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Phytochemical screening, GC-MS analysis and *in vitro* inhibition of alpha-amylase and alpha-glucosidase activities by methanol extract of *Psidium guajava* leaves and fractions

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Abstract
Decreasing post-prandial blood glucose levels by retarding the activities of principal carbohydrate-metabolizing enzymes such as α-amylase and α-glucosidase is one approach in the management of diabetes mellitus. The leaves of *Psidium guajava* are used to manage and control a number of diseases including diabetes mellitus. This study was aimed at evaluating *in vitro*, the inhibitory capabilities of the crude methanol extract, n- hexane, dichloromethane and aqueous fractions of the methanol extract of *Psidium guajava* leaves on the activities of α-amylase and α-glucosidase and also screening the phytochemical constituents and analyzing the bioactive components of the most potent fractions using Gas Chromatography - Mass Spectrometry (GC-MS) analysis. All fractions of the methanol extract had inhibitory effects on the activities of α-amylase and α-glucosidase with the dichloromethane fraction having the highest percentage inhibition on the activity of α-glucosidase, having 92.52% inhibition at 100µg/ml and the crude methanol extract had the highest percentage inhibition of α-amylase activity, having 89.2% inhibition at 100µg/ml. Phytochemical screening of the most potent fractions (crude methanol extract for α-amylase and dichloromethane fraction for α-glucosidase respectively) revealed the presence of phytochemicals such as flavonoids, glycosides, phenolic compounds and condensed tannins. Also, GC-MS analysis of the most potent fractions revealed the presence of compounds such as caryophyllene, phytol, squalene, and 2',6'-dihydroxymethoxychalcone that have been reported to have antidiabetic activities. The results from this work therefore show the potential of the fractions of the methanol extract of *Psidium guajava* leaves in the management of diabetes mellitus.

Keywords: *Psidium guajava*, crude methanol extract, dichloromethane fraction, alpha-glucosidase, alpha-amylase, phytochemical screening, gas chromatography - mass spectrometry (GC-MS)

Introduction
Diabetes mellitus is an endocrine disease that affects a large number of individuals [1]. The characteristics of this disorder include increased blood glucose levels and disorders in carbohydrate metabolism, secondary to total or partial deficiency of insulin, a hormone involved in carbohydrate metabolism [2]. This disorder is one that is characterized by shortage of insulin production and its function or both [3].

The population of diabetic individuals has increased over the years [4]. In addition to insulin supplementation, diabetes mellitus treatment includes many oral hypoglycemic agents, exercise and regulated diet [4]. A therapeutic approach which may be helpful in managing diabetes mellitus is post-prandial blood glucose reduction. This is done by slowing down the uptake of glucose by inhibiting the activity of principal enzymes involved in carbohydrate metabolism [4]. These enzymes include alpha-glucosidase and alpha-amylase. The digestion of carbohydrate foods is delayed by inhibitors of these enzymes resulting in a marked decrease in glucose absorption rate thereby reducing post-prandial blood glucose rise [5]. Examples of inhibitors of these principal carbohydrate-metabolizing enzymes used in managing diabetes mellitus are voglibose, acarbose and miglitol [6]. These drugs however have some gastrointestinal side effects which include diarrhoea and abdominal pain in diabetics [7]. Therefore, it is needful to identify other inhibitors of these enzymes especially from natural origin whose side effects are milder.

Several plants and plant products have been recommended and used for diabetes mellitus management worldwide but there is little knowledge on the basis of the mechanism of their function. Thus, these products need to be scientifically evaluated so that their antidiabetic properties can be verified [4].

Guava plant (*Psidium guajava*) belongs to the Myrtaceae family. Studies have revealed that *Psidium guajava* is used in different places in the world in treatment of diseases, for diabetes

mellitus and hypertension management, as an anti-inflammatory agent, pain relief, treatment of wounds and also for the reduction of fever. Some countries known to have long history of medicinal use of *Psidium guajava* are Mexico and some other countries in Central America, Asia and Africa [8]. This study was aimed at investigating *in vitro*, the inhibitory capabilities of the crude methanol extract and fractions of methanol extract of *Psidium guajava* leaves on the activities of alpha-amylase and alpha-glucosidase and also screening the phytochemical constituents of the most active fractions and analyzing the bioactive compounds using GC-MS.

Materials and methods

Collection and Identification of *Psidium guajava* leaves

Fresh leaves of *Psidium guajava* were collected from the Department of Biochemistry, University of Benin, Benin City, Nigeria. The leaves were identified at the Department of Plant Biology and Biotechnology, University of Benin, with voucher number UBHp378. The leaves were dried under shade for one week before they were reduced to powder.

Preparation of Methanol Extract of *Psidium guajava* leaves and its different Fractions

Dry powder of *Psidium guajava* leaves was macerated using methanol for 72 hours. The mixture was filtered using Whatmann (grade 1) filter paper. The filtrate was concentrated to dryness using water bath at 66ºC. The crude methanol extract was subjected to partition chromatography using n-hexane and dichloromethane. The n-hexane fraction and dichloromethane fractions were collected and what was left was the aqueous fraction. Altogether, there were three fractions (n-hexane, dichloromethane and aqueous fractions) and the crude methanol extract.

In vitro inhibitory assay for alpha-amylase activity

Exactly 20µL of Porcine alpha-amylase enzyme was incubated with 200µL of 0.02M phosphate buffer and plant extracts in concentration ranging from 20-100µg/mL for 10 minutes at room temperature. This was followed by the addition of 200µL of starch in all the test tubes. The reaction was terminated with 400µL DNS reagent in each test tube and was placed in boiling water bath for 5 minutes after which the tubes were cooled and diluted with 15mL of distilled water. The absorbance was then read at 540nm. Control samples were prepared without plant extracts. Acarbose at various concentrations (20-100µg/mL) was used as standard. The results were given as percentage inhibition and were calculated with the formula [9]:

$$\frac{Abs_{540}(control) - Abs_{540}(extract)}{Abs_{540}(control)} \times 100$$

where:
Abs_{540} = absorbance at 540nm.
IC_{50} values were derived from plots of percentage inhibition against concentration. The reference alpha-amylase inhibitor was acarbose. All tests were done in triplicate.

In vitro inhibitory assay for alpha-glucosidase activity

Alpha-glucosidase enzyme from yeast was dissolved in 100mM phosphate buffer at pH 6.8. This was used as the enzyme extract. Exactly 320µL of phosphate buffer was taken into each test tube followed by the addition of different concentrations of the plant extract (20µg/mL, 40µg/mL, 60µg/mL, 80µg/mL and 100µg/mL) in the respective test tubes. Exactly 20µL of enzyme solution was added to each test tube and the mixture was left to stand for 10minutes before addition of 200µL of the enzyme's substrate (p-Nitrophenyl-α-D-glucopyranoside). The mixture was left to stand for 15minutes before addition of 3mL NaOH to each test tube. Absorbance was taken at 410nm. Acarbose at various concentrations (20-100 µg/mL) was used as standard. The results were given as percentage inhibition, which was calculated with the formula [9]:

$$\frac{Abs_{410}(control) - Abs_{410}(extract)}{Abs_{410}(control)} \times 100$$

where:
Abs_{410} = Absorbance at 410nm.
IC_{50} values were derived from plots of percentage inhibition against concentration. The reference alpha-glucosidase inhibitor was acarbose. All tests were done in triplicate.

Qualitative Phytochemical screening

Phytochemical screening was carried out according to standard methods by Khandelwal [10], Kokate [11], Trease and Evans [12], Sofowora [13] and Ayoola *et al.* [14].

Procedure

Saponin Test (Froth test)

To 0.5mL of sample solution was added 5mL of distilled water. The mixture was shaken vigorously. Formation of froth showed that saponins were present.

Flavonoids Test

To 2mL of sample solution, an equivolume of dilute ammonia was added. A yellow colour showed the presence of flavonoids.

Steroids Test

To 2mL of sample solution was added 2mL of acetic anhydride and 2mL H_2SO_4. Colour change from violet to blue or green showed that steroids were present.

Phenolic compounds

To 2mL of sample solution, 5mL of water was added and warmed at 45 ^{0}C-50 ^{0}C. Then 0.3% $FeCl_3$ was added. Green or blue colour formation showed that phenols were present.

Hydrolysable tannins

To 2mL of sample solution was added 2mL of 0.1% ferric chloride. A blue colour formation showed that hydrolysable tannins were present.

Condensed tannins

To 2mL of sample solution was added 2mL of 0.1% ferric chloride. A brownish green colouration showed the presence of condensed tannins.

Cardiac glycosides (Keller-Killiani test)

To 2mL of sample solution was added 5mL of water followed by addition of 2mL glacial acetic acid containing drops of ferric chloride. 1mL of H_2SO_4 was added along the side of the tube. Formation of brown ring at the interface showed the presence of cardiac glycosides. A violet ring may also appear below the brown ring.

Test for glycosides (Fehling's test)
Diluted Fehling's solution A and B was boiled for 1minute. To the clear blue solution, 8 drops of the sample solution was added. The mixture was then boiled for 5 minutes. Formation of brick-red colour showed the presence of glycosides.

Anthraquinones
To 3mL of sample solution was added 5mL of chloroform. This was mixed for 5 minutes, resulting in the formation of two layers. Presence of bright pink colour in the aqueous layer showed the presence of anthraquinones.

Reducing sugar
A sample solution (2mL) was made alkaline by addition of 20% NaOH solution. This was boiled in an equivolume of Benedict qualitative solution. The formation of a brick red precipitate showed the presence of reducing sugar.

Starch
To 2mL of sample solution, 2mL of iodine solution was added. A blue-black colouration showed the presence of starch.

Alkaloids
Dragendorff's test: To 2mL of sample solution was added few drops of draggendorff's reagent. Formation of orange-brown precipitate showed the presence of alkaloids.

Gas Chromatography - Mass Spectrometry (GC-MS) analysis
Gas Chromatography - Mass Spectrometry analysis was carried out on two fractions of the *Psidium guajava* leaves extract (dichloromethane fraction and crude methanol extract). The oven temperature was set at 80 ^{0}C as initial temperature to hold for 2 minutes at 10 ^{0}C per minute to the temperature of 240 ^{0}C to hold for 6 minutes. Helium was used as the carrier gas. The column used was HP5MS Agilent technologies of length 30mm, internal diameter 0.320mm and thickness 0.25µm. The volume of sample injected was 1µL and the carrier gas was injected at 2mL per minute. The injector temperature was 250 ^{0}C. The compounds present were identified by comparing retention indices and also comparing the mass spectra of each of the compounds with those of the National Institute for Standards and Technology (NIST) library database.

Statistical analysis
Results were given as mean ± standard deviation and IC_{50} values were generated by a plot of percentage inhibition against concentration. Analysis of variance (ANOVA) followed by LSD Post Hoc test was carried out to obtain values. Differences between the mean of percentage inhibition by crude methanol extract and other fractions of the methanol extract as well as that of acarbose were considered to be significant at $p < 0.05$.

Results and discussion
This study was aimed at assessing the inhibitory capabilities of fractions of crude methanol extract of *Psidium guajava* leaves on activities of α-amylase and α-glucosidase and the results are shown in Tables 1 and 2.
The crude methanol extract of the guava leaves extract gave highest percentage inhibition of alpha-amylase activity at all the different concentrations (Table 1 and Figure 1) compared with the n-hexane, dichloromethane and aqueous fractions, having 89.20% inhibition at 100 µg/mL, while the n-hexane fraction, dichloromethane fraction and aqueous fractions had 68.22%, 86.69% and 66.52% inhibitions respectively at 100µg/mL. The percentage inhibitions by the crude methanol extract showed no significant increase or decrease ($p > 0.05$) at all concentrations when compared with the standard drug acarbose.

Table 1: Percentage alpha-amylase activity inhibition by crude methanol extract, n-hexane fraction, dichloromethane fraction and aqueous fraction of *Psidium guajava* leaves at varying concentrations (values are expressed as mean ± SD, n = 3).

Conc. (µg/ml)	% Inhibition By crude Meth. extract	IC_{50} (µg/ml) crude Meth. extract	% Inhibition by n-Hex. fraction	IC_{50} (µg/ml) n-Hex. fraction	% Inhibition by Dichl. fraction	IC_{50} (µg/ml) Dichl. fraction	% Inhibition By Aque. fraction	IC_{50} (µg/ml) Aque. fraction	% Inhibition by Acar.	IC_{50} (µg/ml) Acar.
20	72.41±1.92		35.69±11.37		48.53±1.48		27.79±11.00		65.46±4.81	
40	89.57±2.34		33.20±16.81		80.06±0.33		38.63±2.36		65.47±0.76	
60	94.17±0.93	13.81	46.14±6.80	68.67	83.86±1.43	20.93	42.86±33.46	69.63	71.73±1.24	15.27
80	88.46±4.68		55.04±3.08		75.94±0.53		57.71±15.82		71.83±7.90	
100	89.20±3.95		68.22±3.36		86.69±1.55		66.52±8.95		78.71±1.68	

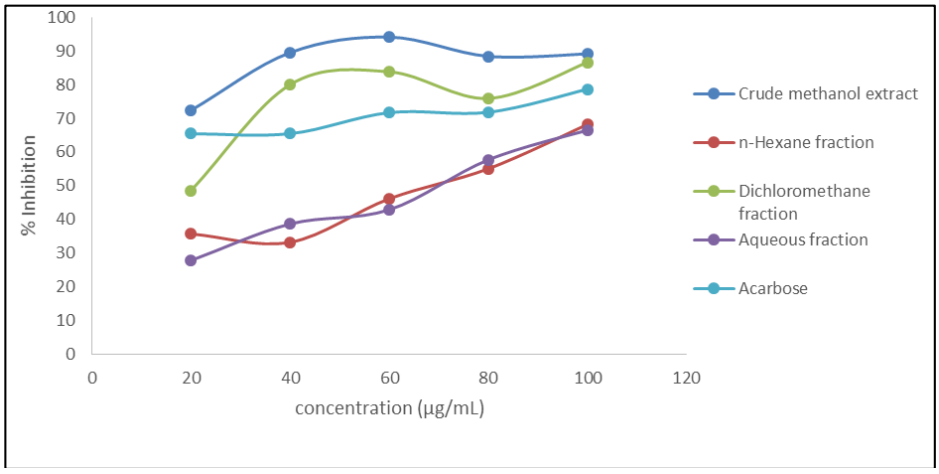

Fig 1: Percentage alpha-amylase inhibition by fractions of *Psidium guajava* leaves and acarbose (values are expressed as mean ± SD, n = 3).

The dichloromethane fraction of *Psidium guajava* leaves gave the highest percentage inhibition of alpha-glucosidase activity when compared with the n-hexane, crude and aqueous fractions having 92.52% inhibition at 100µg/mL, while the n-hexane, crude and aqueous fractions had 77.39%, 74.60% and 60.47% inhibitions respectively at 100µg/mL (Table 2 and Figure 2). The percentage inhibitions by the dichloromethane fraction showed significant increase in percentage inhibition at all concentrations ($p < 0.05$), when compared with the percentage inhibition by the standard drug, acarbose.

Table 2: Percentage alpha-glucosidase activity inhibition by crude methanol extract, n- hexane fraction, dichloromethane fraction and aqueous fraction of *Psidium guajava* leaves at varying concentrations (values are expressed as mean ± SD, n = 3).

Conc. (µg/ml)	% Inhibition By crude Meth. extract	IC_{50} (µg/ml) crude Meth. extract	% Inhibition by n-Hex. fraction	IC_{50} (µg/ml) n-Hex. fraction	% Inhibition by Dichl. fraction	IC_{50} (µg/ml) Dichl. fraction	% Inhibition by Aque. fraction	IC_{50} (µg/ml) Aque. fraction	% Inhibition by Acar.	IC_{50} (µg/ml) Acar.
20	33.94±0.61		57.84±0.98		62.21±1.43		61.63±0.52		31.84±6.35	
40	48.08±1.02		70.68±2.05		63.37±0.98		66.97±0.30		40.20±0.98	
60	48.08±1.02	62.21	68.63±0.00	17.28	74.51±3.92	16.07	64.71±0.00	16.22	41.18±0.00	69.81
80	65.16±1.52		74.39±3.80		79.29±1.11		64.17±0.54		59.48±1.30	
100	74.60±1.87		77.39±1.04		92.52±1.61		60.47±2.29		64.03±3.24	

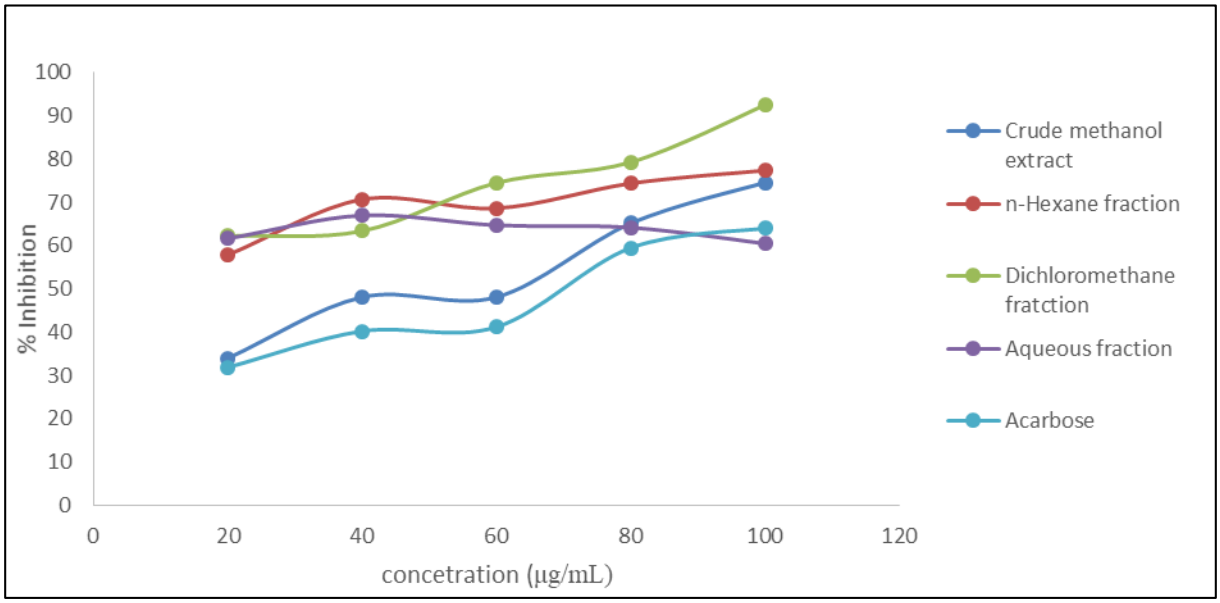

Fig 2: Percentage alpha-glucosidase inhibition by fractions of *Psidium guajava* leaves and acarbose (values are expressed as mean ± SD, n = 3).

Phytochemical screening of the fractions that had the highest percentage of α-amylase and α-glucosidase inhibition i.e. the crude methanol extract and dichloromethane fraction respectively showed that the crude methanol extract contained moderate amounts of flavonoids, glycosides, phenolic compounds, reducing sugar, condensed tannins and steroids (Table 3). Studies have shown that a synergistic action of these phytochemicals is capable of reducing blood glucose levels [15]. The dichloromethane fraction contained moderate amount of flavonoids and little amounts of phenolic compounds and condensed tannins. Studies have shown that phenolic compounds are inhibitors of alpha-glucosidase and alpha-amylase activities, inhibiting alpha-glucosidase more strongly than alpha-amylase. They can thus be used as means to reduce post-prandial blood glucose rise with less side effects [16].

Table 3: Qualitative phytochemical analysis of dichloromethane fraction and crude methanol extracts of the leaves of of *Psidium guajava*.

Phytochemicals	Dichchloromethane fraction	Crude methanol fraction
Flavonoids	++	++
Saponins	-	-
Reducing sugar	-	++
Glycosides	-	++
Phenolic compounds	+	++
Hydrolysable tannins	-	-
Condensed tannins	+	++
Steroids	-	++
Alkaloids	-	-
Cardiac glycosides	-	-
Anthraquiones	-	-
Starch	-	-

KEYS: Moderately present = + +; Slightly present = +; Not present = -

Tables 4 and 5 show the retention time, peak area percentage of the crude methanol extract and dichloromethane fraction of crude extract of the leaves of *Psidium guajava* respectively. The major compounds identified in the crude methanol extract were caryophyllene, with 18.22% peak area; δ-armophene, with 10.67% peak area; β-bisabolene with 10.21% peak area; Ar-curcumene, with 8.79% peak area; β-bisabolol, with 8.75% peak area; β-copaene, with 7.34% peak area and phytol, with 6.09% peak area (Table 4 and Figure 3).

Table 4: Results of Gas Chromatography- Mass Spectrometry (GC-MS) analysis of the crude methanol extract of *Psidium guajava* leaves.

Peak	Retention time (minutes)	Molecular formula	Compound name	Molecular weight	Peak Area (%)
1.	12.603	$C_{15}H_{24}$	α–Copaene	204	4.99
2.	13.289	$C_{15}H_{24}$	Caryophyllene	204	18.22
3.	14.010	$C_{15}H_{22}$	Ar-Curcumene	202	8.79
4.	14.371	$C_{15}H_{24}$	β-Bisabolene	204	10.21
5.	14.405	$C_{15}H_{24}$	Bisabolatriene	204	4.75
6.	14.600	$C_{15}H_{24}$	β-Sesquiphellandrene	204	5.54
7.	14.640	$C_{15}H_{24}$	δ-Amorphene	204	10.67
8	15.063	$C_{15}H_{22}O$	Nerolidol	222	2.58
9.	16.126	$C_{15}H_{24}$	Acoradiene	204	4.77
10.	16.350	$C_{15}H_{24}$	β-Copaene	204	7.34
11.	16.711	$C_{15}H_{22}O$	β – Bisabolol	222	8.75
12.	23.709	$C_{20}H_{40}O$	Phytol	296	6.09
13.	29.511	$C_{16}H_{14}O_4$	5-hydroxy-7-methoxyflavanone	270	4.49
14.	31.846	$C_{30}H_{50}$	Squalene	410	2.79

Caryophyllene has been shown to have significant antidiabetic effect [17]. Caryophyllene, a natural sesquiterpene, modulates the metabolism of carbohydrate [17]. Phytol has also been shown to be effective in the management of metabolic disorders that accompany diabetes mellitus [18]. Squalene has been shown to be beneficial for diabetes [19] while bisabolol has been shown to have ulcer protective properties [20].

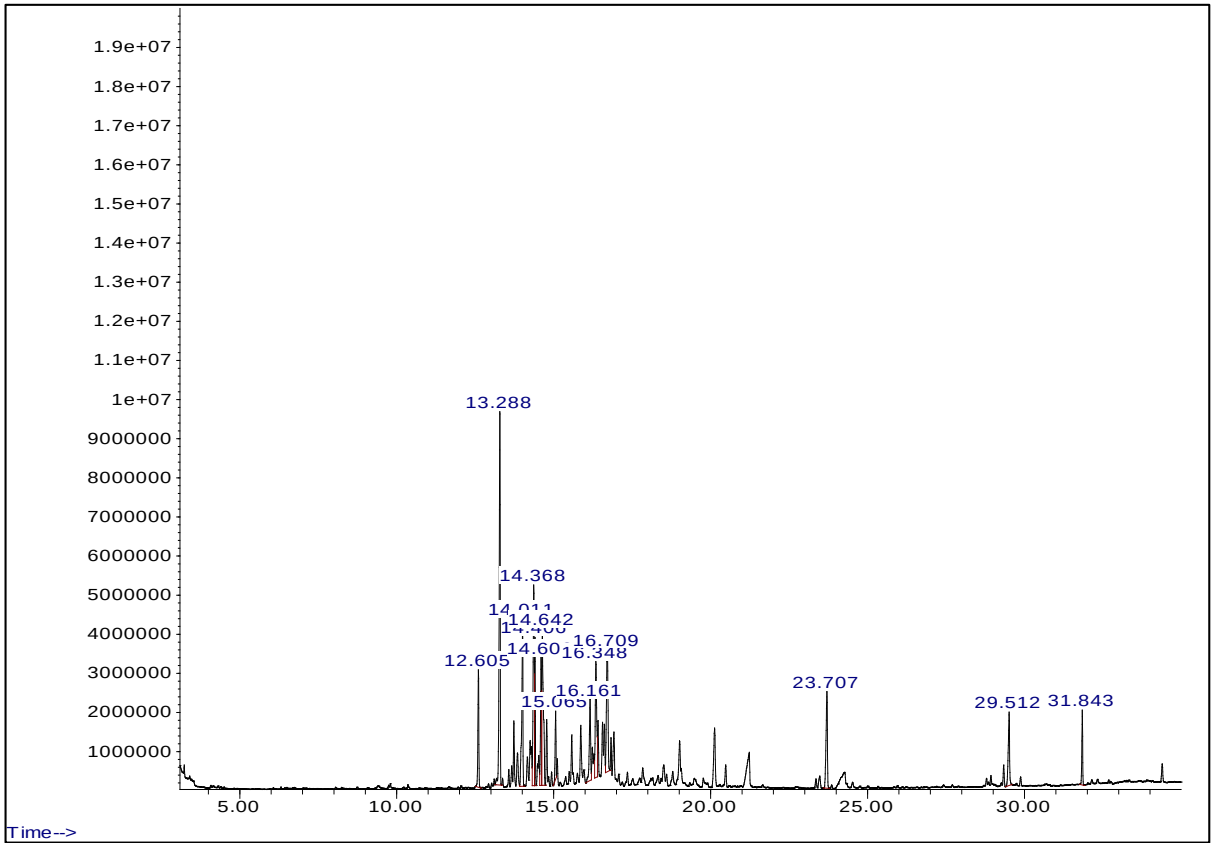

Fig 3: Chromatogram of crude methanol extract of *Psidium guajava* leaves

The dichloromethane fraction gave the following compounds as major components: 2^1,6^1-dihydroxy-4^1-methoxychalcone, with 13.80% peak area; caryophyllene, with 12.57% peak area; 1-methylindene, with 8.18% peak area; pseudocumene, with 7.23% peak area and α-copaene with 7.13% peak area (Table 5 and Figure 4). 2^l,6^l-dihydroxy-4^l-methoxychalcone exhibits hypoglycaemic and anti-hyperglycaemic activities in diabetic rats [21].

The results obtained from this experiment indicated that of the three fractions generated from the methanol extract of *Psidium guajava* leaves and the crude methanol extract, the crude methanol extract had the highest alpha-amylase inhibition at all concentrations. The dichloromethane fraction

of methanol extract of guava leaves gave the highest percentage inhibition of alpha-glucosidase at all concentrations. The experiment indicated the presence of phytochemicals which in synergistic activity may be responsible for the antidiabetic effect of the crude extract and the dichloromethane fraction of the methanol extract of *Psidium guajava* leaves. Phytochemical screening showed moderate amounts of glycosides, flavonoids, phenolic compounds, condensed tannins and steroids in the crude methanol extract (Table 3).

Phytochemical screening of the dichloromethane fraction showed presence of flavonoids in moderate amount while phenolic compounds and condensed tannins showed little presence (Table 3). The presence of bioactive compounds such as caryophyllene, phytol and squalene, in the crude methanol extract which have been shown to have antidiabetic activities [19, 18, 17] was also observed in this experiment (Table 4) while the dichloromethane fraction revealed presence of bioactive compounds such as caryophyllene and 2^{l},6^{l}-dihydroxy-4^{l}-methoxychalcone (Table 5) which have been shown to have antidiabetic activities [21].

Table 5: Results of Gas Chromatography- Mass Spectrometry (GC-MS) analysis of the dichloromethane fraction of methanol extract of *Psidium guajava* leaves.

Peak	Retention time (minutes)	Molecular formula	Compound name	Molecular weight	Peak Area (%)
1.	3.682	CH_2Cl_2	Methylene chloride	84	19.45
2.	6.526	C_9H_{12}	Pseudocumene	128	7.23
3.	8.620	$C_{13}H_{18}O$	1-Allyl-2,3,4,5-tetramethyl benzene	238	3.12
4.	9.129	$C_{10}H_{12}$	P-Allyltoluene	132	4.00
5.	9.713	$C_{10}H_{10}$	1-methylindene	128	8.18
6.	12.574	$C_{15}H_{24}$	α-Copaene	204	7.13
7.	13.238	$C_{15}H_{24}$	Caryophyllene	204	12.57
8.	13.970	$C_{15}H_{22}$	Ar-Curcumene	202	4.61
9.	14.313	$C_{15}H_{24}$	β-Bisabolene	204	2.59
10.	14.600	$C_{15}H_{22}$	Trans-calamenene	202	5.03
11.	16.282	$C_9H_{12}O$	5-methylene-1,3a,4,5,6,6a-hexahydropentalen-1-ol	136	2.17
12.	18.954	$C_{10}H_{14}O_2$	Epidolichodial	165	3.95
13.	20.064	$C_{15}H_{26}O_2$	4.4.8trimethyltricyclo[6.3.1(1, 5)]dodecane-2,9-diol	238	6.17
14.	29.474	$C_{16}H_{14}O_4$	2^{l},6^{l}-dihydroxy-4^{l}-methoxychalcone	270	13.80

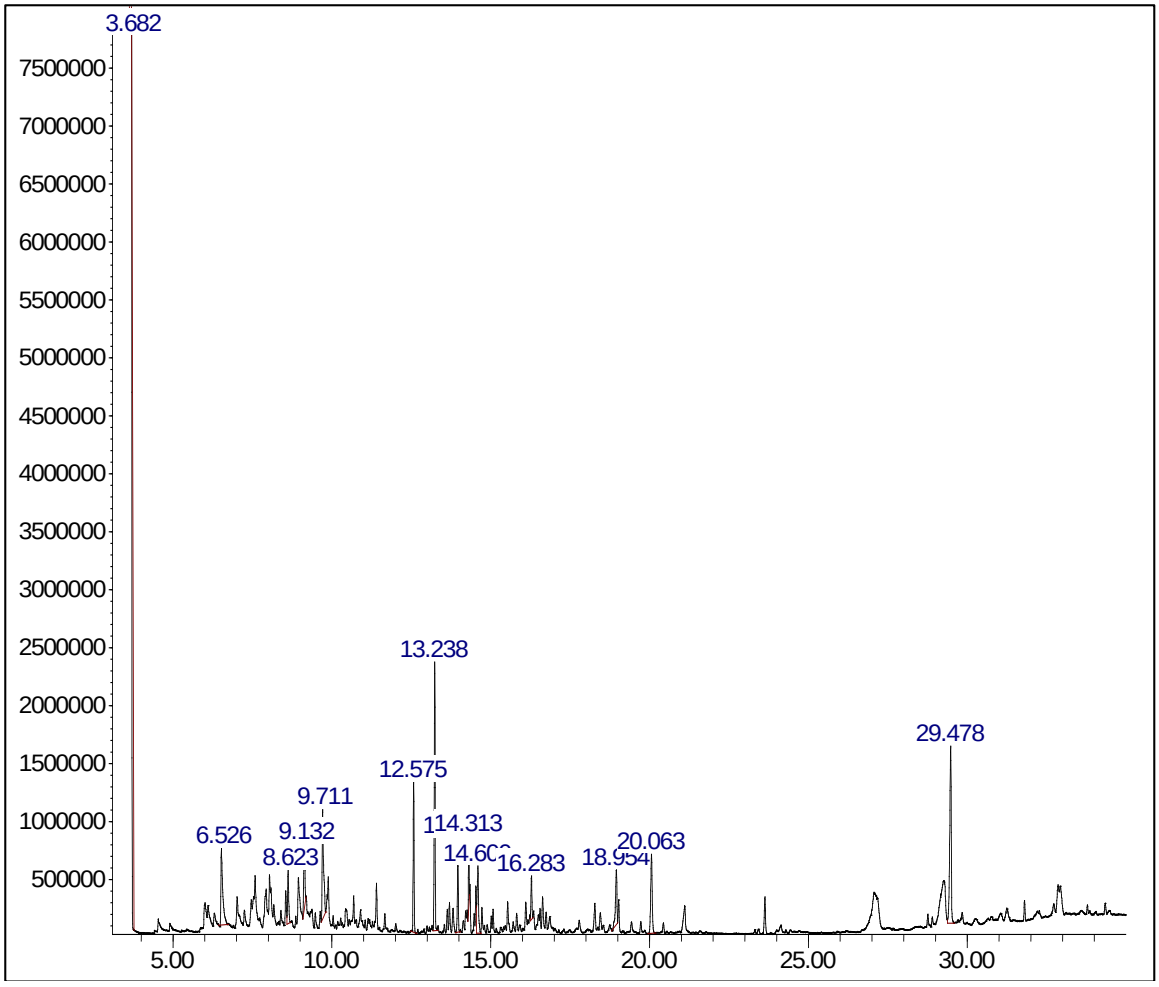

Fig 4: Chromatogram of dichloromethane fraction of methanol extract of *Psidium guajava* leaves.

Conclusion

This study has further confirmed that guava leaves are useful as an antidiabetic agent as different fractions generated using different solvents (dichloromethane and n-Hexane) were capable of inhibiting the activities of the principal carbohydrate-metabolizing enzymes (alpha-amylase and alpha-glucosidase). The reduction of their activities helps to reduce post-prandial hyperglycaemia. This is one of the mechanisms of action of the leaves of *Psidium guajava* in reducing blood sugar. This justifies the use the leaves of *Psidium guajava* in ethnomedicine as an antidiabetic tool. Also this study showed that the crude methanol extract of guava leaves inhibits the activity of alpha-amylase more than the dichloromethane, aqueous and n-hexane fractions while the dichloromethane fraction was shown to be most potent in the inhibition of the activity of alpha-glucosidase. Also the presence of phytochemicals and bioactive compounds which have been shown to be potent antidiabetic agents gives an idea of agents that are likely responsible for the antidiabetic properties of *Psidium guajava* leaves.

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