernard 1991). Various solvent systems were tried from high polar to low polar combinations. Chloroform, methanol and water ratios; 70:20:10, 60:30:10 and chloroform and methanol were tried at ratios, 60:40, 65:35, 70:30, 80:20, 85:15, 90:10, 95:5 and lastly 100 percent chloroform gave better separation. For the water extracts chloroform and methanol at ratios, 60:40 gave better separation.

Analytical Thin Layer Chromatography

Thin layer chromatography plates were manually prepared in the laboratory on a glass 20cm X 20cm sizes. Solvent system obtained above was used in the analytical TLC. Samples were spotted on the TLC plates using fine capillary tubes. Forty spots were spotted separately and allowed to dry. The spotted plate was then placed in the developing chamber, spotted end down; the solvent level was made below the spots. The solvent then slowly rose in the adsorbent by capillary action. When the solvent front has moved to within about 1cm of the top end of the adsorbent each plate was removed and the position of the solvent front marked. The plates were visualized under ultraviolet light of 254nm and 365nm wave length.

Determination of Bio-active Component(s) using Bio-autography

Bio-autography was employed on the TLC fractions of each extract to test the antibacterial activity.Two millilitres of methanol were added to each of the scrapped band from the dried TLC fractions in order to dissolve them. Filter paper discs were soaked to absorb the compounds contained. The discs were removed and allowed to dry and thereafter were tested for antibacterial activity on the test bacteria. The antibacterial activity was determined by observing presence or absence of zones of inhibition formed around the discs(Bauer*et al.,* 1966).

Gas Chromatography Mass Spectrophotometry Analysis

Gas Chromatography Mass Spectrophotometry analysis was carried out on the most bioactive components of the TLC fractions of each among the extract identified during the Contact by Autography Test. Gas Chromatography Mass Spectrophotometry analysis was carried out for the bioactive volatile compounds on QP-2010 Plus, Shimadzu, Japan with the following specification; Column Oven Temperature 80.0 °C, Injection Temperature 250.00 °C, Injection Mode Split, Flow Control Mode Linear Velocity, Pressure 108.0 kPa, Total Flow 6.2 mL/min, Column Flow 1.58 mL/min, Linear Velocity 46.3 cm/sec, Purge Flow 3.0 mL/min, Split Ratio 1.0. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained (Dukes, 2013).

Brine Shrimp Lethality Assay (BSLA)

Brine shrimp eggs were obtained from Chemistry Research Laboratory, Bayero University Kano. Filtered artificial seawater was prepared by dissolving 38g of sea salt in 1 liter of distilled water for hatching the shrimp eggs. The sea water was poured into small improvised container (hatching chamber) made from a type of sponge case that has cover. It was wrapped (the sponge case) with a black masking tape with a hole from the top for light penetration. Shrimp eggs were added into the dark side of the chamber while the lamp above was meant to attract the hatched shrimp. Two days were allowed for the shrimp to hatch and mature as nauplii (larva). After two days, when the shrimp larvae were ready, 4mL of the artificial seawater was added to each test tube and 10 brine shrimps were introduced into each tube. Thus, there were a total of 30 shrimps per dilution. Then the volume was adjusted with artificial seawater up to 5mL per test tube. The test tubes were left uncovered under lamp. The number of surviving shrimps were counted and recorded after 24 hours. Using probit analysis, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. LC_{50} of less than 100ppm was considered as potent (active) Gufta *et al.,*(1996). As mentioned by Meyer *et al.,*(1982), LC_{50} value of less than 1000µg/mL is toxic while LC_{50} value of greater than 1000 µg/mL is non-toxic.

The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

RESULT AND DISCUSSION

The result of the thin layer chromatography of the aqueous, ethanolic and chloroform extract of *P. guajava* is presented in (Table 1). According to the result TLC profile of *P. guajava* revealed 3, 4 and 6 bands respectively for the aqueous, ethanolic and chloroform extracts. The TLC was performed on 20 x 20 silica gel plates. Other parameters considered include appearance of each band at 254nm and 365nm wave length as well as weight of the scrapped band prior to been dissolved likewise the weight of the extract (mg) recovered from the stationary phase.

Table 1A: TLC Separation of Extracts of *P. guajava* on Silica gel

Extracts	Rf No.	Rf Value	Appearance of the band under UV at		Weight of the scrapped band(mg)	Weight of the extract recovered from the band(mg)
			254nm	365nm		
Aqueous	1	0.1272	Dark Brown	Brown	22.87	10.16
	2	0.7572	Purple	Brown	34.01	11.96
	3	0.9769	Orange	Yellow	23.56	10.74
Ethanolic	1	0.1316	Brown	Light brown	65.22	26.10
	2	0.1404	Green	Green	19.00	8.02
	3	0.1520	Purple	Light purple	20.02	7.89
	4	0.1637	Pale yellow	Pale yellow	19.87	9.24
Chloroform	1	0.1389	Brown	Brown	50.23	20.10
	2	0.1667	Light green	Light Green	23.09	10.11
	3	0.2778	Green	Green	42.34	17.89
	4	0.3611	Dark green	Dark Green	55.01	19.32
	5	0.5278	Orange	Pale orange	20.61	8.12
	6	0.7222	Yellow	Yellow	19.22	10.99

The result of the direct contact bio-autography conducted on both the aqueous, ethanolic and chloroform extracts of *P. guajava* against the test bacteria showed that, out of thirteen (13) fractions tested only one (1) showed activity on the test bacteria as shown in (Table 2). However, the level of the activity varies with the bacteria. While the only bioactive fraction (Rf3) appeared active against all the 3 bacteria, the zones of inhibition produced are not uniform. *S.* Typhi showed weak sensitivity, while *S.* ParatyphiA moderately sensitive, *S.* ParatyphiB showed strong sensitivity.

Table 2: Result of Bioautographic Assay of *P. guajava*

Solvent	**Bacteria**	**Rf1**	**Rf2**	**Rf3**	**Rf4**	**Rf5**	**Rf6**
Water	*Salmonella* Typhi	-	-	+	N	N	N
	Salmonella Paratyphi A	-	-	++	N	N	N
	Salmonella Paratyphi B	-	-	+++	N	N	N
Ethanol	*Salmonella* Typhi	-	-	-	-	N	N
	Salmonella Paratyphi A	-	-	-	-	N	N
	Salmonella Paratyphi B	-	-	-	-	N	N
Chloroform	*Salmonella* Typhi	-	-	-	-	-	-
	Salmonella Paratyphi A	-	-	-	-	-	-
	Salmonella Paratyphi B	-	-	-	-	-	-

Key: + = weakly active, ++ = moderately active, +++ = strongly active, N = limit of the retention factor

GC-MS analysis of the most active fraction (Rf3) as mentioned earlier showed that in the total ion chromatograms (TIC) different constituents including fatty acids, heterocyclic compounds and esters among others were found.

Result of the GC-MS analysis of the Rf3 of the water extract of *P. guajava* is contained in Table 3. All the compounds have been, effectively matched and identified. The major constituents were oleic acid (Peak 6, 35.56% peak area), Palmitic acid (Peak 2, 20.26% peak area) followed by stearic acid (peak 7, 9.84% peak area). Kabara, (1978) reported that fattv acids such as oleic, palmitic, stearic, myristic, linoleic and linolenic acids were active against *Clostridium perfringens* and *Staphylococcus pyogens.* Some *in vitro* studies have indicated that the fatty acid composition could either directly or indirectly affect the aflatoxin contamination (Burrow *et al.,* 1997).

Reports by Dilika *et al.*(2000) suggested that long-chain unsaturated fatty acids, including linoleic acid, are well known to inhibit bacteria like *E. coli*. Sheu *et al.,*(1975) reported that long-chain fatty acids have higher antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria. The difference in the fatty acid sensitivity between Gram-positive and Gram-negative bacteria may result from the impermeability of the outer membrane of Gram negative bacteria since it is an effective barrier against hydrophobic substances. Linolenic, linoleic and palmitic acids isolated from *Schotia brachypetala, Pelargonium* sp. and *Pentanisia prunelloides*, respectively, were found to have antibacterial activity (McGraw *et al.,* 2002).

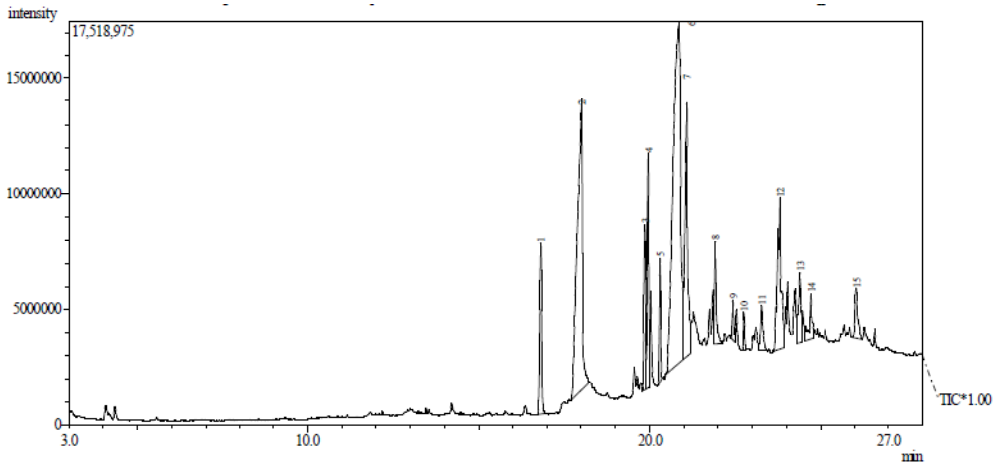

Figure 1: GC-MS chromatogram of Rf3

Table 3: GC-MS Analysis of Rf3

Peak No.	Retention time (min)	% composition By area	Compound name	Molecular Formula	Chemical structure
1	16.819	4.30	Pentadecanoic acid	$C_{17}H_{34}O_2$	
2	18.014	20.26	Palmitic acid	$C_{16}H_{32}O_2$	
3	19.857	3.50	9, 12-octadienoic acid	$C_{19}H_{34}O_2$	
4	19.953	5.04	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	
5	20.302	2.23	Octadecanoic acid	$C_{19}H_{38}O_2$	
6	20.846	35.56	Oleic acid	$C_{18}H_{36}O_2$	
7	21.079	9.84	Stearic acid	$C_{18}H_{36}0_2$	
8	21.912	2.61	2-piperidinone	$C_9H_{16}BrNO$	
9	22.432	0.79	Decyl fluoride	$C_{10}H_{21}F$	
10	22.751	0.57	Eicosanoic acid	$C_{21}H_{42}O_2$	
11	23.263	1.37	Arachic acid	$C_{20}H_{40}O_2$	
12	23.799	7.68	Z-17-Nonadecen-1-ol acetate	$C_{21}H_{40}O_2$	
13	24.382	2.59	2,3-dihydroxypropyl palmitate	$C_{19}H_{38}O_4$	
14	24.703	1.67	n-Docosanoic acid methyl ester	$C_{23}H_{46}O_2$	
15	26.062	1.99	Brassidic acid	$C_{22}H_{42}O_2$	

The result of the brine shrimp lethality assay is presented in (Table 4). From the result all the extracts were toxic, chloroform extracts (31.112µg/mL) was most toxic followed by ethanolic extracts (39.903µg/mL) and lastly water extracts (49.534µg/mL).From phytochemical screening carried out by numerous researchers including Mohammed, (2016), presence of alkaloids and steroids was reported in *P. guajava* leaves. So the observed cytotoxic action may be due to the presence of these compounds. Again, reports exist on the role of alkaloids and steroids in cytotoxic activity of plant extracts (Badami *et al.,* 2003). However, phenolics and flavonoids are also known to show cytotoxicity in Hoechst 33258 fluorescence assay by inhibiting cellular DNA in a concentration-dependent manner (Chang *et al.,* 2000).

Table 4: Brine Shrimp Lethality Assay of the Extracts

Plant Extract	Organic solvent	Concentration (ppm or µg/ml)	No. of Shrimps	No. of Survivors	% Mortality	LC_{50} (µg/mL) Brine Shrimp Lethality
P. guajava	Aqueous	1000	10	0	100	49.534
		100	10	3	70	
		10	10	9	10	
	Ethanol	1000	10	0	100	39.903
		100	10	2	80	
		10	10	9	10	
	Chloroform	1000	10	0	100	31.112
		100	10	2	80	
		10	10	8	20	

CONCLUSION

From the findings of this research it can be concluded that leaves extracts of*P. Guajava* contain some bioactive compounds. The major compounds identified were oleic acid, Palmitic acid and stearic acid. However, the cytotoxicity study carried out showed that the extracts are toxic.

Recommendation

More research is encouraged to determine the chronic toxicity status of the *P. guajava* leaves extracts.

Conflict of interest

We declare that we have no conflict of interest.

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