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Enzyme Inhibitory Activities, Phytochemical Screening and Gc-Ms Analysis of *FicusExasperataVahl*Leaf Extract and Fractions

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Abstract: *Alpha (α) amylase and alpha (α) glucosidase are carbohydrate hydrolyzing enzymes known to play profound roles in carbohydrate breakdown. Inhibition of these enzymes are important mechanisms in modulating postprandial hyperglycemia due to their ability to delay carbohydrate digestion. The aim of this study was to determine the α-amylase and α-glucosidase inhibitory potential of Ficusexasperata (F.exasperata) leaf extract and fractions in vitro. Dried leaves of F.exasperata were extracted with methanol and the methanol extract was further fractionated with solvents of varied polarity (dichloromethane and hexane). The α-amylase and α- glucosidase inhibitory assays of the extract and fractions were determined in vitro by reacting different concentrations of the extract and fractions with starch solutions and p-nitrophenyl-α-d-glucopyranoside (pNPG) respectively. Absorbance were determined spectrophotometrically at wavelengths 540 nm and 410 nm respectively. Phytochemical screening of the extract and fractions were determined. Bioactive compounds present in the extracts with highest inhibitory activity against the enzymes were identified using GC-MS analysis. The results showed that the aqueous fraction and methanol extract had the most effective inhibition with IC_{50} values of 140.86 µg/mL and 32.47 µg/mL for α-amylase and α-glucosidase respectively. Phytochemical screening of the extract and fractions revealed the presence of glycosides, flavonoids, alkaloids, eugenols, phenolics, terpenoids and high amount of saponins. The GC-MS analysis of the aqueous fraction and crude methanol extract of F.exasperata leaves identified the presence of eight and seven bioactive compounds respectively. These bioactive compounds present in F.exasperata leaves have α-amylase and α- glucosidase inhibitory potentials and thus validates its use in the management of diabetes and as a mechanism of action.*

Keywords: *FicusexasperataVahl, α-amylase, α-glucosidase, phytochemicalscreening, GC-MS*

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I. Introduction

FicusexasperataVahl is tropical and sub-tropical African medicinal shrub grown mostly uncultivated in the rainforest region [1]. Its leaves, bark and root are known to possess numerous sanative properties against a variety of ailments like hypertension, microbial and venereal infections [2], arthritis, stomach disorders, ulcer, haemorrhoids, inflammation and delivery induction[3,4].Its ability to improve glucose tolerance and lower plasma triacylglycerol and β-OH butyrate levels in alloxan induced diabetic rats were also reported [5,6]. However, paucity of details is available on the mechanism by which it exhibits its hypoglycemic activity. Carbohydrate hydrolyzing enzymes such as alpha amylase and alpha glucosidase are vital enzymes known to play profound roles in carbohydrates breakdown due to their ability to hydrolyze starch to small and easily absorbable forms like glucose. However, when the amount of available glucose is in excess, due to the conversion of starch to glucose by these enzymes, the body creates adjustment by stimulating the hormone insulin, to help mop off the excess glucose moieties and store it as glycogen-energy source. Although, in cases of insulin deficiency or insulin resistance, with excess alpha amylase activity, hyperglycemia results [7], a disease condition referred to as diabetes mellitus. Hence, inhibition of these enzymes could be an important mechanisms in modulating postprandial hyperglycemia due to their ability to delay carbohydrate digestion[7].Phytochemicals are important secondary compounds beneficial to plants and humans due to the diverse range of activities they elicits [8]. Hence, this study was aimed at evaluating the *in vitro* alpha (α) amylase and alpha (α) glucosidase inhibitory activities of the crude methanol extract and fractions of *FicusexasperataVahl*leaf, identifying the phytochemicals and phytoconstitutents present in the fractions with highest inhibitory activities against these enzymes.

II. Materials And Method

Plant material

Fresh *Ficusexasperata* leaves (FEL) obtained from its tree at a botanical garden, University of Benin were identified by the taxonomist of the Department of Plant Biology and Biotechnology, University of Benin,

Benin City, Nigeria. A voucher number of the plant (UBH- F319) was given and a sample deposited. The leaves were rinsed with tap water to eliminate impurities and air dried at room temperature (24°C) for two weeks until crispy leaves were gotten. The dried leaves were pulverized using a milling machine (Viking Exclusive Joncod, USA) and weighed using analytical weighing balance (Ohaus Corporation, United States).

Sample extractions

Solvent- solvent cold maceration extraction of the plant was carried out by soaking 889.048 g of the pulverized *Ficusexasperata* leaves in analytical 99.55% methanol (JHD, China) solvent for four days and stirred intermittently during the period. The resultant methanol extract was concentrated using a rotary evaporator (model no: RE52-3, Search Tech Instruments, England) at 50°C and weighed giving a weight of 43.06 g and a percentage yield of 4.84%. About 36.65 g of the obtained crude methanol extract of FEL was fractionated using n-hexane (Lobachemie, India), dichloromethane (Lobachemie, India) solvents and distilled water based on differences in their polarity in order to obtain the hexane fraction, dichloromethane fraction and aqueous fraction. The collected fractions were concentrated using a water bath (Gallenphamp, England) at 60°C, stored in sterile samples bottles and refrigerated for biochemical analysis.

***In vitro* enzyme inhibitory activities for alpha amylase**

The alpha amylase inhibitory assay was done according to the method of [9] with slight modification. Two hundred microliter (200 µL) of 0.02M phosphate buffer (pH 6.8), 20µl alpha amylase enzyme (Aobox, China) in 0.02 M sodium phosphate buffer (0.5 mg/ml) and varied concentrations of 20-100 µg/mL of extract were incubated at 25°C for 10 minutes. After pre-incubation, 200 µL of 1% starch solution (JHD, China) was added in all test tubes and the reaction mixture was allowed to stand for ten minutes. The reaction was terminated with 400 µL of 3,5-dinitrosalicylic acid (JHD, China) a colouring agent and further incubated at 100°C for 5 minutes in a water bath (Uniscope SM801A, Surgifriend Medical, England) to give an orange colouration. The contents in the test tubes were cooled and diluted with 10 mL of distilled water to give a light yellow colouration. The absorbance was read at 540 nm using a UV-Visible spectrophotometer (Labtech, United States). Positive control was prepared using the same procedure except that the (1 mg/mL) plant extracts were replaced with (1 mg/mL) Acarbose (a known inhibitor) at varied concentration of 20- 100 µg/mL. The control samples were prepared using the same procedure except that the plant extract and acarbose were replaced with 1 mL of distilled water. The percentage inhibition was estimated according to the formula shown below [10].

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{540}\text{ (control)} - \text{Abs}_{540}\text{ (extract)}}{\text{Abs}_{540}\text{ (control)}} \times 100$$

The IC_{50} (inhibition concentration at which 50% inhibition of the enzyme activity occurred) values were ascertained from plots of percentage inhibition versus inhibitor concentration and were calculated by non-linear regression analysis from the mean inhibitory values. Acarbose was used as the reference α-amylase inhibitor. All tests were done in triplicate.

***In vitro* enzyme inhibitory activities for alpha glucosidase**

The reaction assay was performed according to the method described by [9]. The enzyme solution used for the assay was 0.5mg/mL concentration (0.5 mg of enzyme in 1 mL of 100mM sodium phosphate buffer). It was prepared by dissolving 100mg (0.1g) of alpha glucosidase (from *Saccharomyces cerevisiae*; Kem Light Lab., Pvt Ltd., India) in 200 ml of 100mM phosphate buffer. Three hundred and twenty microliter (320 µL) of 100 mM phosphate buffer was pipetted into each test tube. Varying concentrations of the plant extract from 20 µg/mL- 100 µg/mL were placed in respective test-tubes. Twenty microliter (20 µL) of 0.5 mg/mL alpha glucosidase enzyme was added to the respective test tubes and left to stand for 10 minutes at room temperature. After incubation, 200µLof ρ-nitrophenyl- alpha-d-glucopyranoside substrate (Santa Cruz Biotechnology, USA) was added. The reaction mixture was thereafter terminated by adding 3mL of 50mM NaoH (Lobachemie, India) and the absorbance was read at 410 nm.

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{410}\text{ (control)} - \text{Abs}_{410}\text{ (extract)}}{\text{Abs}_{410}\text{ (control)}} \times 100$$

The IC_{50} (inhibition concentration at which 50% inhibition of the enzyme activity occurred) values were ascertained from plots of percentage inhibition versus inhibitor concentration and were calculated by non-linear regression analysis from the mean inhibitory values. Acarbose was used as the reference α-glucosidase inhibitor. All tests were done in triplicate.

Qualitative estimation of phytoconstituents

Phytochemical screening was carried out to identify the presence of constituents such as steroids, glycosides, eugenols, terpenoids, alkaloids, saponins, flavonoids, phenolics and tannins in the crude methanol extract and fractions (hexane, dichloromethane and aqueous) of *Ficusexasperata*Vahl leaves by the method described by [11,12,13]

Gas chromatography-Mass spectrometry (GC-MS) analysis of *FicusexasperataVahl*leaves.

GC-MS analysis of the leaf extract and fraction with the highest inhibitory activity against alpha amylase and alpha-glucosidase was determined using Agilent technologies (model number 7890) GC system coupled with a mass spectrometer Agilent technologies (model number 5975). The column (Hp-5ms model) dimensions were 30 m length x 0.32 mm internal diameter x 0.25 µm thickness. The carrier gas used was helium gas with 10 µL of the sample volume injected. The initial oven temperature used for the analysis was 80°C to hold for 2 minutes. At intervals of a minute, 10°C was added to the initial temperature until a final temperature of 240°C was reached and it remained unchanged for 6 minutes. Compounds identification were based on comparison of the mass spectra with the National Institute Standard and Technology (NIST) data base.

Statistical analysis

Results were expressed as mean ± SEM of triplicate determinations. The data were analyzed by one way analysis of variance (ANOVA) followed by Duncan test to determine the level of significance which was expressed at 5% confidence interval ($P \leq 0.05$).

III. Results

Alpha amylase inhibitory activity of *F. exasperata*

Alpha amylase inhibitory activities of crude methanol extract and its fractions (aqueous, dichloromethane and hexane) of *F.exasperata*leaves are shown in figure 1 and table 1. There was a concentration dependent increase in percentage inhibition for dichloromethane, aqueous and hexane fractions at 20-80 µg/mL while at 100 µg/mL there was no change in percentage inhibition for dichloromethane fraction. Though, a reduction that was not statistically different was observed for the aqueous fraction at 100 µg/mL and at 80 µg/mL for hexane fraction.

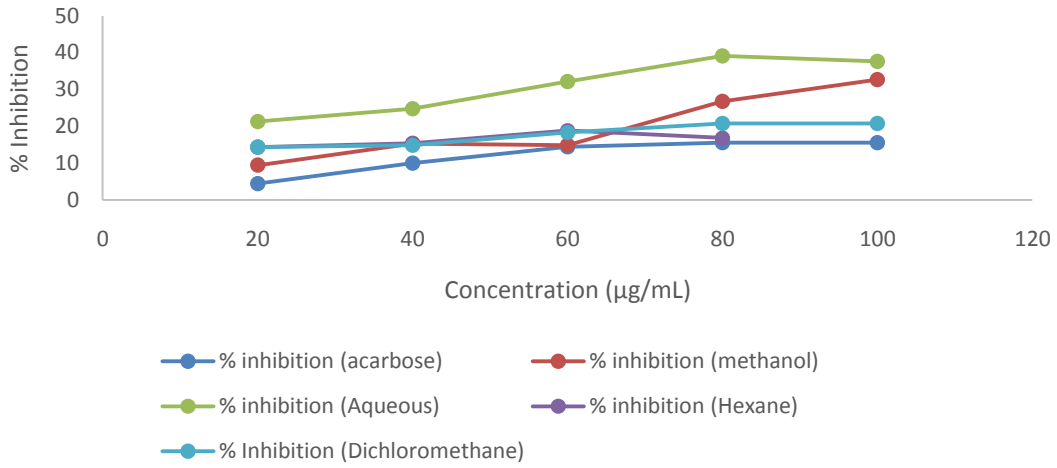

Figure 1: % inhibition of alpha amylase enzyme by *F. exasperata* methanol extract, its fractions: aqueous, dichloromethane, hexane and acarbose (reference alpha amylase inhibitor).

Table 1: Percentage inhibition and IC_{50} values of porcine alpha amylase by crude methanol extract, dichloromethane fraction, aqueous fraction and n-hexane fraction at varying concentrations.

Concentration (µg/mL)	% Inhibition by Crude Methanol extract	IC_{50} (µg/mL) Crude Methanol extract	% Inhibition by Dichloromethane fraction	IC_{50} (µg/mL) Dichloromethane fraction	% Inhibition by Aqueous fraction	IC_{50} (µg/mL) Aqueous fraction	% Inhibition by Hexane fraction	IC_{50} (µg/mL) hexane fraction
20	9.41± 0.50[a]		14.36±0.50[a]		21.29±0.50[a]		14.36±1.49[a]	
40	15.35±1.49[b]	164.31	14.85±0.99[a]	402.32	24.75±1.98[a]	140.86	15.35±0.50[a]	668.75
60	14.85±0.00		18.32±0.50[b]		32.18±3.47		18.81±0.00[b]	

	[b]				[b]			
80	26.73±0.00[c]		20.79±0.99[b]		39.11±1.49 [b]		16.83±0.00[a] [b]	
100	32.67±0.00 [d]		20.79±0.99[b]		37.62±0.00 [b]			

Data are expressed as mean ± SEM. Values with same superscript are not significantly different while those with different superscripts are significantly different at $p \leq 0.05$

Alpha glucosidase inhibitory activity of *F.exasperata*.

Alpha-glucosidase inhibitory activities of crude methanol extract and its fractions (aqueous, dichloromethane and hexane) of *F. exasperate* leaves are shown in figure 2 and table2. Results showed that alpha glucosidase inhibition activities of the extract and fractions of *F.exasperata*leaves revealed a concentration dependent decrease in percentage inhibition especially for crude methanol extract and hexane fraction, while the decrease observed in dichloromethane, aqueous and acarbose inhibitory activities were not concentration dependent. However, it was also revealed that the crude methanol extract had the strongest inhibitory activity as attested to by its lowest half maximal inhibition concentration (IC_{50}) value of 32.47µg/mL, followed by its hexane fraction of 35.10µg/mL and its aqueous and dichloromethane fractions of 94.32µg/mL and 97.81µg/mL respectively.

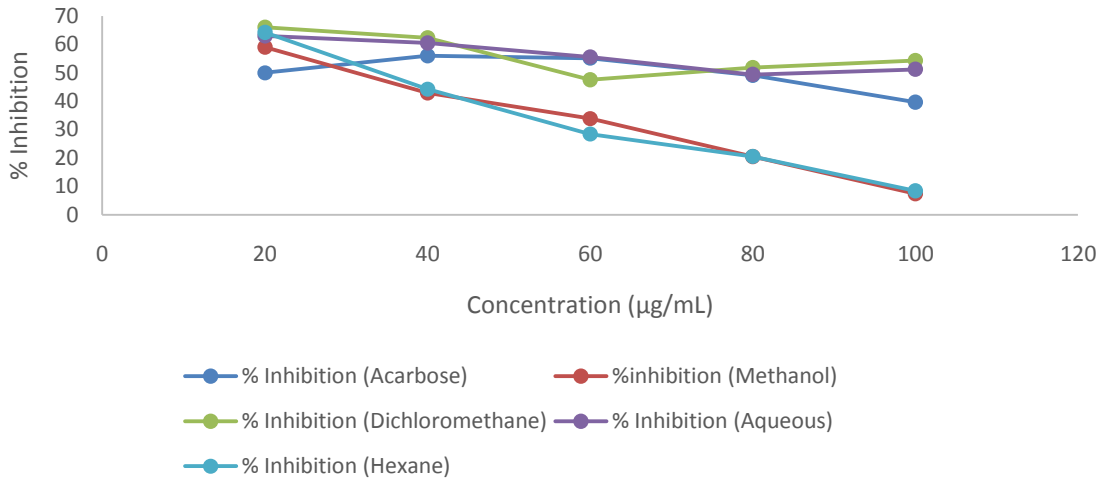

Figure 2: % inhibition of alpha glucosidase enzyme by *F. exasperata* methanol extract, its fractions: aqueous, dichloromethane, hexane and acarbose (reference alpha amylase inhibitor).

Table 2: Percentage inhibition and IC $_{50}$ values of alpha glucosidase by crude methanol extract, dichloromethane fraction, aqueous fraction and n-hexane fraction at varying concentrations.

Concentration (µg/ml)	% Inhibition by crude Methanol extract	IC_{50} (µg/mL) Crude Methanol extract	% Inhibition by Dichloromethane fraction	IC_{50} (µg/mL) Dichloromethane fraction	% Inhibition by Aqueous fraction	IC_{50} (µg/mL) Aqueous fraction	% Inhibition by Hexane fraction	IC_{50} (µg/mL) hexane fraction
20	59.00±0.00[a]		66.05±0.62[a]		62.97±1.24[a]		64.21±0.00[a]	
40	42.86±0.00[b]		62.35±0.62[b]		60.49±0.00[a]		44.21±1.05[b]	
60	33.85±9.31[c]	32.47	47.53±0.62[c]	97.81	55.56±1.24[b]	94.32	28.42±2.10[c]	35.10
80	20.50±0.00[d]		51.85±0.00[d]		49.39±1.24[c]		20.53±1.58[d]	
100	7.45±0.62[e]		54.32±0.00[c]		51.24±0.62[c]		8.42±0.00[e]	

Data are expressed as mean ± SEM. Values with same superscript are not significantly different while those with different superscripts are significantly different at $p \leq 0.05$.

Phytochemical Screening

The phytochemical screening of the extract and fractions denoted the presence of flavonoids, phenolics, alkaloids and saponins, in all the extracts as shown in table 3.

Table 3: Phytochemical evaluation of crude methanolic extract, hexane fraction, dichloromethane fraction and aqueous fraction of FEL

Phytochemicals	Methanol extract	Hexane extract	Dichloromethane extract	Aqueous extract
Glycoside	+	-	+	+
Tannins	-	-	-	-
Flavonoids	+	+	+	+
Phenolics	+	+	++	++
Terpenoids	-	-	-	+
Alkaloids	+	+	+	+
Eugenols	+	-	+	+
Steroids	-	-	-	-
Saponins	++	++	+	++

Key: (+) = Present
(++) = Highly present
(-) = Absent

Gas chromatography-mass spectrometry (GC-MS) analysis of *F. exasperata*

The GC-MS analysis of the aqueous fraction and crude methanol extract of FEL revealed the presence of eight and seven bioactive compounds respectively as shown in Tables 4 and 5 respectively. The retention time (RT), molecular formula (M/F), molecular weight (MW) and relative abundance which was expressed as peak area % of the identified compounds were also shown. Figures 3 and 4 also reflected the chromatogram of the respective fraction and extract.

Table 4: Phytocomponents Identified by GC-MS analysis of aqueous fraction of *F. exasperata*

S/N	Retention Time (min)	Peak Area (%)	Name of Chemical Component	Molecular Formula	Molecular weight
1	11.041	6.58	1,2,3,4-Butanetetrol	$C_4H_{10}O_4$	123
2	13.896	8.42	4-Hydroy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl) cyclohe-2-enone	$C_{13}H_{18}O_3$	222
3	15.509	4.98	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
4	16.293	5.72	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
5	18.038	12.75	9,12-octadecadienoic acid(z,z)-methyl ester	$C_{19}H_{36}O_2$	294
6	18.147	14.64	9-octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296
7	18.594	6.11	Methyl stearate	$C_{19}H_{38}O_2$	298
8	19.000	36.81	9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	280

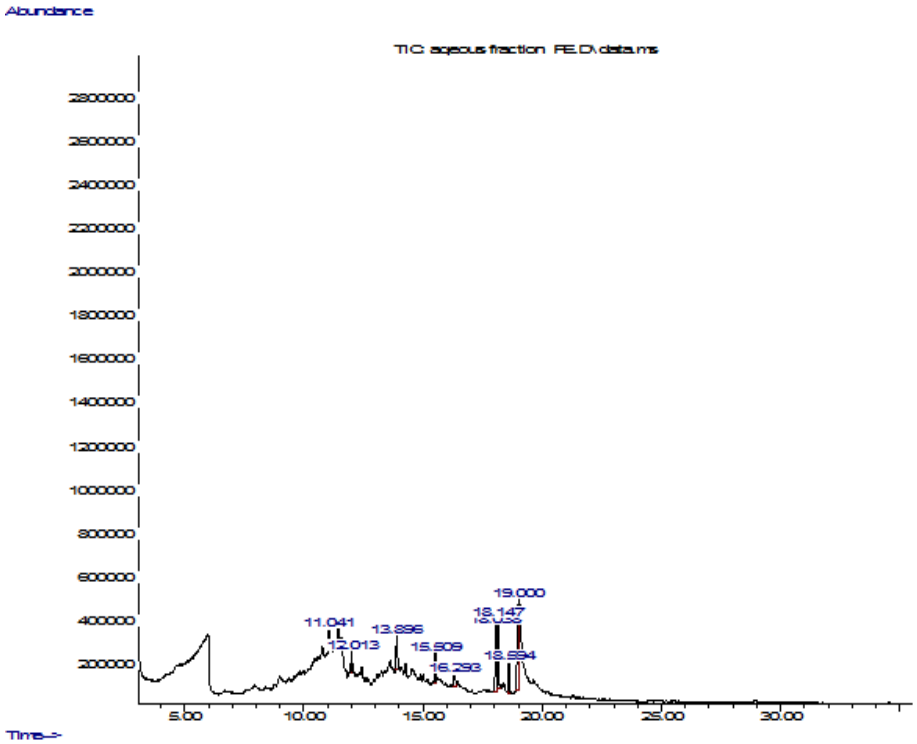

Figure 3: The GC-MS Analysis of aqueous fraction of *Ficusexasperata* leaves.

Table 5: Phytocomponents Identified by GC-MS analysis of crude methanolic extract of *F. exasperata*leaves

S/N	Retention time(min)	Peak Area (%)	Name of compounds	Molecular formula	Molecular Weight
1	14.313	1.44	Biocyclo(3.1.1)heptane-2,3-diol, 2,6,6-trimethyl	$C_{10}H_{18}O_2$	170
2	15.521	13.93	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
3	18.084	33.14	9,12-Octadecadienoic acid(z,z)- methyl ester	$C_{19}H_{34}O_2$	294
4	18.204	31.43	9-octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296
5	18.410	6.06	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292
6	18.616	12.95	Methyl stearate	$C_{19}H_{38}O_2$	298
7	19.663	1.05	Methyl-10-trans,12-cis-octadecadienoate	$C_{19}H_{34}O_2$	294

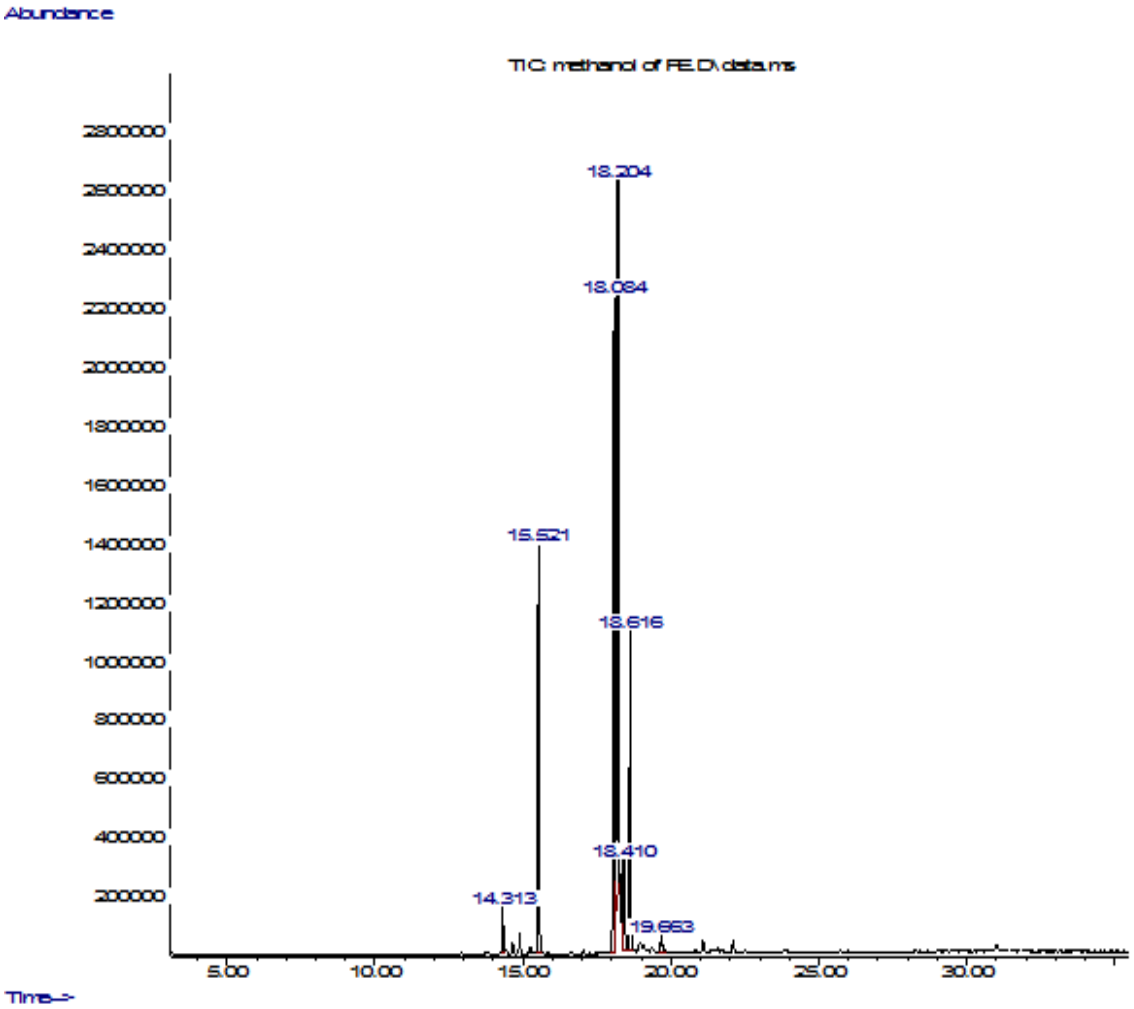

Figure 4: The GC-MS Analysis of crude methanol extract of *Ficusexasperata*leaves

IV. Discussion

The alpha amylase inhibitory activities of the crude methanol extract reflected a concentration dependent increase in percentage inhibition from 20-100 µg/mL except at 60 µg/mL, where a reduction that was not statistically different was observed. The concentration dependent increase observed in this extract is almost similar to that observed in the aqueous fraction of *Salaciaoblonga*[14]. The percentage inhibition is indirectly proportional to inhibitory concentration at 50 %. Therefore it was observed that aqueous fraction had the most effective anti-alpha amylase activity (140.86 µg/mL), the crude methanol extract had an appreciable inhibition (164.31 µg/mL) while the dichloromethane (402.32 µg/mL) and hexane fractions (668.75 µg/mL) had least inhibitory activities. The graph in figure 1 depicted plot of percent alpha amylase inhibition of the plant extract and fractions as a function of concentration in comparison with acarbose (reference drug).

The alpha glucosidase inhibitory activities of the crude methanol extract and hexane fraction showed a low IC_{50} values of 32.47µg/mL and 35.10 µg/mL respectively which could be as a result of the concentration dependent decrease that ranged from 59.00±0.00 to 7.45±0.62% and 64.21± 0.00 to 8.42±0.00% respectively,

when compared to the dichloromethane fraction, aqueous fraction and acarbose (positive control) whose inhibitory activities were not concentration dependent.

Glycosides and eugenols were absent in hexane fraction but present in the other fractions and extract. Hence, the excellent alpha amylase inhibitory activity (140.86 µg/mL) observed in the aqueous fraction could be due to the high amount of phenolics and presence of terpenoids found in the fraction. Studies have reported that phenolics exhibit a wide range of therapeutic activities against diseases such as diabetes, partly due to their antioxidant properties [15] which helps in the neutralization of free radicals and decreased oxidative stress. Decreased oxidative stress in pancreatic cells aid proliferation of beta cells of the pancreas, which result in increased insulin secretion and consequent decrease in blood glucose level due to glucose uptake into the extra hepatic tissues [16].The presence of terpenoids could also be attributed to the alpha amylase inhibitory potential of the aqueous fraction, as terpenoids have been implicated in the treatment of diabetes and diabetic complications via several biological activities on glucose absorption, glucose uptake and insulin secretion [17]. Also, terpenoids like oleanolic and ursolic acid have been known to inhibit pancreatic alpha amylase [18] while oleanic acid and maslinic acid are known to exert their anti-diabetic properties via inhibition and gene expression, reduction of carbohydrate hydrolyzing enzymes like alpha amylase and alpha glucosidase. [17]. The alpha glucosidase inhibitory properties of the crude methanol extract could be due to the synergistic activities of glycosides, flavonoids, phenolics, alkaloids, eugenols and saponins present in the crude methanol extract, as a study has reported that eugenol can decrease blood glucose levels by inhibiting alpha glucosidase[19].

The GC-MS analysis of the fractions revealed that **t**he highest alpha amylase inhibitory properties observed in the aqueous fraction of *F. exasperata*leaves could be largely due to the relative abundance of n-Hexadecanoic acid (5.72%) and 9,12-octadecadienoic acid (36.81%). These compounds present in the aqueous fraction of this study have been known to be potent anti-diabetic agents [14,20], due to their antioxidant properties as reported by [21,22,23]. The highest alpha glucosidase inhibitory potential ascertained in the crude methanol extract of *F. exasperata* leaves could be due to the relative abundance of 9,12-Octadecadienoic acid(z,z)-methyl ester (33.14%), 9-octadecenoic acid, methyl ester (31.43%), Methyl stearate (also known as octadecanoic acid, methyl ester or stearic acid, methyl ester) (12.95%) and Hexadecanoic acid, methyl ester (13.93%). These compounds are known to possess alpha glucosidase inhibitory activities [20, 24,25 ,26] alongside their other biological activities.

V. Conclusion

This study shows that the aqueous fraction and crude methanol extract of *Ficusexaperata* leaves were the most potent in alpha amylase and alpha glucosidase inhibition respectively due to their low IC_{50} enzyme inhibitory values. Phytochemical evaluation of the fractions and extract revealed the presence of flavonoids, eugenols, alkaloids, glycosides, phenolics and high amount of saponins in both the aqueous fraction and crude methanol extract. The GC-MS analysis of these samples revealed the presence of bioactive compounds like 9,12-Octadecadienoic acid(z,z)- methyl ester; 9-Octadecenoic acid methyl ester; Hexadecanoic acid, methyl ester and Methyl stearate which have proven to be effective anti-diabetic agents. Hence, this study validates the scientific and traditional use of *Ficusexasperata* in the management of diabetes mellitus. In addition, its ability to effectively inhibit alpha amylase and alpha glucosidase enzymes is one of its mechanisms of action.

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