

Original article:**CHEMICAL COMPOSITION, TOXICITY AND ANTIOXIDANT ACTIVITIES OF ESSENTIAL OILS OF STEM BARK OF NIGERIAN SPECIES OF GUAVA (*PSIDIUM GUAJAVA* LINN.)**Taiye R. Fasola¹, Ganiyat K. Oloyede^{2*} and Babalola S. Aponjolosun¹¹ Department of Botany and Microbiology, University of Ibadan, Nigeria² Natural products/Medicinal Chemistry Unit, Department of Chemistry, University of Ibadan, Nigeria

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ABSTRACT

Essential oil from the stem bark of Nigerian species of *Psidium guajava* of the family Myrtaceae was obtained by hydro-distillation using an all-glass Clavenger apparatus. GC and GC/MS analysis were carried out on the essential oil and was found to contain 62 compounds constituting 99.98 % of the total oil composition. The principal constituents are hydrocarbons, amines, amides and esters with 3,6-dioxo-2,4,5,7-tetraoctane,2,2,4,4,5,5,7,7-octamethyl (11.67 %) and cyclononane (10.66 %) dominating the total essential oil. Brine shrimp lethality test was carried out to determine the toxicity of the oils to living organisms (shrimps). LC₅₀ value (µg/ml) of 1.0009 obtained showed that the essential oil of *P. guajava* stem bark was toxic. The antioxidant property of essential oil was investigated by measuring the decrease in absorption at 517 nm of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) in a UV/visible spectrophotometer. The oil showed better activity as a radical scavenger than α-tocopherol. The oil activity was 71.83 % at 0.2 mg/ml and the absorption is stoichiometric with respect to the number of electron taken up. Thus, the results of this study showed that the essential oil from *P. guajava* was not only toxic; it possessed antioxidant activity, which could exert beneficial actions against pathological alterations caused by the presence of highly reactive free radicals. The toxicity of the oil can be taken advantage of in the therapy of diseases involving cell or tumor growth.

Keywords: Toxicity, antioxidant, essential oils, *Psidium guajava*, GC/GC-MS, 1,1-diphenyl-2-picrylhydrazyl

INTRODUCTION

Free radicals are chemical species that contain one or more unpaired electrons. They are produced when oxidation reactions occur in cells but excess production starts chain reactions and causes serious damage to biological macromolecules. Antioxidants terminate these chain reactions by removing the excess free radical inter-

mediates, and inhibit other oxidation reactions by being oxidized themselves. Natural antioxidants are substances from plants or animals sources that can delay or terminate the process of oxidation. Natural compounds with anti-oxidative properties include flavonoids, lignans, tannins, essential oils and terpenoids (Gutteridge, 1988; Hochstein and Atallah; 1988; Halliwell, 1989; Potterat, 1997). As a result of preva-

lence of oxidative stress, studies involving antioxidant chemistry is gaining much relevance in recent times (Feher et al., 1986; Aruoma et al., 1989; Belch et al., 1989; Kehrer, 1993; Sies, 1997; Lee et al., 2001).

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 (Oliver-Bever, 1986; Iwu, 1993; Burkill, 1997; Olajide et al., 1999). Also much biological work has been carried out on *P. guajava* and many compounds of medicinal importance have been isolated (Basols and Demole, 1994; Sen et al., 1995; Kavimani et al., 1997; Nadkarni and Nadkarni, 1999; Tona et al., 1999; Vieira et al., 2001; Paniandy et al., 2000; Abdelrahim et al., 2002; Arima and Danno, 2002; Michael et al., 2002; Lozoya et al., 2002). However, in this present study we report the chemical constituents of essential oil from the stem bark of Nigerian species of *P. guajava*. The oil was obtained by hydro-distillation and analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The toxicity of the essential oil was determined by brine shrimp lethality test (BST) which is a bench top bioassay for elementary cyto-toxicity study. *P. guajava* oil was screened for free-radical scavenging activity by *in-vitro* assessment on 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). DPPH radical becomes deep violet colour in alcoholic solutions and gives strong absorption at 517 nm in visible spectroscopy but disappears in the presence of a free radical scavenger. The anti-oxidative potential of the oil was compared with known antioxidant standards - ascorbic acid, butylated hydroxyanisole and α -tocopherol.

MATERIAL AND METHODS

Plant materials

Psidium guajava stem bark was collected in August 2009 behind Tedder Hall at the University of Ibadan, Oyo State, Nigeria and authenticated at the Herbarium of

the Department of Botany and Microbiology of the institution. The volatile oil was immediately collected from the fresh plant material by hydro-distillation using an all-glass scavenger apparatus.

Reagents

Hexane and methanol (BDH chemicals); 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) were obtained from Sigma Chemical Co (Germany).

Reference standards: Ascorbic acid, Butylated hydroxyanisole (BHA) and α -tocopherol for antioxidant activity.

Major equipments used

UV-visible spectrophotometer (Unico1 200 & Perkin Elmer lambda 25 models), GC-Mass spectrophotometer (Agilent Technologies), Hydro-distiller - Clavenger apparatus.

Isolation of essential oils

The oil was obtained from chopped fresh plant sample (300 g) of *P. guajava* by hydro-distillation on a Clavenger type apparatus for 4 hours in accordance with the British pharmacopoeia specifications (1980). Small amount of hexane was intermittently added to aid the trapping of the oil during the process. The essential oil was collected and stored at 4 °C in order to prevent loss of oil by volatilization until analysis. The oil yield was calculated relative to the dry matter.

Analysis of the essential oils

Gas chromatography

GC-MS analyses of the essential oil was analyzed on an Agilent Technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683B series device. An Agilent (9091)-413:325 °C HP-5 column (30 m x 320 μ m x 0.25 μ m) was used with helium as carrier gas at a flow rate of 3.3245 ml/min. The GC oven temperature was initially programmed at 50 °C (hold for 1 min) and finally at 300 °C (hold for 5 min) at a rate of 80 °C/min while the trial temperature was 37.25 °C. The column heater was set at

250 °C in a split less mode while the pressure was 10.153 psi with an average velocity of 66.45 cm/sec and a hold-up time of 0.75245 min. Mass spectrometry was run in the electron impact mode (EI) at 70eV. The percentage compositions were obtained from electronic integration measurements using flame ionization detector (FID), set at 250 °C. The peak numbers and relative percentages of the characterized components are given in Table 1.

Gas chromatography–mass spectrometry

The essential oils were analysed by GC-MS on an Agilent Technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683B series device. An Agilent (9091)-413:325 °C HP-5 column (30 m x 320 µm x 0.25 µm) was used with helium as carrier gas at a flow rate of 3.3245 ml/min. GC oven temperature and conditions were as described above. The injector temperature was at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 30 to 500.

Identification of components

The individual constituents of the oil were identified on the basis of their retention indices determined with a reference to a homologous series of *n*-alkanes and by comparison of their mass spectral fragmentation patterns (NIST 08.L database/chemstation data system) with data previously reported in literature (McLafferty and Stauffer, 1989; Adams, 1995; Joulain and König, 1998).

Brine shrimp lethality test

The brine shrimp lethality test (BST) was used to predict the toxicity of the oils and was conducted according to the methods of Meyer et al. (1982), Falope et al. (1993) and Oloyede et al. (2010) using Brine shrimp eggs obtained from Ocean Star International, Inc. Company, USA. The shrimp's eggs were hatched in sea water for 48 h at room temperature. The nauplii (harvested shrimps) were attracted to one side of the vials with a light source. Solutions of the oils were made in DMSO, at varying

concentrations (1000, 100 and 10 µg/ml) and incubated in triplicate vials with the brine shrimp larvae. Ten brine shrimp larvae were placed in each of the triplicate vials. Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. After 24h, the vials were examined against a lighted background and the average number of larvae that survived in each vial was determined. The concentration at which fifty percent of the larvae (LC₅₀) were killed was determined using the Finney computer programme.

Free radical scavenging activity

Scavenging effect on DPPH

The free radical scavenging assay has been efficiently used as an adequate parameter for selecting medicinal plants with antioxidant properties. A solution of 0.5 mM 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) in methanol was prepared. The free radical scavenging activity of the oil at 0.1 and 0.2 mg/ml was determined by measuring the decrease in absorption after 10 min of incubation at 517 nm in a UV-visible spectrophotometer. The actual decrease in absorption was measured against that of the control. The same experiment was carried out on ascorbic acid, butylated hydroxyanisole (BHA) and α -tocopherol which are known as antioxidant agents. All test and analysis were run in triplicates and the result obtained was averaged (Koleva et al., 2002; Masuda et al., 2003; Ayoola et al., 2008). The activities were also determined as a function of their % Inhibition which was calculated using the formula;

$$\% \text{ Inhibition} = \{A_c - A_s / A_c\} \times 100$$

A_c = Absorbance of the control
 A_s = Absorbance of the sample.

Statistical analysis

Statistical analysis was carried out with Statistical Package for Social Sciences (SPSS). The data was expressed as the mean \pm standard deviation and a probability of less than 0.05 ($p < 0.05$) was considered to be statistically significant. Graph was drawn using Microsoft Office excel, 2007 software.

RESULTS AND DISCUSSION

GC-MS analysis of the essential oils

Essential oils from freshly collected stem bark of *P. guajava* were obtained by means of hydro-distillation. The yield of the 300 g hydro-distilled oil was 0.60 % (w/w). The essential oil, colourless, with characteristic smell was analyzed by GC and GC/MS systems using a polar column. The peaks showing the relative abundance of the chemical components with retention time is presented in Figure 1.

Overall, 62 constituents representing 99.98 % of essential oils were identified in the essential oils. Individual constituents of the oil were identified on the basis of their retention indices determined with a reference to a homologous series of *n*-alkanes and by comparison of their mass spectral fragmentation patterns (NIST 08.L database/chemstation data system) with data

previously reported in literature. The results of these analyses are presented in Table 1. *P. guajava* stem bark essential oils from the Nigerian soil are hydrocarbons, amines, amides and esters dominated. The following compounds had the highest % peak area, 3,6-dioxa-2,4,5,7-tetraoctane,2,2,4,4,5,5,7,7-octamethyl (11.67 %), cyclononane (10.66 %), pyridazin-3(2H)-one,4-amino-5-chloro-2-phenyl (9.35 %), pyridazin-3(2H)-one, 4-diacetylamino-5-Chloro-2-phenyl (7.35 %), tetramethyldecane (5.97 %) and N-methylrhodanine (5.01 %) while the remaining compounds (see Table 1) appeared in minute quantities. The results obtained from this analysis compare favourably well with compounds early identified in other parts of *P. guajava* essential oil (Bassols and Demole, 1994; Li et al., 1999; Paniandy et al., 2000; Begum, 2002).

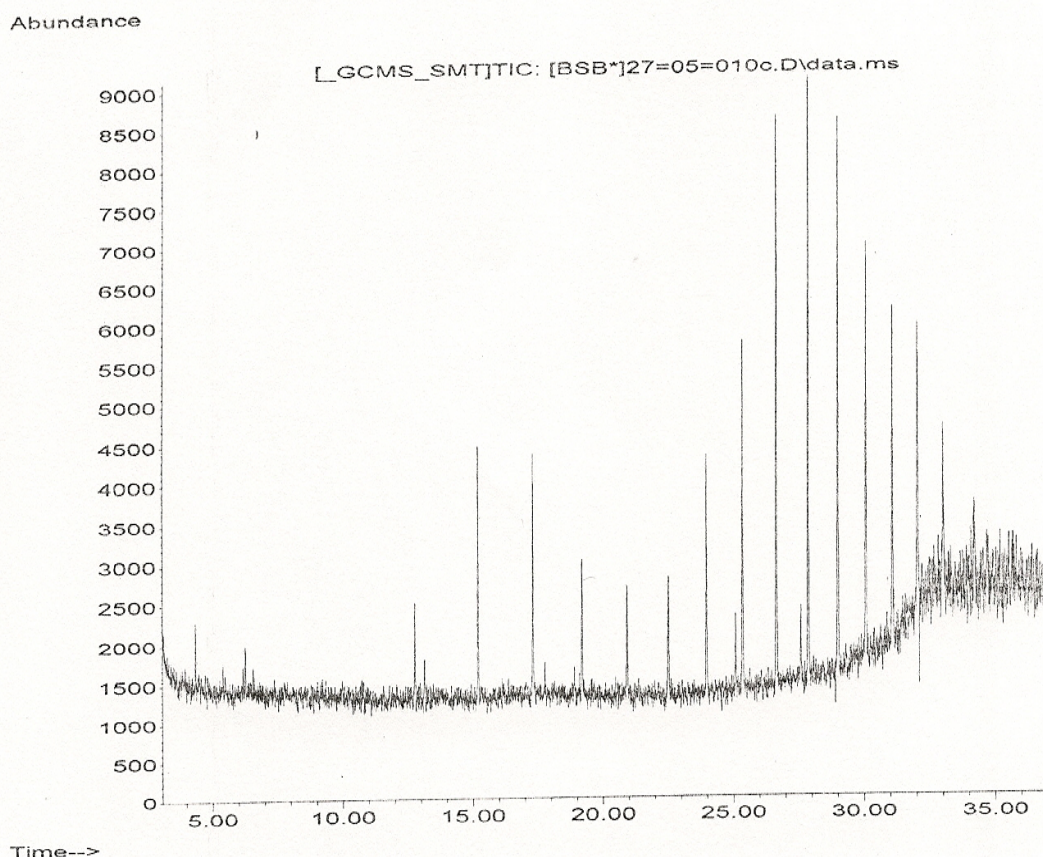


Figure 1: The peaks of the compounds in *Psidium guajava* stem bark oil at different retention time from the Gas chromatography-Mass spectrometric (GC-MS) analysis

Table 1: Compounds obtained from GC/GC-MS analysis of *P. guajava* stem bark essential oil*

S/N	Retention time (min)	Chemical composition	% Area
1	4.349	4-Ethylphenethylamine	0.92
2	5.412	1-Heptene	0.38
3	12.772	Pyrrolo [1,2-c][1,3,2]diazaphosphorine-4-carbonitrile 1, 5, 6, 7-tetrahydro-1-(methylthio)-3-(4-morpholinyl)-, 1-sulfide	1.19
4	12.915	Carbamic acid, N-(4-chlorophenyl)-,4-nitrophenyl ester	0.36
5	13.144	Benzoic acid, 2-ethoxy-, ethyl ester	0.45
6	15.190	Dodecamethyl-2-[p-Fluorophenyl]-8-methylcinchoninic acid	4.02
7	15.552	Hexamethyldecane	0.61
8	17.306	Hexamethyldodecane	3.25
9	19.183	3H-3a-Azacyclopenta[a]indine-2-carbonitrile, 3-oxo-1-(piperidine-1-yl)-4, 5, 6, 7-tetrahydro	2.09
10	20.920	9-Amino-7-mercapto-5, 6, 8, 10-tetra-benzo[b]fluoren-11-one	0.90
11	22.497	2-(4-Chlorophenyl)-5,7-dimethylimidazo[1,2-a]pyridine-8-carbonitrile	1.57
12	23.972	trans-4-Dimethylamino-4'-methoxychalcone	4.29
13	24.573	N-Methyl-7-azabicyclo (2, 2, 1) hept-2-ene	0.44
14	25.085	Phthalic acid, monoamide, N-ethyl-N-(3-methylphenyl)-butyl ester	0.39
15	25.360	Tetramethyldecane	5.97
16	26.643	Pyridazin-3(2H)-one, 4-amino-5-chloro-2-phenyl	9.35
17	27.868	3, 6-Dioxa-2,4,5,7-tetraoctane,2,2,4,4,5,5,7,7-octamethyl	11.67
18	28.924	Cyclooctane, hexamethyl	0.59
19	28.997	Cyclononane	10.66
20	30.050	2-Amino-2-oxo-acetic acid, N-[3,4-dimethylphenyl]-, ethyl ester	2.08
21	30.064	N-Methylrhodanine	5.01
22	31.070	Pyridazin-3(2H)-one, 4-diacetylamino-5-chloro-2-phenyl	7.35
23	31.997	4-Chloro-2-trifluoromethylbenzo[h]quinoline	0.35
24	32.030	5-(p-Aminophenyl)-4-(O-tolyl)-2-thiazolamine	3.17
25	32.116	2-1-Phenyl ethylidene-hydrazono-3-methyl-2,3-dihydrobenzothiazole	2.08
26	32.425	Pyrido[3, 4-d]imidazole,1,6-dicarboxylic acid	0.40
27	32.518	2-Methoxy-6-nitro-4-phenyl –quinazoline	0.36
28	32.817	Acrylophenone,3,3-diphenyl-,semicarbazone	0.92
29	32.993	4H-Pyrido [1,2-a] pyrimidine-3-carbonamide, 1,6,7,8-tetrahydro-1,6-dimethyl-4-oxo	0.45
30	33.282	Chroman-4-one,2,3-dehydro-3-hydroxy-2-(4-dimethylaminophenyl)	0.46
31	33.651	2-Anilino-6-methyl-6,7,8,9 tetrahydro-5H 1,2,4-triazolo[1,5-c]1,3-diazepin-5-ylidenecyanamide	0.36
32	33.671	8-Chloro-5H-indole[1,2-b]quinoline-10,11-dione	0.49
33	33.817	Chroman-4-one, 2,3-dehydroxy-2-(4-dimethylaminophenyl)	0.41
34	33.897	5-(p-Aminophenyl)-4-(p-tolyl)-2-thiazolamine	0.54
35	33.950	1-Phenazinecarboxylic acid, 6-(1-methoxyethyl)-, methyl ester	0.72
36	34.003	Benzofuran-6-ol-3-one,2-(4-ethoxycarbonyl) benzylidene	0.52

S/N	Retention time (min)	Chemical composition	% Area
37	34.069	3(2H)-Benzofuranone, 6-methoxy-2-[(3-methoxyphenyl) methylene]-, (E)-	0.60
38	34.162	14 α -Morphinan, 7,8-didehydro-3-methoxy-17-methyl-6-methylene	1.18
39	34.225	Hydrocinnamic acid, p-methoxy- β -methyl-, methyl ester	0.62
40	34.355	9, 10-Dimethylantracene	0.39
41	34.398	4-Acetylamino-(1,2:3,4)bis (oxadiazolo) benzene 3,8-dioxide	0.53
42	34.431	1,2-Dihydroantra[1,2-d]thiazole-2, 6, 11-trione	0.48
43	34.631	4-(Anisylideneamino) cinnamic acid	0.63
44	34.664	5H-Cyclohepta[b] pyridine-3-carbonitrile,6,7,8,9-tetrahydro-2-amino-4-(2-fluorophenyl)-	0.53
45	34.803	trans-4-Dimethylamino-4'-methoxychalcone	0.85
46	34.890	Quinazoline, 6-methoxy-4-methylthio-2-phenyl	0.44
47	34.923	4, 5-Ethylene-8, 9-dimethoxy-6-phenanthridone	0.56
48	35.076	6, 7-Benzo-phenothiazine-5, 5-dioxide	0.57
49	35.135	1-Allylglycine, n-heptafluorobutyl-,nonyl ester	0.42
50	35.172	Pyrazol-3(2H)-one, 4-(2-furfurylidenamino)-1,5-dimethyl-2-phenyl-	0.45
51	35.302	1-Methylene-2-(pyridine-4-ylcarbonyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline	0.47
52	35.345	3H-3a-Azacyclopenta[a]indene-2-carbonitrile,3-oxo-1-(piperidine-yl)-4,5,6,7-tetrahydro	0.61
53	35.428	Glycine, N-(4-thylbenzoyl)-, tetradecylester	0.38
54	35.474	Pyridine, 3-(5-ethyl-1,2,4-oxadiazol-3-yl)-2-methoxy-6-phenyl	0.47
55	35.720	3(2H)-Benzofuranone, 6-methoxy-2-[(3-methoxyphenyl) methylene]-,(E)-	0.67
56	36.364	2, 2, 2-Trifluoro-N-[2(1-hydroxy-2,2,6,6-tetramethy-piperidin-4-yl)-ethyl]-acetamide	0.40
57	36.398	Megestrol acetate	0.42
58	36.530	Dibenz [b, d] cyclohepten,2,3,4-trimethoxy-	0.68
59	36.570	4-[6-Methoxypyridazinyl-3-amino]1,2-naphthoquinone	0.60
60	36.594	Benz[e]azulen-3(3aH)-one,4,6a,7,8,9,10,10a,10b-octahydro-3a,8,10a-trihydroxy-5-(hydroxymethyl)-2, 10-dimethyl-, [3aR-(3a α ,6a α ,8 β ,10 β ,10a β ,10b β)]-	0.44
61	36.826	2-(14-Carboxytetradecyl)-2-ethyl-4,4-dimethyl-1,3-oxazolidine-N-oxyl	0.57
62	37.225	Di (1, 2, 5-oxadiazolo) [3, 4-b;3,4-E] pyrazine,4,8-diacetyl	1.26
		Total %	99.98

* GC/GC-MS analysis of essential oil of *P. guajava* stem bark

Toxicity analysis

The LC₅₀ value of 1.0009 μ g/ml (Table 2) obtained from Brine shrimp toxicity test showed that the essential oil of *P. guajava* was toxic. The toxicity was determined using brine shrimp larvae (*Artemia salina*) nauplii which are living organisms with no advanced nervous system. The

dominance of hydrocarbons in the essential oil accounted for the toxicity level. Its usage at high concentration should therefore be monitored. The result obtained suggests that *P. guajava* stem bark essential oil is of relevance in medicine as it has been established by other workers that secondary metabolites from plants which are active med-

icinally are most times toxic to brine shrimp larvae (Aiyelaagbe et al., 2010; Onocha and Ali, 2010; Oloyede et al., 2010).

Table 2: Brine shrimp lethality test of the essential oil of *P. guajava* stem bark

Conc (µg/ml)	Essential oil of <i>P. guajava</i> stem bark	
	Survivor	Dead
1000	0	30
100	0	30
10	5	25
Control	30	0
LC ₅₀ (µg/ml)	1.0009	

*LC₅₀ = the lethal concentration to half of the test organisms LC₅₀ < 1000 = Toxic, Upper Limit 28.0904, Lower Limit 1.101471

Antioxidant activity

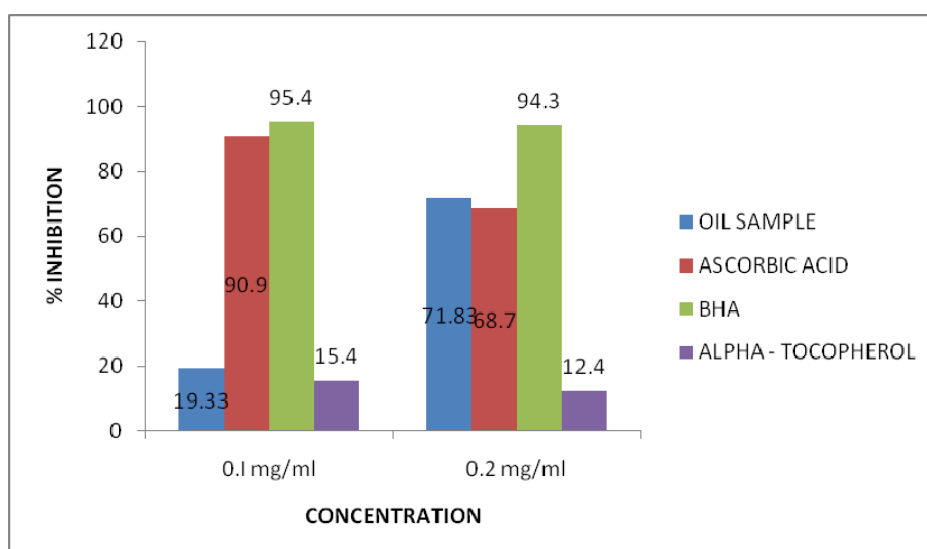
The essential oil samples were screened using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The antioxidant activity of the volatile oil was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH. The volatile oil scavenged DPPH in a concentration-dependent manner as shown in Table 3. There is decrease in absorbance measurement as the concentration is increased.

It was observed that the % inhibition of essential oil of *P. guajava* stem bark at 0.1 and 0.2 mg/ml (19.33 % and 71.83 % respectively) were lower than the % inhibition of two of the standards, ascorbic acid (90.9 % and 68.7 %) and BHA (95.4 % and 94.3 %) but higher than the % inhibition of α-tocopherol (15.4 % and 12.4 %). The % inhibition of the essential oils increased with the increase in the concentration of the oils (Figure 2).

Table 3: Scavenging effect of *P. guajava* essential oils and standards on DPPH

Conc. (mg/ml)	Oil sample	Ascorbic acid	BHA	α-tocopherol
0.1	0.797 ± 0.001	0.0843 ± 0.010	0.0370 ± 0.006	0.6800 ± 0.029
0.2	0.575 ± 0.001	0.2893 ± 0.128	0.0460 ± 0.008	0.7040 ± 0.003

*Absorbance of essential oil of the stem barks of *P. guajava*, ascorbic Acid, BHA and α-tocopherol at 517 nm.



*Absorbance of control = 0.988 ± 0.001

Figure 2: % Inhibition of *P. guajava* essential oil and known antioxidants at absorbance_{517nm} on DPPH*

This suggests that *P. guajava* stem bark has weak activity as a proton donor in the reaction involving DPPH but its activity as an OH radical scavenger is promising. One of the most important effective defenses of a living body against diseases is the removal of OH radical. The hydroxyl radical is a very reactive free radical formed in biological systems (Hochstein and Atallah, 1988) and reacts with phospholipids, sugars, amino acids, organic acids and DNA bases found in living cells (Halliwell and Gutteridge, 1984) therefore natural products with antioxidant activity capable of scavenging OH radical might contribute towards the total and partial alleviation damages caused by oxidative reactions (Lin et al., 1995). Thus the result of this study (Table 3 and Figure 2) shows that *P. guajava* oil possesses antioxidant activity which could exert beneficial actions against pathological alterations caused by the presence of hydroxyl radical. This result agrees favorably with previous reports about the antioxidant potential of Guava fruit (Jimenez-Escrig et al., 2001; Lim et al., 2006; Lugasi et al., 1999).

CONCLUSIONS

The stem bark of *P. guajava* essential oil from the Nigerian soil contains mostly hydrocarbons, amines, amides and esters. The antioxidant activity of the volatile oil was measured in terms of hydrogen donating ability, using the stable radical 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). DPPH is incompatible with oxidizing agents and strong bases. Reduction in absorbance is due to the pairing of the odd electron of the radical, indicating free radical scavenging activity. The oil from guava stem bark was found to have weak activity as a proton donor in the reaction involving DPPH. However, the activity is comparable to α -tocopherol which is known to be a good scavenger of hydroxyl radical. This clearly suggests that essential oil from the stem bark of *P. guajava* oil has the ability to scavenge OH radical. Also brine shrimp

lethality test revealed that the essential from the bark of *P. guajava* was toxic at varied degrees with reference to 1000 $\mu\text{g/ml}$. It can therefore be inferred that *P. guajava* essential oils contain medicinally important secondary plant metabolites.

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