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Antidiabetic and antioxidant properties of *Fagara zanthoxyloides* root-bark ethyl acetate fraction: in-vitro enzyme inhibition and GC-MS analysis

Christian Amah[1]*, Parker Joshua[2], Ikechukwu Okoro[3], Favour Iyidiegwu[2], Chibuike Brendan[4], Linus Nsude[5], Ugochukwu Nweke[6]

[1]Department of Biochemistry, Faculty of Natural and Applied Sciences, State University of Medical and Applied Sciences, Nsukka 412111, Nigeria. [2]Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka 410001, Nigeria. [3]Department of Medical Biochemistry, College of Health Sciences, David Umahi Federal University of Health Sciences, Uburue, Uburu 491105, Nigeria. [4]Department of Chemical Sciences, Faculty of Natural and Applied Sciences, Spiritan University Nneochi, Nneato Road, Leru 441111, Nigeria. [5]Department of Genetics and Biotechnology, Faculty of Natural and Applied Sciences, State University of Medical and Applied Sciences Igbo-Eno, Nsukka 412111,Nigeria. [6]Department of Pharmacognosy and Natural Medicine, University of Uyo, Uyo 520003, Nigeria.

***Corresponding to:** Christian Amah, Department of Biochemistry, State University of Medical and Applied Sciences, Lgbo-Eno, Nsukka 412111, Nigeria. E-mail: amachrist28@gmail.com.

Author contributions

Amah C was responsible for obtaining funding, developing the methodology, conducting the research, writing the first draft of the manuscript, and handling peer review during the analysis and interpretation of the data. Joshua P contributed to the manuscript's conceptualization, provided supervision, and participated in manuscript editing. Okoro I and Brendan C contributed to the methodology, wrote the original draft, edited the manuscript, and were involved in analyzing and interpreting the data. Iyidiegwu F, Nsude L, and Nweke U focused on editing the manuscript. All authors critically revised and finalized the manuscript for publication.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

EAFFZRB, ethyl acetate fraction of *Fagara zanthoxyloides* root-bark; DPPH, 2-diphenyl-1-picrylhydrazl; FRAP, ferric reducing antioxidant power; GC-MS, Chromatography-Mass Spectometry; ROS, reactive oxygen species; TAC, total antioxidant capacity.

Citation

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Abstract

The application of therapeutic herbs in treatment of common infections and diseases has gained popularity in developing nations due to its cost-effectiveness and purported lower side effects compared to synthetic drugs. *Fagara zanthoxyloides* Lam also known as *Zanthoxylum zanthoxyloides*, is an indigenous plant used widely as chewing stick for tooth cleaning in West Africa. The root-bark macerations, decoctions or infusions are the most valuable organs widely taken to treat malaria and diabetes by local populations, traditional healers and local pharmaceutical firms. Searching alternative, more efficient, safer, and less expensive anti-diabetic natural products from plants has received great attention, hence this research delved into the inhibitory effects of the ethyl acetate fraction of *F. zanthoxyloides* root-bark (EAFFZRB) on in-vitro α-amylase and α-glucosidase enzymes. Standard biochemical methods were used to extract and assess the in-vitro antioxidant such as 2, 2-diphenyl-1-picrylhydrazl (DPPH), total antioxidant capacity (TAC) and Ferric Reducing Antioxidant Power (FRAP). The sample was combined at different concentrations (10, 20, 40, 80, 160, 320, and 640 µl/dl). The α-amylase, and α-glucosidase activities of EAFFZRB sample was combined at different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL). The phyto-constituents in EAFFZRB were identified using Gas Chromatography-Mass Spectrometry (GC-MS) technique. The plant fraction showed a significant ($P < 0.05$) increase in the inhibition of α-amylase IC_{50}= 0.726 and α-glucosidase IC_{50} = 0.877 at varying concentrations, comparable to the reference drug Acarbose IC_{50} = 0.728 and IC_{50} = 0.852 respectively. EAFFZRB demonstrated significant ($P < 0.05$) inhibition at different concentrations on DPPH (66.55 ± 1.57), TAC (0.47 ± 0.17) and FRAP (3.64± 0.14), similar to the effect of the standard ascorbic acid. The GC-MS analysis of EAFFZRB revealed a total of 12 volatile organic compounds such as; Phenol-3-methoxy-2,5,6-trimethyl, 2-Hydroxy-5- nitrobenzylbromide, 2,6-Dimethyl-4-(3-nitrophenyl)pyridine, 1H-Benz[e] indene,2-methyl and others. The study confirmed the in-vitro anti-diabetic and anti-oxidant properties of EAFFZRB, supporting the traditional use of the plant which might have acted additively or in synergy towards potentiating of antidiabetic effect observed, and it could be beneficial to the pharmaceutical industry if utilized.

Keywords: *Fagara zanthoxyloides*; α-amylase and α-glucosidase; anti-oxidant; GC-MS

Background

Ongoing research is necessary to discover effective and affordable treatments for a broad range of human illnesses. Plants offer a diverse array of healing compounds and show great potential for developing new medications [1]. Natural antioxidants are essential for preventing or treating conditions caused by free radicals. These highly reactive chemicals can harm cells, organelles, deoxyribonucleic acid, and other bio-molecules, leading to diseases like cancer, cardiovascular issues, and neurodegenerative disorders [2].

Antioxidant molecules possess the ability to suppress or halt free radical processes, which can delay or prevent cellular damage. Antioxidants are found in both the extracellular and intracellular regions, existing in enzymatic and non-enzymatic forms [3]. Enzyme-based antioxidants break down and eliminate free radicals through a multi-step process. With the assistance of cofactors such as copper, zinc, manganese, and iron, these antioxidant enzymes first convert harmful oxidative products into H_2O_2 and then into water. Non-enzymatic antioxidants interrupt the chain reactions that generate free radicals. Reactive oxygen species (ROS) and reactive nitrogen species are produced by various activities, including normal metabolic processes, increased environmental exposure, and higher levels of food xenobiotics. ROS and reactive nitrogen species contribute to oxidative stress in several pathophysiological conditions [3]. Oxidative stress causes changes in the body's cellular components, leading to various health issues. Antioxidants can effectively combat oxidative stress by enhancing cellular defenses. Regular metabolism generates ROS such as superoxide radicals ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) [4, 5].

Diabetes mellitus manifests through symptoms such as high blood sugar, abnormal lipid levels, and oxidative stress, making it the most prevalent endocrine disorder [6,7]. It affects the eyes, skin, kidneys, nerves, and blood vessels, leaving afflicted people susceptible to long-term effects [8].Diabetes mellitus also leads to various other complications, notably diabetic ketoacidosis resulting from excessive ketone body production, heightened susceptibility to extremity-related infections, increased cardiovascular disorder risk, electrolyte balance disturbances, retinopathy, and other issues like skin abscesses, weight loss, weakness, and debilitation [9].

Inhibiting α-amylase and α-glucosidase to lower intestinal glucose reabsorption is one of the treatment objectives presently being investigated for type 2-diabetes mellitus.Alpha-amylase (α-1,4-glucan-4-glucanohydrolases) is produced by the salivary gland and pancreas and is essential for the breakdown of complex carbohydrates into different sugars in the intestinal mucosa. Afterwards, these sugars are converted into monosaccharides and taken up by the circulation. There's a higher incidence of postprandial hyperglycemia due to the quick breakdown of dietary carbohydrate. Controlling human pancreatic alpha-amylase activity in the small intestine is crucial for the management of type-2 diabetes since studies have linked it to greater postprandial glucose levels [10]. Inhibitors of pancreatic α-amylase cause a reduction in the rate of glucose absorption and delay the digestion of carbohydrates, which lowers postprandial serum glucose levels.The side effects associated with α-amylase and α-glucosidase inhibitors, such as hypoglycemia, diarrhea, flatulence, and colon bloating, limit their utility in the treatment of diabetes and its complications [11].

Diabetes-related complications are frequently caused by decreased viability and functionality of pancreatic β-cells [12]. Because of their diverse actions on islet β-cells, flavonoids have been linked in numerous studies to possible diabetes management agents [13]. Polyphenolic antioxidant phytochemical substances such as luteolin, rutin, quercetin, apigenin, cinnamic acid, and catechin were found in the ethyl acetate fraction of *F. zanthoxyloides* root-bark, as demonstrated by the high performance liquid chromatography fingerprint analysis conducted by Amah et al [14]. Finding novel treatments for diabetes that have fewer or no side effects is critical right now. Although insulin and other artificially synthesized anti-diabetes drugs like Acarbose, metformin, Glibenclamide and voglibose are effective for the management of diabetes, they are far from satisfying the urgency for their enormous price costs or undesirable side effects. Plant-based medicines are gaining more attention for the treatment of sickness because they are more affordable, more tolerant, and have fewer side effects than conventional anti-diabetic drugs [15, 16].

F. zanthoxyloides Lam, also known as *Zanthoxylum zanthoxyloides*, is a native plant widely used as a teeth-cleaning chewing stick in West Africa [17]. Numerous studies have documented its various effects, including anti-malarial activity, anti-inflammatory properties, antimicrobial action, and anticancer potential [18–24].

Ethnopharmacological investigations have been achieved on this plant (Figure 1) and several active compounds such as vanillic acid and hydroxy-2-methyl-benzoic acid and derivatives were isolated from the leaf, stems-bark and roots-bark extracts [25].The root-bark is the most valuable organs used by local populations, traditional healers and local pharmaceutical firms. Root or stem-bark macerations, decoctions or infusions are widely taken to treat malaria, diabetes, fever, sickle cell anaemia and general body weakness [26].

The stem-bark and root-bark contain alkaloids of the benzophenanthridine, furoquinoline and aporphine types. From the stem-bark are fagaronine and chelerythrine [27]. Root-bark was isolated from fagaronine, dihydroavicin, chelerythrin, oxychelerythrin (benzophenanthridines), skimmianin and 8-methoxydictamine (furoquinolines), as well as magnoflorine, berberine, tembetarin and N-methyl-corydine (aporphines). These compounds as an antioxidant enhances regeneration or revitalization of damaged pancreatic beta cells, and protecting against further damage, enhancing insulin synthesis and secretion from the beta-cells, decreasing glucose absorption from gastro-intestinal system, increasing insulin sensitivity of the tissues, possessing of insulin mimicking effects, and changing the activity of some enzymes involved in glucose metabolism.

Although many ethnobotanical investigations were performed on the anti-diabetic plants in African countries and beyond [28–31]. It should be noted that few studies have been carried out on those used in Nigeria. Aloke et al. and Amah et al. reported in vivo antidiabetic of *F. zanthoxyloides* root-bark in animal model [14, 32]. From the report, Amah et al. conducted a pilot study on in vivo experiment and found that ethyl acetate fraction of the *F. zanthoxyloides* root-bark extract gave higher activity in glucose reduction [14].

This study was motivated by the lack of data on the in vitro effects of *F. zanthoxyloides* extracts on α-amylase and α-glucosidase, as well as its antioxidant properties. Our findings demonstrate that the ethyl acetate fraction of *F. zanthoxyloides* root bark has significant in vitro antidiabetic effects. These results support the traditional use of this plant in managing diabetes and provide valuable information for selecting effective complementary treatments.

Methods

Plant materials

The identity of the root-bark was validated and certified by Mr. Alfred Ozioko, a taxonomist at the Research Center of the International Center for Economic and Drug Development (InterCEDD) in Nsukka, Enugu State. Also, the plant sample was placed in the herbarium for future use and issued Voucher No. InterCEDD/901 [14]. Additionally, the identity and legitimacy of the plant sample were verified by cross-referencing it with the records stored in the databases of http://www.theplantlist.org and http://www.ipni.org.

Ethical consideration

The Institutional Ethics and Biosafety Committee, Faculty of Biological Sciences, University of Nigeria, Nsukka (Reference No. UNN/FBS/EC/1057) approved this study.

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Figure 1 (A) Section of *F.zanthoxyloides* tree. (B) Shed-dried root-bark of *F. zanthoxyloides*

Extraction procedure

Fresh *F. zanthoxyloides* root-barks were collected and cleaned. After being cut into slices, the plant components were dried in the shade, with regular turns to avoid deterioration. A mechanical grinder was used to grind the dried root bark into a powder. In a maceration flask, a prescribed quantity of powdered root bark (3,500 g) was soaked in 10 liters (4 bottles) of pure ethanol. After being stirred occasionally for 72 h, the mixture was filtered through a muslin cloth into a flask with a flat bottom. Whatman No. 1 filter paper was used for additional filtration in order to get rid of any fine residues. After that, the filtrate was concentrated at 45 ˚C in a rotary evaporator to get the plant's crude ethanol extract. In order of increasing polarity, the crude extract was fractionated using n-Hexane first, ethyl acetate second, and ethanol third. This involves the use of coloum chromatography and slica gel pore size (200–400 mesh). The extract was mixed thoroughly with the silca gel; the mixture was packed in a coloum clamped in a retort stand. The slical gel and extract serves as stationary phase while the solvents were the mobile phase to elute the sample (n-Hexane, ethyl acetate and ethanol fractions). The fractions were subjected to a high vacuum and rotatory evaporation until they were completely dried. The fraction that exhibited the highest activity in glucose reduction was sent for gas chromatography-mass spectrometry analysis [14].

Equipment

The Department of Biochemistry and several scientific shops in Nsukka supplied the equipment. Among them are the following: Jenway Spectrophotometer (E312 Model) (Staffordshire, UK), Colorimeter EI Scientific Co. (Delhi, India), Measuring Cylinder, Pyrex (Darmstadt, Germany), Weighing Balance, Vickas Ltd (Darmstadt, Germany), Centrifuge, Vickas Ltd (Darmstadt, Germany), Refrigerator, Thermocool (London, England), Oven (Gallenkamp, Germany), Water Bath (Darmstadt, Germany), Mechanical Grinder, Vickas Ltd (Darmstadt, Germany), Rotary evaporator, Vickas Ltd (London, England), Muslin cloth, and Flat Bottomed flask Pyrex (Darmstadt, Germany). Agilent 6,890 gas chromatograph (Los Angeles, CA, USA, including an HP 88 capillary column with a film thickness of 100 m × 0.25 µm, a flame ionization detector, and an automatic injector on the column.

Reagents materials

The investigation employed analytical grade chemicals and were obtained from the following sources: May and Baker (London, England), Qualikems (Delhi, India), Qualikems (London, England); Fluka (Darmstadt, Germany), and Sigma-Aldrich, Inc. (St. Louis, MO, USA). Commercial kits and products from Teco (TC) and Randox Laboratories-US, Ltd. (Kearneysville, WV, USA) were used as assay reagents.

In-vitro alpha-amylase inhibition assay

Alpha-amylase inhibition assay was carried out according to Kwon et al. [33]. Soluble starch (substrate) and porcine pancreatic α-amylase (EC 3.2.1.1) were used in this experiment. The porcine pancreas produces α-amylase is a single polypeptide chain, and it has been demonstrated in previous research that although α-amylase from the porcine pancreas is a monomeric enzyme, it undergoes some proteolysis to form smaller associated fragments that maintain their activity [34, 35]. The primary role of pancreatic α-amylase is to break down starch in the body. In humans, the process of starch digestion begins with salivary amylase in the mouth. Starch is broken down into oligomers, which are then further broken down by pancreatic α-amylase into maltose, maltotriose, and low-molecular-weight maltooligosaccharides in the small intestine. The plant fraction and the standard Acarbose at concentration (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) in various dilutions and 500 µl of 0.5 mg/mL α-amylase solution were incubated for 10 min at 37 ˚C in a 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl). A 0.02 M sodium phosphate buffer containing 500 microliters of a 1% starch solution was then added. A 15min incubation period at 37 ˚C was followed by the addition of 1.0 mL of DNSA color reagent, which is a solution of 1%, 3, 5-dinitrosalicylic acid, and 12% sodium potassium tartrate in 0.4 M NaOH, to terminate the reaction. After being heated to a boiling temperature for 5 min, the reaction mixture was allowed to cool before being diluted with 10 mL of deionized water. At 540 nm, the absorbance was measured. The percentage α-amylase inhibition was computed using the mean absorbance of three separate tests, as stated below:

$$\text{Alpha amylase inhibition (\%)} = \frac{\text{A540 control} - \text{A540 sample}}{\text{A540 control}} \times 100$$

In vitro alpha-glucosidase inhibition assay

The alpha-glucosidase inhibitory activity was tested using the method described by Kim et al., using para-nitrophenylglucopyranoside (PNPG) as the substrate and Bacillus stearothermophillus α-glucosidase (EC 3.2.1.20) as the enzyme. In brief, 15 min were spent treating five units of aliquot of α-glucosidase with plant fraction and Acarbose at concentration (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) [10]. As a substrate, 20 mM phosphate buffer containing 3 mM PNPG was added in order to start the hydrolytic reaction at pH 6.9. To terminate the hydrolytic reaction, 2 milliliters of 0.1 M Na_2CO_3 were added after it had run for 20 min at 37 ˚C. The absorbance of the yellow p-nitrophenol generated during the hydrolysis of PNPG was measured at 400 nm.Each test was run in triplicate, and the percentage of α-glucosidase was determined using the mean absorbance as follows:

$$\text{Alpha glucosidase inhibition (\%)} = \frac{\text{A400 control} - \text{A400 sample}}{\text{A400 control}} \times 100$$

In-vitro antioxidant activity

Determination of 2,2-diphenyl-1-picrylhydrazl scavenging activity

To assess the plants ability to scavenge the radicals 2, 2-diphenyl-1-picrylhydrazl (DPPH), the Brand-Williams et al. methodology was employed. Spectrophotometrically, the stable free radical DPPH can be found at 517 nm in a purple hue [36]. A colorless substance known as 1, 1-diphenyl-2-picryl hydrazine is created when antioxidants degrade DPPH. The ethanol solution containing 0.1 mM DPPH was made. Three milliliters of this solution were added to the sample, with concentrations of 10, 20, 40, 80, 160, 320 µl/dl. Because ascorbic acid has a limited metal-chelating ability at varying concentrations and a strong reducing capacity at different concentrations, it was chosen as the reference compound in this experiment. A spectrophotometer (UV-VIS Shimadzu UV 2450) was used to measure the absorbance at 517 nm after the mixture was well shaken and allowed to remain at room temperature in the dark for 30 min. Using various ascorbic acid concentrations, a concentration vs. % inhibition linear graph was generated.

We utilized the following formula to determine the percentage DPPH scavenging effect:

$$\%\ \text{Inhibition} = \frac{\text{Blank} - \text{Extract or standard}}{\text{Blank}} \times 100$$

Where the absorbance in the absence of the sample was indicated by Extract or Standard, and Blank represented the absorbance of the control sample.

Ferric reducing antioxidant power assay (FRAP)

The antioxidant capacity of the plant fraction that reduces ferric iron was ascertained using the modified method of Oyaizu [37]. It first reacts with ferric chloride to produce potassium ferrocyanide, which subsequently mixes with other substances that can lower energy to form ferric-ferrous complex, which peaks in absorbance at 700 nm. The sample was combined at different concentrations (10, 20, 40, 80, 160, 320, and 640 µl/dl) with one milliliter of potassium ferricyanide (1% by volume) and one milliliter of sodium phosphate buffer (pH 6.6). For 20 min, the mixture was incubated at 50 ˚C. One milliliter of 10% w/v trichloroacetic acid was added, and the mixture was centrifuged for 10 min at 3000 rpm. A UV-VIS Shimadzu UV 245031-32 was used to test the absorbance at 700 nm. 1.5 milliliters of deionized water, 0.1 milliliters of 0.1% ferric chloride, and 1.5 milliliters of the upper layer were added. We experimented with the combination for 10 min. Increased reducing power is indicated by higher absorption. The data are shown as the mean value ± standard deviation, and the assays were performed in triplicate. By comparing the sample concentration to the absorbance at 700 nm, one may determine the sample concentration that produced 0.5 of absorbance (IC_{50}). The standard was ascorbic acid.

$$\text{Increase in reducing power}\ (\%) = \frac{\text{Abstest} - \text{Abs blank}}{\text{Abs blank}} \times 100$$

Where absorbance of blank is represented by Abs blank and absorbance of test solution by Abs test. In this case, the reduction of Fe $[(CN)_6]_3$ to $Fe[(CN)_6]_2$ results in the creation of an intense Perl's Prussian blue complex, which, when the reducing power is increased, yields significant absorbance at 700 nm.

Total antioxidant capacity (TAC) by phosphomolybdate assay

The method established by Prieto et al. was used to evaluate the sample's overall antioxidant capacity [38]. Three milliliters of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were mixed with 0.3 milliliters of the plant sample at concentration (10, 20, 40, 80, 160, 320, and 640 µl/dl). For 90 min, the reaction solution-containing tubes were incubated at 95°C. Once the combination had cooled to room temperature, its absorbance at 695 nm was measured using a UV-VIS spectrophotometer and compared to a blank. 0.3 mL of alcohol was used as the blank instead of the sample. The quantity of gram equivalents of ascorbic acid represents the total antioxidant activity. The calibration curve was created by mixing ascorbic acid with alcohol.

GC-MS analysis

The GC-MS analysis of the EEFZRB was carried out using a GC-MS-QP2010 (Shimadzu, Japan) outfitted with an AOC-20s headspace sampler and an AOC-20i autoinjector with an MS analysis capillary column (30 mm length × 0.25 mm diameter and 0.25 µm film thickness). The injector was operating in split injection mode at 250 ˚C. This was the temperature program that ran: 80 ˚C for 3 min, and then 10 ˚C per minute until 280 ˚C. Pure (99.999%) helium (40.5 cm/s linear velocity) was utilized as the carrier gas, and a constant column flow of 1.21 mL/min (a total flow of 16.3 mL/min) was employed. Before analyte molecules are eluted into the mass spectrometer (MS) for detection, the sample mixture is separated by gas chromatography (GC). The carrier gas, flowing continuously through the GC and into the MS, transports the separated compounds, which are then detected by the MS. While GC and other detection methods provide peak intensity and retention time, mass spectrometry adds mass data, enhancing the identification and quantification of molecules. The ions are analyzed in the MS detector to determine their relative abundance and mass-to-charge ratio (m/z). The resulting mass spectrum displays the signal strength corresponding to each fragment's m/z ratio, facilitating chemical identification through the unique "fingerprint" of the molecule.

Statistical analysis

Using the Statistical Product and Service Solution (SPSS) version 20.0, the collected data were analyzed usingone-way analyses of variance (ANOVA). The findings were shown as Mean ± SD. Mean values with $P < 0.05$ were considered significant. The mean values were separated using Post Hoc Tests and Homogeneous subsets (Duncan).

Results

In-vitro percentage inhibition at different concentration of the EAFFZRB and Acarbose on alpha amylase enzyme

The result of the Table 1 showed a significant ($P < 0.05$) different in the percentage inhibition of EAFFZRB at concentrations dependent manner, as the concentration increases, the percentage inhibition increases. This means that the inhibition on the EAFFZRB increases with the increase in the concentrations having maximal effect at 1.0 mg/mL and the result was in line with the standard Acarbose used as reference.

Table 1 In-vitropercentage inhibition at different concentration of the EAFFZRB and Acarbose on alpha amylase enzyme

Concentrations (mg/mL)	EAFFZRB (%)	Acarbose (%)
0.2	144.89 ± 0.82 ^{e}	57.71 ± 0.47 ^{e}
0.4	55.24 ± 4.26 ^{d}	62.34 ± 0.86 ^{d}
0.6	56.60 ± 2.12 ^{c}	71.60 ± 0.70 ^{c}
0.8	63.50 ± 1.03 ^{b}	79.12 ± 0.80 ^{b}
1.0 (IC_{50})	67.43 ± 0.90 ^{a} (R^2 = 0.726)	82.04 ± 1.35 ^{a} (R^2 = 0.728)

The findings are presented as Means ± SD (n = 3). Mean values with different letters as superdcripts down the column are considered significantly different at $P < 0.05$, while mean values with the same letters as superscripts down column are considered non-significant at $P < 0.05$.

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In-vitro percentage inhibition at different concentration of the EAFFZRB and Acarbose on alpha glucosidase enzyme

The result of the Table 2 had a significant ($P < 0.05$) different in the percentage inhibition of EAFFZRB at concentrations dependent manner. The EAFFZRB on the concentrations showed a preference/respect on alpha glucosidase. This result is comparable to the standard Acarbose having maximal effect at 1.0 mg/mL.

Percentage inhibition of DPPH scavenging activities on the EAFFZRB against standard ascorbic acid

The DDPH result of EAFFZRB indicates a non significant ($P > 0.05$) increases in the inhibition at concentrations of 10 and 640 µl/dl compared to other concentrations Table 3. Meanwhile, standard ascorbic acid revealed a significant ($P > 0.05$) increases in the inhibition at 10, 20 and 80 µl/dl compared to other concentration. Though the EAFFZRB and ascorbic acid did not follow concentration dependent manner, they exhibits maximal inhibition at (10 and 640 µl/dl) and (200 and 80 µl/dl) respectively.

Effect of TAC scavenging activities on the EAFFZRB against standard ascorbic acid

The TAC result of EAFFZRB was high at 160 µl/dl concentration when compared to other concentrations Table 4. The EAFFZRB showed a significant ($P < 0.05$) decreases in the inhibition at non concentrations dependent at 20, 40, 80, 320 and 640 µl/dl compared to the concentration at 160 µl/dl. The standard ascorbic acid revealed a significant ($P > 0.05$) increases in the inhibition at 10, 20 and 80 µl/dl compared to other concentration.

Effect of FRAP scavenging activities on the EAFFZRB against standard ascorbic acid

The FRAP result of EAFFZRB was observed to be significantly ($P < 0.05$) higher in the inhibition at concentrations of 10 and 80 µl/dl compared to other concentrations Table 5. Non significant ($P > 0.05$) different was observed at 20, 40, 160, 320 and 640 µl/dl. This is comparable to the standard ascorbic acid used which had a significant ($P > 0.05$) increases in the inhibition at 10, 20 and 80 µl/dl compared to other concentration.

Table 2 In-vitro percentage inhibition at different concentration of the EAFFZRB and Acarbose on alpha glucosidase enzyme

Concentrations (mg/mL)	EAFFZRB	Acarbose
0.2	43.69 ± 4.47 ^{e}	45.41 ± 0.92 ^{e}
0.4	46.38 ± 2.42 ^{d}	62.23 ± 3.74 ^{d}
0.6	49.33 ± 1.78 ^{c}	73.24 ± 1.40 ^{c}
0.8	71.77 ±2.88 ^{b}	80.26 ± 1.38 ^{b}
1.0 (IC_{50})	83.48± 1.76 ^{a} (R^2 = 0.877)	87.82 ± 2.39 ^{a} (R^2 = 0.852)

The findings are presented as Means ± SD (n = 3). Mean values with different letters as superdcripts down the column are considered significantly different at $P < 0.05$, while mean values with the same letters as superscripts down column are considered non-significant at $P < 0.05$.

Table 3 DPPH scavenging effect of the EAFFZRB

Concentrations (µl/dl)	EAFFZRB (%)	Ascorbic Acid (%)
10	166.57 ± 1.35^{a}	92.60 ± 1.78 ^{b}
20	61.23 ± 1.49 ^{d}	93.20 ± 2.49 ^{b}
40	64.89 ± 0.88 ^{b}	87.49 ± 11.47 ^{c}
80	67.83 ± 0.65 ^{c}	93.15 ± 1.63 ^{b}
160	62.73 ± 0.75 ^{d}	90.43 ± 0.63 ^{a}
320	64.66 ± 0.88 ^{b}	90.94 ± 0.40 ^{a}
640 (IC_{50})	66.55 ± 1.57 ^{a} (R^2 = 0.367)	90.33 ± 1.20 ^{a} (R^2 = 0.361)

The findings are presented as Means ± SD (n = 3). Mean values with different letters as superdcripts down the column are considered significantly different at $P < 0.05$, while mean values with the same letters as superscripts down column are considered non-significant at $P < 0.05$.

Table 4 TAC scavenging effect of the EAFFZRB

Concentrations (µl/dl)	EAFFZRB (%)	AscorbicAcid (%)
10	0.25 ± 0.02 ^{c}	92.60 ± 1.78 ^{b}
20	0.30 ± 0.02 ^{a}	93.20 ± 2.49 ^{b}
40	0.39 ± 0.01 ^{a}	87.49 ± 11.47 ^{c}
80	0.32 ± 0.00 ^{a}	93.15 ± 1.63 ^{b}
160	0.47 ± 0.17 ^{b}	90.43 ± 0.63 ^{a}
320	0.39 ± 0.04 ^{a}	90.94 ± 0.40 ^{a}
640 (IC_{50})	0.36 ± 0.01 ^{a} (R^2 = 0.687)	90.33 ± 1.20 ^{a} (R^2 = 0.361)

The findings are presented as Means ± SD (n = 3). Mean values with different letters as superdcripts down the column are considered significantly different at $P < 0.05$, while mean values with the same letters as superscripts down column are considered non-significant at $P < 0.05$.

Table 5 FRAP scavenging effect of the EAFFZRB

Concentrations (µl/dl)	EAFFZRB (%)	Ascorbic Acid (%)
10	3.06 ± 0.07 [b]	92.60 ± 1.78 [b]
20	2.55± 0.13 [a]	93.20 ± 2.49 [b]
40	2.85 ± 0.49 [a]	87.49 ± 11.47 [c]
80	3.64 ± 0.14 [b]	93.15 ± 1.63 [b]
160	2.73 ± 0.21 [a]	90.43 ± 0.63 [a]
320	2.66 ± 0.19 [a]	90.94 ± 0.40 [a]
640	2.66 ± 0.01 [a]	90.33 ± 1.20 [a]
(IC_{50})	(R^2 = 0.295)	(R^2 = 0.361)

The findings are presented as Means ± SD (n = 3). Mean values with different letters as superdcripts down the column are considered significantly different at $P < 0.05$, while mean values with the same letters as superscripts down column are considered non-significant at $P < 0.05$.

Phytochemical compounds identified in EAFFZRB using GC-MS analysis

Phyto-constituents were analyzed for volatile organic compounds using GC-MS. The GC-MS analysis of EAFFZRB identified a total of 12 compounds. Table 6 shows the constituents of EAFFZRB, including their retention time, molecular formula, molecular weight, and peak area. Figure 2 represents the chromatogram. The following chemical constituents identified in the GC-MS analysis of EAFFZRB were:
1-Phenyl-1-(trimethylsilyloxy)ethylene;
Cyclopentanecarboxamide;
Dodecanamide, N,N-diethyl;
Isopropylbarbituric acid;
Oxypurinol;
trans-1,4-Cyclohexanedihydroxamic acid;
3,6-Dihydro-6-hydroxymethyl-2-phenyl-2H-1,2-oxazine;
2-Chloro-4,4-dimethylhexane;
1H-Benz[e]indene, 2-methyl;
2,6-Dimethyl-4-(3-nitrophenyl)pyridine;
Hydroxy-5-nitrobenzyl bromide;
Phenol, 3-methoxy-2,5,6-trimethyl.

Discussion

The use of herbal medications to treat diabetes has long been promoted, but few of these claims have been substantiated by successful commercial formulations. Plant-based drugs and functional meals that alter physiological effects are becoming more and more popular as ways to prevent and treat obesity and diabetes. These notable developments in the use of oral hypoglycemic medicines to treat diabetes have prompted further research into improved drug formulations. Several disadvantages and unfavorable side effects plague the synthetic medications available today.

The findings showed that there were substantial differences ($P < 0.05$) in the percentage inhibition between the α-amylase and α-glucosidase concentrations on the EAFFZRB (Table 1 and 2). However, the inhibitory effect of EAFFZRB on the concentrations demonstrated a preference/respect for α-amylase and α-glucosidase. This suggests that the inhibition of the fraction increases with concentration, peaking at 1.0 mg/mL, and it appears this level of inhibition was comparable to the effects of the standard drug Acarbose. This was also evidenced by comparing their IC_{50} values. EAFFZRB inhibited α-amylase (IC_{50} = 0.726) more effectively than α-glucosidase (IC_{50} = 0.877) as compared to the standard drug Acarbose with α-amylase (IC_{50} = 0.728) and α-glucosidase (IC_{50} = 0.852) as shown in Figure 3 and Figure 4 respectively. The results obtained in this study agree with those of a number of other studies [39–41]. Numerous medicinal plants and their different parts can reduce the plasma glucose concentration. This EAFFZRB's ability is due to its phyto-constituents like Phenol-3-methoxy-2,5,6-trimethyl, 2-Hydroxy-5-nitrobenzylbromide, 2,6-Dimethyl-4-(3-nitrophenyl) pyridine,1H-Benz[e]indene,2-methyl and others present in plant fraction, which have been evaluated for their potential to inhibit α-amylase and α-glucosidase [42]. These Phytochemicals are regarded as essential compounds with good antioxidant properties. EAFFZRB is a generous example with both α-amylase and α-glucosidase inhibitory potential, which leads to the minimum absorption of monosaccharide-glucose.

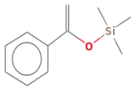
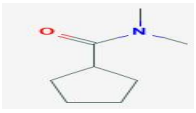
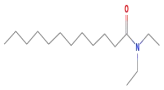
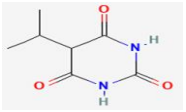
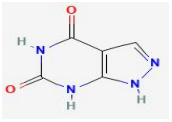
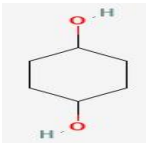
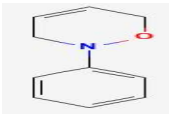
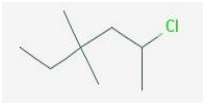
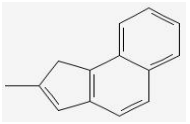
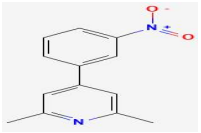
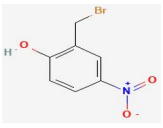
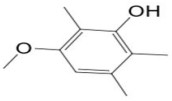
Inhibiting the digestive enzymes α-amylase and α-glucosidase is a useful strategy for regulating blood glucose levels in people with type 2 diabetes and those who are borderline because it can effectively reduce the rise in blood glucose that occurs after a meal [43].The mechanism of action of several antidiabetic drugs, such as Acarbose, is to prevent the hydrolysis of carbohydrates by digestive enzymes such as pancreatic, salivary, and intestinal brush border α-glucosidase [44]. One approach to early-stage diabetes management involves lowering postprandial hyperglycemia. To achieve this, the digestive tract's α-amylase and α-glucosidase enzymes which hydrolyze carbohydrates are inhibited, which postpones the absorption of glucose [45]. Ascarbose is a microbiological pseudo-tetrasaccharide that is commonly used as an oral hypoglycaemic medication to manage postprandial hyperglycemia in conjunction with other antidiabetic medicines. It is an inhibitor of α-glucosidase and α-amylase. However, gastrointestinal side effects such as; diarrhea, flatulence (gas), and abdominal discomforts are described when using Acarbose as an oral hypoglycaemic drug. These side effects are purportedly caused by the excessive inhibition of pancreatic α-amylase by Acarbose [33]. It has been observed that, in contrast to Acarbose, plant-derived inhibitors of α-amylase and α-glucosidase have greater inhibitory action against α-glucosidase and less inhibitory effect against α-amylase activity [33]. This shows that for the treatment and control of postprandial hyperglycemia, plant extracts and their constituents may be effective therapeutic agents with fewer adverse effects than Acarbose.

In comparison to other concentrations, the DDPH result of EAFFZRB shows non-significant ($P > 0.05$) increases in the inhibition at concentrations of 10 and 640 µl/dl (Table 3). In contrast to other concentrations, standard ascorbic acid demonstrated a significant ($P > 0.05$) increase in inhibition at 10, 20, and 80 µl/dl. DPPH is a commonly used method to assess how well different antioxidant substances scavenge free radicals [3]. Since ascorbic acid, also known as vitamin C, is a water-soluble free radical scavenger, it has been used as the standard in this study. Furthermore, it combines elements that help replenish vitamin E in cell membranes by donating reducing equivalents with vitamin E. Vitamin C transfers an electron to the lipid radical, changing it into the ascorbate radical, which halts the lipid peroxidation chain process [32, 46].Vitamin C is an antioxidant with therapeutic capabilities that also plays a role in multiple metabolic processes, wound healing, osteogenesis, detoxification, iron absorption, collagen production, and preventing blood clotting [47, 48]. The DPPH is one of the most trustworthy free radicals and is frequently used to evaluate the radical scavenger properties of natural foods [49]. A quick and simple technique to manually examine the antioxidant content is to use the DPPH test method.

This methodology may be applied to both liquid and solid samples,

and it considers the entire antioxidant capacity of the sample instead of just one antioxidant. The DPPH test is based on the stable 2, 2-diphenyl-1-picrylhydrazyl free radical's ability to react with hydrogen donors [50, 51]. The basis of the DPPH test method is the decrease of the stable free radical DPPH. The odd-electron free radical DPPH is most absorbable at 517 nm (purple). When antioxidants react with DPPH, they reduce the stable free radical to DPPH-H, which lowers absorbances in comparison to DPPH, by pairing it with a hydrogen donor (such as an antioxidant that scavenges free radicals)

Table 6 Compounds identified in EAFFZRB using GC-MS analysis

Peak No.	Name of compound	Retention time (min)	Molecular formula	Molecular weight (g/mol)	Peak area (%)	Structures of compound
1	1-Phenyl-1-(trimethylsilyloxy)ethylene	5.798	$C_{11}H_{16}OSi$	192.33	1.22	
2	Cyclopentanecarboxamide,N,N-diheptyl	6.813	$C_8H_{15}NO$	141.21	1.47	
3	Dodecanamide,N,N-diethyl	8.640	$C_{16}H_{33}NO$	255.44	1.03	
4	Isopropylbarbituricacid	9.757	$C_7H_{10}N_2O_3$	170.17	1.90	
5	Oxypurinol	10.366	$C_5H_4N_4O_2$	152.11	13.22	
6	Trans-1,4-Cyclohexanedihydroxamic acid	16.406	$C_6H_{12}O_2$	116.16	1.11	
7	3,6-Dihydro-6-hydroxymethyl-2-phenyl-2H-1,2-oxazine	18.487	$C_{10}H_{11}NO$	161.20	12.80	
8	2-Chloro-4,4-dimethylhexane	23.867	$C_8H_{17}Cl$	148.67	3.64	
9	1H-Benz[e]indene,2-methyl	26.355	$C_{14}H_{12}$	180.24	19.37	
10	2,6-Dimethyl-4-(3-nitrophenyl)pyridine	37.724	$C_{13}H_{12}N_2O_2$	228.25	2.85	
11	2-Hydroxy-5-nitrobenzylbromide	42.952	$C_7H_6BrNO_3$	232.03	9.99	
12	Phenol-3-methoxy-2,5,6-trimethyl	68.179	$C_{10}H_{14}O_2$	166.22	31.41	

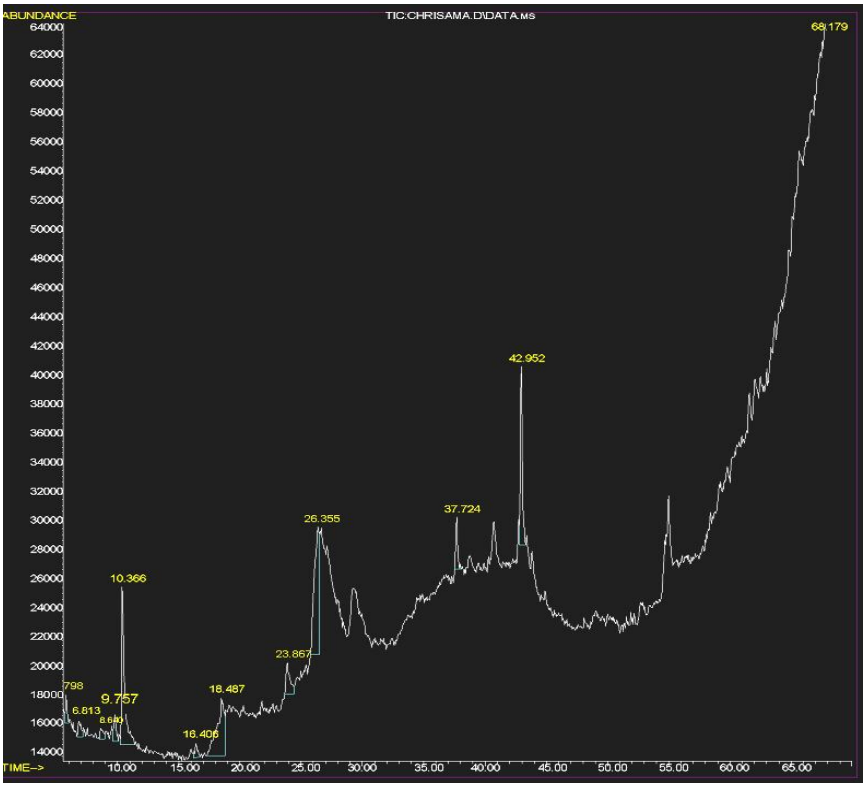


Figure 2 GC-MS Chromatogram of the essential compounds found in EAFFZRB

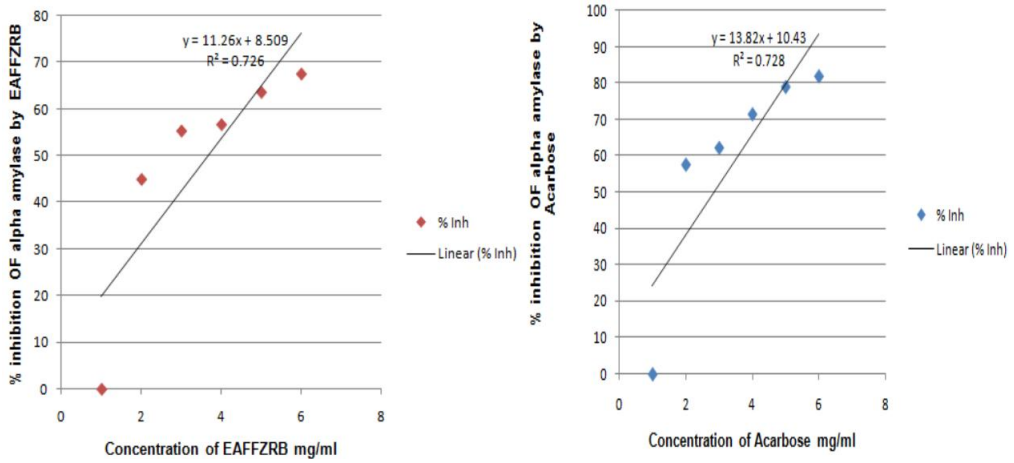


Figure 3 The percentage inhibition graph of alpha amylase against concentrations of EAFFZRB and the Acarbose from the standard curve (IC_{50}) respectively

[51]. The radical that forms the DPPH-H form decolorize (turns yellow) depending on how many electrons are got. Higher reducing power is correlated with greater decolorization. This assay is the most commonly used paradigm to evaluate a new drug's ability to scavenge free radicals. The DPPH radical has a strong UV-visible (UV-Vis) absorption spectrum. When a solution of DPPH is mixed with a substance that may donate a hydrogen atom, violet, or the pale yellow color of the picryl group present, is lost, resulting in the reduced form (diphenyl picryl hydrazine; nonradical) [52].

In Table 4, the TAC result for EAFFZRB revealed a high concentration of 160 µl/dl when compared to the other concentration reported. The EAFFZRB showed a significant ($P < 0.05$) decrease in the inhibition at non-concentration-dependent levels at 20, 40, 80, 320, and 640 µl/dl in comparison to the concentration at 160 µl/dl. The TEAC test was first developed in 1993 by Miller and colleagues as a rapid and simple method of determining the total antioxidant capacity (TAC) [53]. The test measures how well antioxidants neutralize the 2-azinobis (3-ethylbenzthiazolin-6-sulfonic acid) (ABTS•+) stable radical cation, a blue-green chromophore with maximum absorption at 734 nm, whose intensity diminishes in the presence of antioxidants. ABTS can generate ABTS•+ in the presence of strong antioxidants. The degree to which the blue-green hue is discolored measured as a sudden reduction in absorbance to 734 nm depends on the length of the reaction, the intrinsic antioxidant activity, and the sample concentration. The TEAC test was used to measure the total antioxidant capacity of pure chemicals, physiological fluids, and vegetal materials. The TEAC test can be mechanized and modified for use with microplates and flow injection procedures, just like other radical neutralization techniques [53].

At concentrations of 10 and 80 µl/dl, the FRAP result of EAFFZRB demonstrated a considerably ($P < 0.05$) greater inhibition in comparison to other concentrations (Table 5). At 20, 40, 160, 320, and 640 µl/dl, there were non-significant changes ($P > 0.05$). The antioxidant ability of EAFFZRB was evaluated using the FRAP assay.

This assay converts ferric ions to ferrous ions in the presence of an antioxidant (or reducing agent), resulting in the formation of a blue-colored ferrous tripyridyltriazine complex (Fe^{2+}-TPTZ) at pH 3.5. The change is monitored spectrophotometrically at 593 nm [54]. There were indications of antioxidant power in the EAFFZRB.The assumption that flavonoids and phenolics are very potent antioxidants is supported by Figures 5–7, which displayed a high association between DPPH, TAC, and FRAP values [14]. After being effectively employed to evaluate the antioxidant activity of plasma, FRAP is another approach used to compute total antioxidant activities. It is used to measure the antioxidant activity of diverse biological samples and pure chemicals [55–57]. Since antioxidant and antiradical characteristics are mostly attributed to phenolic compounds, it makes sense to believe that a fraction's effectiveness is proportional to the amount of phenolic compounds it contains [58, 59].

For assessing the overall antioxidant capacity of various foods and plant extracts, the ferric chloride hexahydrate ($FeCl_3$•$6H_2O$) assay is a useful technique. Its foundation is the reduction of 2, 4, 6-tripyridyl-s-triazine (TPTZ) complexes using ferric chloride hexahydrate, which is almost colorless. When the solution has totally decreased to a brownish tint, blue ferrous complexes will eventually form in it. The results of the FRAP test were also often used to assess the total antioxidant activity of biological fluids, such as plasma, urine, saliva, follicular fluid, alveolar liquid, seminal material, tears, cerebrospinal fluid, and feces, as well as the effects of age, gender, disease, medical treatment, and conventional and alternative therapies. In addition, the FRAP test was used on samples from a range of animals, insects, and marine species in the biomonitoring and supplementary studies [59].

This investigatio-n established a correlation between the phytoconstituents and the in vitro antioxidant (DPPH, TAC and FRAP) activities of EAFFZRB. Therefore, these antioxidant actions may be the cause of the antidiabetic impact. Similar findings were seen in another investigation [60]. The etiology of certain diseases is caused by the overproduction of oxidants (reactive oxygen species and reactive nitrogen species) in the human body. Scavenging these oxidants is believed to be a useful strategy for reducing the degree of oxidative stress experienced by organisms. EAFFZRB contains antioxidant phytochemicals, which are thought to be the cause of its health advantages [61, 62]. The plant's potent antioxidant and free radical scavenging properties, which underpin other bioactivities and health advantages such the prevention of diabetes and obesity, have been demonstrated by the suppression of EAFFZRB [62]. The antioxidant properties observed in EAFFZRB, as shown in Table 6, can be attributed to the presence of various distinct antioxidant compounds. Notable compounds include 1-Phenyl-1-(trimethylsilyloxy)ethylene, Cyclopentanecarboxamide, Dodecanamide (N,N-diethyl), Isopropylbarbituric acid, Oxypurinol, trans-1,4-Cyclohexanedihydroxamic acid, 3,6-Dihydro-6-hydroxymethyl-2-phenyl-2H-1,2-oxazine, 2-Chloro-4,4-dimethylhexane, 1H-Benz[e]indene (2-methyl), 2,6-Dimethyl-4-(3-nitrophenyl) pyridine, 2-Hydroxy-5-nitrobenzylbromide, and Phenol (3-methoxy-2,5,6-trimethyl). Additionally, various parts of the plant have been reported to possess hypoglycemic pharmacological properties [14]. Thus, the antioxidant activities in EAFFZRB are likely due to these essential phytoconstituents with significant antioxidant potential.

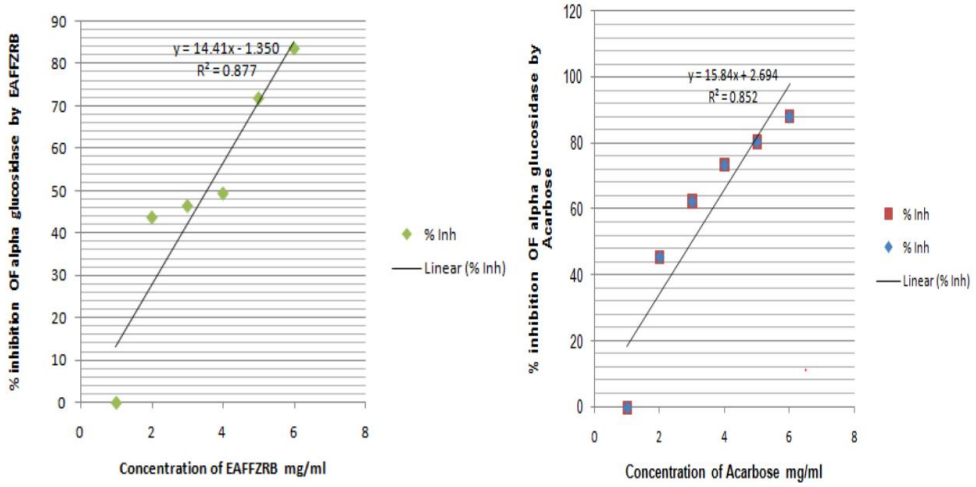


Figure 4 The percentage inhibition graph of alpha glucosidase against concentrations of EAFFZRB and the Acarbose from the standard curve (IC_{50}) respectively

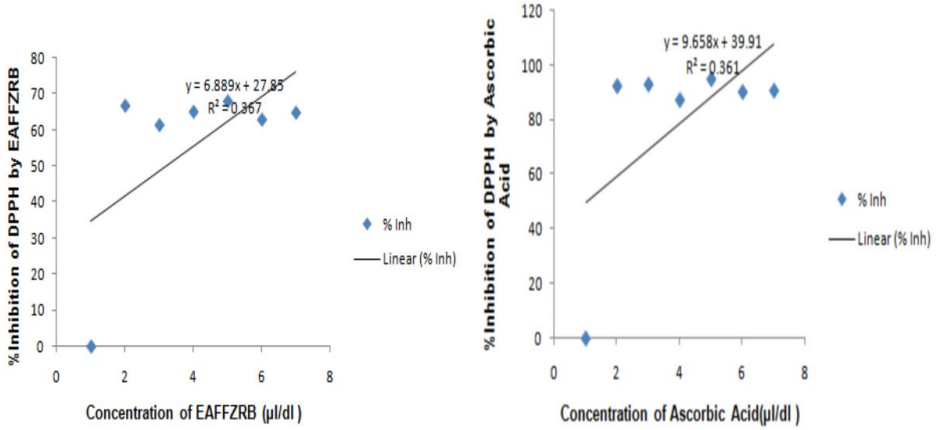


Figure 5 The percentage inhibition graph of DPPH against concentrations of EAFFZRB and the Acarbose from the standard curve (IC_{50}) respectively

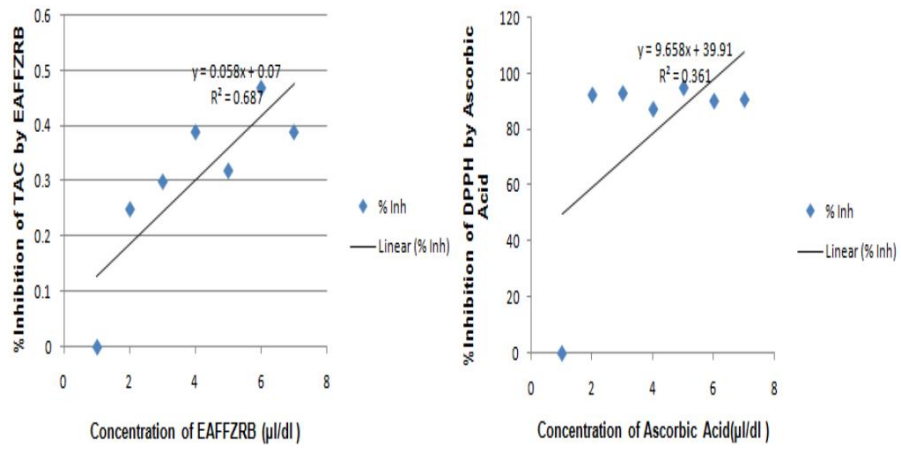


Figure 6 The percentage inhibition graph of TAC against concentrations of EAFFZRB and the Acarbose from the standard curve (IC_{50}) respectively

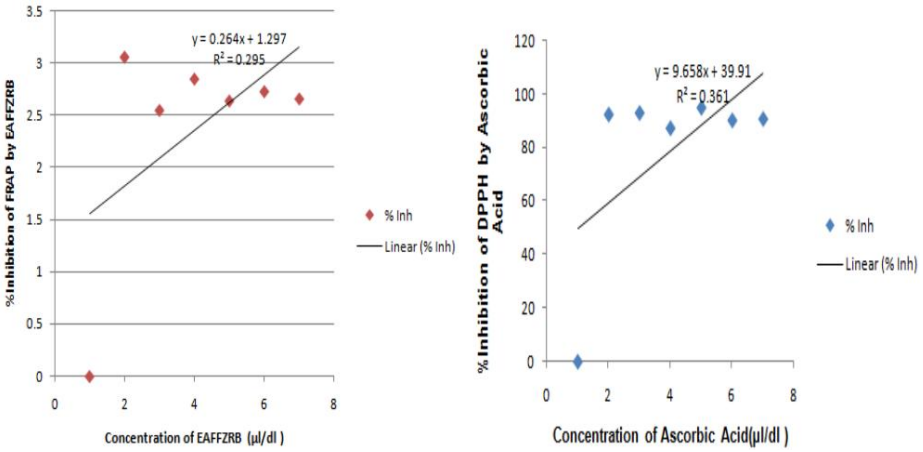


Figure 7 The percentage inhibition graph of FRAP against concentrations of EAFFZRB and the Acarbose from the standard curve (IC_{50}) respectively

Conclusion

The plant fraction's ability to scavenge free radicals was demonstrated to be significant and substantial when compared to the usual medication in this investigation. Furthermore, the ethylacetate fraction of *F. zanthoxyloides* root-back exhibited strong in-vitro antidiabetic effects. The outcomes validate the plant's practice of managing and treating diabetic mellitus with its root bark. The findings of this study will also provide scientific information to individuals in selecting effective complementary medicines as potential anti-diabetic agents. In order to successfully create an active medication or drugs for the management and treatment of diabetes mellitus and its associated consequences, further research should be directed toward comprehending the molecular mechanism (s) behind the antidiabetic function of the ethyl acetate fraction of *F. zanthoxyloides*.

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