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Bioassay guided isolation and compounds identification of the anti-diabetic fractions of (rosemary) *Rosmarinus officinalis* leaves extract

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Abstract

Background Diabetes mellitus is a metabolic disease characterized by prolonged elevated blood glucose levels. It is a common health problem with a high mortality and morbidity to the human race. A number of medicinal plants such as rosemary (*Rosmarinus officinalis*) have been used for the treatment of diabetes. Most of the anti-diabetic conventional drugs have been found to have some side effects and there is therefore need to explore new sources of anti-diabetic drugs. The aim of the current study was to investigate the possibility of getting anti-diabetic compounds from *R. officinalis* that can be used as leads for drug discovery.

Methodology *R. officinalis* leaves were macerated in 50% methanol in dichloromethane and the crude extract fractionated by column chromatography. The obtained fractions were subjected to an in-vitro alpha-amylase inhibition assay. The anti-hyperglycemic potential of the fractions was evaluated in diabetic induced Wistar rats. The most potent fractions were analyzed by gas chromatography-mass spectrophotometry (GC-MS) for identification of the compounds.

Results A total of 21 chromatographic fractions were assembled with different alpha- amylase inhibition activity. Eleven of the fractions had more than 30% alpha-amylase inhibition activity. The ethyl acetate fraction had the highest inhibition potential (LC_{50} of 2.8 µg/mL). The anti-diabetic assay in rats showed that fractions (F1) and (F4) had highest blood glucose reduction of 44.5 ± 0.4 and 52.8 ± 1.3%, respectively ($p < 0.05$). GC-MS analysis of fractions F1 and F4 showed the presence of 21 and 23 compounds in F1 and 23, respectively.

Conclusion This study has demonstrated that *R. officinalis* crude extract fractions obtained from 50% methanol in dichloromethane possesses alpha-amylase inhibitory and anti-hyperglycemic activities as well as secondary metabolites with varying chemical structures. The hexane and hexane/ethyl acetate (8/2) fractions showed most potent alpha-amylase inhibition with high anti-hyperglycemic activity giving hope of a possibility of obtaining lead compounds for new anti-diabetic drugs.

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Keywords Alpha-amylase, Anti-diabetic, Chromatography, Secondary metabolites, Fractions, *Rosmarinus officinalis*

Introduction

Herbal natural products have been used as medicinal therapies since ancient days [1]. There is a current upsurge by researchers focusing on studying of more on plants for drug discovery for various ailments because of their less side effects [2, 3]. Many clinically approved drugs have originated from plant isolates gotten by use of chromatographic techniques followed by spectroscopic analysis for their compound identification [4] Diabetes mellitus (DM) is a metabolic disorder demonstrated by uncontrolled high blood sugar characterized by low insulin production or a peripheral insulin intolerance [5]. Untreated DM hyperglycemia can cause extensive damage in different organ system resulting in complications such as nephropathy, retinopathy and neuropathy among other abnormalities [6]. The main complications of the organ systems are due to inflammation characterized with oxidative stress [5]. The insulin hormone, which is produced by pancreatic beta cells is responsible for stimulating the absorption of glucose into cells [7]. It is important to treat DM in order to maintain normal postprandial blood glucose levels [8]. The alpha-amylase enzyme is a known catalyst that helps break into oligosaccharides and monosaccharide glucose cleaving on the α-D- [1, 4] glyosidic bonds [9]. The enzymes, alpha-amylase and alpha-glucosidase, the key enzymes in carbohydrate digestion have been studied in many plant extracts. These enzymes have been widely exploited. intervention for preventing postprandial hyperglycemia in DM status and related metabolic diseases [10, 11]. The ß-cells in the pancreases are key in maintaining the blood glucose levels by producing and synthesizing the insulin hormone. These cells go through apoptotic death if hyperglycemia is prolonged [6]. Many medicinal plants have been reported to have anti-diabetic activity with the ability of restoring insulin secretion or by the action of inhibiting the intestinal absorption of glucose [12, 13]) [14]. Studies have established that more than 1000 plant species have been found to have anti-diabetic efficacy with a wide traditional use, nevertheless few of these have received thorough scientific validation [15]. The current study was focused on studying the anti-diabetic efficacy of rosemary (*Rosmarinus officinalis* L*) an* aromatic plant. This plant has been used for a long time as a food additive, a cosmetic ingredient and also for medicinal purposes such treatment of hyperglycemia, hypertension and atherosclerosis among other ailments [16, 17]. Previous pharmacological studies have shown that *R. officinalis* carnosic acid, rosmarinic acid, and carnosol significantly improved DM by regulating the metabolism of glucose [18]. The safety of *R. officinalis* has been studied in various models and has been classified as "generally safe" or GRAS [19]. Many of the isolated compounds from this plant such rosmarinic acid and carnasoic acid have been found to have low toxicity with oral acute toxicity medial lethal dose LD_{50} of 561 mg/kg and 7100 mg/kg body weight respectively in mice [20, 21]. Compounds from medicinal plants have been known to be the origin of many conventional drugs including, glucose lowering drugs such as, metformin, which was purified from the plant, *Galega officinalis* L [22]. Four categories of compounds derived from *R. officinalis* possessing anti-hyperglycemia activity; hydroxycinnamic acid derivetives, hydroxybenzoic acid derivatives, flavonoids, and phenolic terpenes have been recorded in earlier studies [23, 24]. Supplementing *R. officinalis* leaves in the diet of diabetic induced animals showed significant reduction of hyperglycemia [25]. The aim of this study was to isolate and identify anti-diabetic compounds from *R. officinalis* in hope of getting drug leads that may have considerable anti-diabetic efficacy and safety. The initial step in this study was the extraction of dry powdered leaves using organic solvents followed by fractionation of the crude extract by column chromatography. The fractions were then screened for alpha-amylase activity and the most anti-diabetic ones subjected to GC-MS analysis for the identification of the composition.

Materials and methods

Plant materials

Fresh leaves of *Rosmarinus officinalis* were collected from Lari area of Kiambu County, Kenya in the month of July, 2022. The plant material was botanically identified and authentication done in the Herbarium at the Department of Biology, Faculty of Science and Technology, University of Nairobi and the voucher specimen (ZNK/UON2022/002) deposited in the same herbarium.

Extraction and fractionation

R. officinalis leaves were separated from the stalks, washed using clean water and dried in a room at 25–27°C for 21 days to moisture content of 10–15%. Extraction was done by maceration for 48 h with 50% methanol in dichloromethane and re-extracted for up to 72 h. The filtrate was dried in a rotatory evaporator at 45 °C and stored in sealed containers in refrigerator at 4 °C. Fractionation was done according to standard procedures [26, 27]. A glass chromatographic column (80 cm × 7 cm) was packed with silica gel, mesh 60–120 (Merck, Germany) and stabilized for 24 h. The crude extract (100 g of *R. officinalis*) was adsorbed on silica gel and the dry powder of the adsorbed sample packed in the stabilized

column. The column was eluted using organic solvents with a polarity gradient actualized by mixing hexane with increasing amounts of ethyl acetate and then ethyl acetate with increasing amounts of methanol. Hexane: ethyl acetate mixture in ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 were used. Further fractionation continued with ethyl acetate: methanol in the ratio; 10:0, 8:2. 5:5, 3:7 and 0:10. The amount of eluent collected in each fraction ranged between 100 mL to 200 mL depending on the color observation or TLC profile. The eluates were combined according to their TLC retention factor (Rf). The solvent was removed by evaporation on a rotatory evaporator (45 ºC), transferred into a weighed glass vial and left for further drying prior to weighing. Samples were then stored at 4 ºC before performing the in-vitro alpha-amylase assay and in vivo anti-diabetic analysis in rats.

The in-vitro alpha-amylase inhibition activity evaluation, the LC_{50} determination of the crude extract, the chromatographic fractions and acarbose (the positive control)

The alpha-amylase inhibition assay was done by use of the 3,5-dinitrosalicylic acid (DNSA) method [28]. The combined fractions of *R. officinalis* and the crude extract were dissolved in 10% dimethyl sulfoxide (DMSO). An aliquot of 250 μl of extract (1–10 mg/mL) was mixed with 250 μL of 0.02 M sodium phosphate buffer at pH 6.9 in a tube that contained alpha-amylase (1 mg/mL). The mixture was incubated in a water bath at 25 °C for 10 min, 250 μL of 1% starch dissolved in 0.02 M sodium phosphate buffer (pH 6.9) was added and the components further incubated at 25 °C for 10 min. An aliquot of DNSA reagent (500 μL) was added to stop the reaction and the solution was left in a boiling water bath for 5 min then cooled to room temperature. The final volume was made to 5 mL with distilled water and the absorbance measured at 540 nm using spectrophotometer. The assay was done in triplicates. A control was prepared using the same procedure while replacing the extract with distilled water. The percentage inhibition was calculated using the formula:

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

The concentrations of extracts resulting in 50% inhibition (LC_{50}) were determined graphically by the use of the linear regression curve of % alpha- amylase inhibition against extract concentration.

In vivo evaluation of hypoglycemic effect of extract in streptozotocin (STZ)-induced diabetic animals

The animal study was done with the approval of University of Nairobi Biosafety and Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Nairobi reference, FVM BAUEC/2019/238.

Induction of diabetes in using streptozotocin (STZ)

Wistar male rats weighing between 200 and 300 g (50 in number) were acquired from the animal house, department of Biochemistry, Faculty of Science and Technology, University of Nairobi and acclimatized in the animal house research room for one week. Diabetes was induced using freshly prepared STZ single dose intraperitoneal injection at the rate of 65 mg/kg body weight dissolved in 0.1 M citrate buffer (pH 4.5) [25]. The animals were then put on 10% glucose in drinking water for 6 h to stabilize them. Blood glucose was measured after 72 h using an '*On Call Plus*' glucometer by taking blood from the tail vein. The animals exhibiting glucose level above 15 mmol/L were separated as diabetic. Fasting blood glucose (FBG) level was measured again after 2 and 7 days to confirm the diabetic mellitus (DM) status [29].

Experimental animal design of the STZ diabetic induced Wistar rats for anti-hyperglycemic study

The diabetic rats (blood glucose > 15 mmol/L) were randomly assigned into 14 groups with 3 rats each [19]. The animals were provided with the rodent chow, clean water ad libitum. The animal house was maintained at temperatures of 23–25 °C and a humidity level of 50–60% under 12-hour light/ 12-hour dark. The animals were fasted for 8 h before commencement of treatment. Selected fraction extracts of 300 mg/kg body weight that exhibited alpha -amylase inhibition mean of over 30% were dissolved in 1 mL of 10% Tween 20 and orally administered once to the treatment groups using a metallic gavage. A negative and positive control groups constituting of 10% Tween 20 and 2.5 mg/kg body weight of glibenclamide (GLC) drug respectively were incorporated in the study [30]. The hypoglycemic effect in the treatment groups was assessed by measuring the blood glucose (mmol/L) at different time intervals of 0, 3-, 6-, 12- and 24-hours post treatment administration. Mean percentage glucose reduction/increase was calculated.

Characterization of the fraction of the crude extract of *R. officinalis* leaves with highest hyperglycemic reduction using gas chromatography coupled to mass spectrometry

A 20 µL aliquot of the samples was injected into the gas chromatography coupled to mass spectrometer (GC/MS). The GC/MS analysis was then carried out using an agilent 7890 A gas chromatograph coupled to an agilent 5975 C mass spectrometer equipped with an HP5 capillary column, 30 m long, 0.25 µm i.d., and a 0.25 µm film thickness. The temperature of the column was programmed from 50 °C (2 min) to 120 °C, at a rate of 15 °C per minute, and the final ramp reached 450 °C, at an

increasing temperature rate of 5 °C per minute. Helium grade 5.0 was used as carrier gas at a flow rate of 1 mL/minute. split/splitless (splitless mode) inlet temperature was 280 °C as was that of the mass spectrometry transfer line [31]. The temperature of the ion source was maintained at 230 °C. The mass spectrometer was operated under electron ionization mode at 70 eV mass spectra and the total ion chromatograms were obtained by automatic scanning a mass range (m/z) of 45400. The components were identified by comparing the mass spectrum with those available in the National Institute of Standards (NIST)) spectra library.

Statistical analysis

The mean value and standard deviation of three replicates were analyzed in the excel Microsoft worksheet. This was followed by a one-way analysis of variance (ANOVA) in the applicable assays, followed by a post hoc analysis using Tukey's post t test and Dunnett using Software statistical package for the social sciences (SPSS) version 2016. A $p \leq 0.05$ value was regarded as significant regarded as significant.

Results

The extraction output

The yield of the 50% methanol in dichloromethane crude extract was 19.2% on a w/w basis and the powder was black /green in color. Table 1 shows the output of the fractionated extracts by column chromatography using various eluting solvent systems. A total of 259 vials were collected and combined into 21 fractions on the basis of their thin layer chromatography (TLC) profile. The individual dried fractions weighed between 0.12 and 30.43 g with Rf range of 0.24–0.91 and the colors ranged from white, orange, green black to brown. The highest yield (30.43%) was from the fractions F12 eluted with 20% ethyl acetate in hexane.

In-vitro alpha-amylase inhibition activity of different concentrations of the crude extract, the 21 chromatographic fractions and acarbose (the positive control) and the determined LC_{50}

The results of the alpha-amylase inhibitory potential of the different concentration of the 21 fractions (F1 to F21), that of acarbose and the crude extract are shown in Table 2. The percent inhibition in the highest concentration (1000 µg/mL) ranged from 17.8±1.5 for F14 to 96.3±0.9 in F4. The crude extract exhibited a considerably high alpha-amylase inhibitory potential ranging from 32.1±1.5 to 73.9±0.4 for 50 µg/mL and 1000 µg/mL, respectively, slightly lower than acarbose, the positive control which had 44.4±3.4 and 89.2±2.1 for 50 µg/mL and 1000 µg/mL respectively. A total of 11 fractions of crude *R. officinalis* showed alpha –amylase inhibitory potential of above 30%. The fractions with the highest alpha-amylase inhibitory potential (F4) the hexane: ethyl acetate (8:2) and F1 from hexane (10:0).

Table 1 The column chromatographic fractions from 100 g*R. officinalis* crude leaf extract, their yield in grams (g), their descriptions and their TLC analysis

Fraction	(vial) range	Eluting solvents	Yield	Rf	Description
F1	1 to 4	10:0 hex	0.95	0.48	white and oily
F2	5 to 9	9:1 hex/EtoAC	0.12	0.41	orange paste
F3	9 to 24	9:1 hex/EtoAC	0.25	0.29	orange paste
F4	24–61	8:2 hex/EtoAC	2.22	0.24	orange paste
F5	62–78	7:3 hex/ EtoAC	1.81	0.32	orange paste
F6	78–94	6:4 hex/EtoAC	4.85	0.38	orange paste
F7	95–99	5:5 hex/EtoAc	0.57	0.29	orange paste
F8	100–113	4:6 hex/EtoAC	1.91	0.28	brown/orange
F9	113–127	3:7 hex/EtoAC	0.43	0.34	green/ black
F10	128–135	2:8 hex/EtoAC	0.84	0.39	green/ black
F11	136–139	2:8 hex/EtoAC	0.33	0.42	green/black
F12	140–145	2:8 hex/EtoAC	30.43	0.4	brown/ green
F13	146–162	1:9 hex/EtoAC	22.45	0.55	green/ black
F14	163–178	10:0 EtoAC	14.68	0.56	brown/green
F15	179–188	10:0 EtoAC	0.32	0.61	light green
F16	189–196	8:2 EtoAC/MeOH	0.74	0.71	light green
F17	197–213	5: 5 EtoAC/MeOH	0.54	0.82	orange/ brown
F18	214–242	5: 5 EtoAC/ MeOH	6.49	0.8	orange/ brown
F19	243–248	3: 7 EtoAC/MeOH	1.21	0.82	brown
F20	249–253	3: 7 EtoAC/MeOH	7.75	0.89	brown
F21	254–259	10:0 MeOH	0.38	0.91	brown

Abbreviation: EtoAC (ethyl acetate), hex- (hexane), MeOH- (methanol), Rf- (retention factor), **g**-(grams)

Table 2 The in-vitro alpha-amylase inhibition of the *R. officinalis* crude extract, the 21 chromatographic fractions and the acarbose

	50 µg/mL	200 µg/mL	400 µg/mL	600 µg/mL	800 µg/mL	1000 µg/mL
Acarbose	44.4 ± 3.4	68.4 ± 2.1	64.7 ± 3.8	75.1 ± 6.2	88.3 ± 1.3	89.2 ± 2.1*
Crude	32.1 ± 1.5	38.4 ± 1.6	42.8 ± 2.3	51 ± 0.9	68.6 ± 1.8	73.9 ± 0.4*
F1	42 ± 2.2	58 ± 0.2	67.2 ± 0.7	77.1 ± 0.1	85.4 ± 0.6	93.8 ± 0.1*
F2	8.1 ± 1.9	11 ± 1.8	22.5 ± 2.1	23.1 ± 3.2	23.9 ± 0.7	24.8 ± 0.1
F3	14.1 ± 0.9	18.3 ± 1.8	20.2 ± 1.6	21.9 ± 2.0	22.5 ± 3.1	25.9 ± 1.2
F4	45.8 ± 1.2	66.7 ± 1.7	76.9 ± 0.8	81.2 ± 4.2	89.8 ± 0.8	96.3 ± 0.9*
F5	10.1 ± 0.9	11.3 ± 1.5	15.9 ± 1.2	18.7 ± 2.0	19.6 ± 1.2	21.2 ± 1.1
F6	6.6 ± 3.1	9.2 ± 0.2	10.2 ± 0.9	15.4 ± 1.8	17.4 ± 1.3	20.5 ± 0.6
F7	8.8 ± 2.1	11.3 ± 0.8	14.5 ± 1.3	16.4 ± 1.1	19.2 ± 1.9	24.7 ± 2.3
F8	14.2 ± 1.0	16.8 ± 2.2	18.8 ± 0.7	20.9 ± 0.6	21.8 ± 1.5	23.8 ± 0.9
F9	25.5 ± 1.3	34.7 ± 0.7	45.9 ± 0.8	55.4 ± 2.1	60.6 ± 1.2	68.6 ± 4.2*
F10	5.5 ± 1.5	7.4 ± 1.2	8.9 ± 0.8	15.8 ± 3.8	19.6 ± 2.5	22.5 ± 0.7
F11	11.3 ± 1.8	13.4 ± 2.7	16.9 ± 0.6	17.6 ± 1.8	25.9 ± 1.6	30.5 ± 0.8*
F12	10.8 ± 1.2	15.5 ± 1.7	17.7 ± 3.0	21.5 ± 0.9	27.8 ± 6.1	31.6 ± 1.2*
F13	4.1 ± 0.5	7.8 ± 0.2	11.2 ± 2.6	19.6 ± 1.9	19.0 ± 1.1	19.4 ± 3.8
F14	5.6 ± 0.2	7.3 ± 1.2	7.9 ± 1.4	8.2 ± 0.9	10.1 ± 2.2	17.8 ± 1.5
F15	10.6 ± 3.6	15.8 ± 1.7	19.8 ± 0.2	25.2 ± 1.9	27.8 ± 2.2	29.2 ± 0.8
F16	34.8 ± 2.2	54.7 ± 1.4	60.2 ± 3.1	65.8 ± 0.9	69.2 ± 2.2	72.7 ± 2.1*
F17	9.4 ± 1.6	12.8 ± 0.8	15.2 ± 2.2	18.8 ± 1.0	21.6 ± 2.4	24.6 ± 1.9
F18	39.1 ± 1.1	42.7 ± 0.6	53.9 ± 0.9	61.5 ± 2.9	65.2 ± 1.6	68.5 ± 3.8*
F 19	33.8 ± 2.4	41.4 ± 1.1	54.7 ± 0.4	60.9 ± 2.1	63.4 ± 1.8	65.2 ± 3.1*
F20	3.9 ± 1.7	8.4 ± 2.6	13.2 ± 3.4	17.6 ± 0.5	19.1 ± 0.2	22.8 ± 2.4
F 21	23.8 ± 1.8	25.7 ± 1.5	32.5 ± 2.0	34.1 ± 2.6	34.2 ± 0.3	36.6 ± 0.5*

* Indicates fractions with inhibition above 30.0%. The alpha-amylase inhibition of the *R. officinalis* (methanol: dichloromethane) crude extract, the 21 fractions (F) and the acarbose is expressed as mean percentage ± standard deviation (SD), $n = 3$

The LC_{50} potential of alpha-amylase inhibition of the *R. officinalis* crude extract and the chromatographic fractions in comparison with acarbose

The results of the graphically determined LC_{50} of the crude *R. officinalis* methanol: DCM crude extract and the 11 fractions exhibiting alpha-amylase potential of over 30% in the highest tested concentration (1000 µg/mL) are shown in Table 3. The results reflected a relationship between the percentage inhibition and the LC_{50} values of the extracts; the higher the alpha-amylase inhibition, the more the potency (LC_{50}). The extract from F4 showed the highest LC_{50} followed by F1 with 2.8 (96.3 ± 0.9%) and 110.4 µg/mL (93.8 ± 0.1%) respectively. Acarbose which had an inhibitory potential of 89.2 ± 2.1% showed the highest LC_{50} of 2.5 µg/L. Out of the 11 fractions six fractions had an LC_{50} activity of below 500 µg/mL as follows: F1–110.4, F4–2.8, F8–473.9, F16 -237.3, F18 351.8, and F19–411.9 µg/mL with the crude *R. officinalis* extract demonstrating a moderate LC_{50} of 483.8 µg/mL.

The results showing blood glucose measurements at the different tested intervals, the mean difference and the *p* values are shown in Tables 4 and 5. All the extracts had a significant effect on blood glucose level on the diabetic Wistar rats ($p > 0.05$), except for fraction 21 which showed a non-significant reduction of 0.4 ± 0.2 representing a 2.7% overall reduction (*p* value of 0.122) (Table 5). Fraction F4 showed the highest potential in reducing the blood glucose levels followed by F1 with a mean reduction of 12.5 ± 0.2 and 11.3 ± 3.1 mol/L, respectively, a reduction percentage of 44.5 ± 0.4 and 52.8 ± 1.3, respectively. This is closely compared with the standard drug which gave a percentage blood glucose reduction of 41.2 ± 0.1. Fractions 11 and 12 just like the diabetic control (DMC) caused an elevation of blood glucose by 32.3 ± 0.7 and 39.4 ± 1.4%, respectively, while the increase by the DMC was 20.8 ± 0. Table 5 tabulates the percentage glucose reduction between 2 testing. Neither the *R. officinalis* crude fraction nor any of the chromatographic fractions showed a significant glucose reduction in the diabetic Wistar rats between the time of extract administration and one-hour testing. There was a significant reduction of the blood glucose in the diabetic rats between 1 and 3 h caused by fractions F1, F16, F18, F19 and F21. All the extracts caused a significant blood glucose reduction between 3 h and 6 h except fractions F11 and F16. There was a significant increase of the blood glucose in the rats between 6 and 24 h by fractions 11 and 12 corresponding to 29.0 ± 0.6 and 29.7 ± 0.8, respectively, but all the other extracts caused a significant reduction at this interval except F21 whose *p* value was above 0.05.

Table 3 The LC_{50} of amylase inhibition of the crude extract, the acarbose, and the 21 chromatographic fractions showing the generated graphic equations

	Graphic Equation of the means	LC_{50} (µg/mL)
Acarbose	y = 0.043x + 49.88	2.5**
Crude	y = 0.0457 + 27.89	483.8*
F1	y-0.051x + 44.37	110.4*
F2	y = 0.018x + 9.75	2236
F3	y-0.051x + 15.02	685.9
F4	y = 0.0477x + 49.87	2.8**
F5	Y = 0.012x + 9.87	3343
F6	Y = 0.1 472x + 5.73	3010
F7	Y = 0.0156x + 7.90	2699
F8	Y = 0.096x + 14.51	473.9*
F9	Y = 0.0447x + 7.75	945.3
F10	Y = 0.0191x + 3.57	2444
F11	y-0.02x + 9.12	2044
F12	Y = 0.021x + 9.93	1908
F13	Y = .03682x + 14.12	972.4
F14	Y = 0.0104x + 4.21	4403
F15	Y = 0.0198x + 11.34	1952
F16	Y = 0.034x + 41.93	237.3*
F17	Y = 0.01572x + 9.10	2602
F18	Y = 0.0328x + 38.46	351.8*
F 19	y-0.0337x + 36.12	411.9*
F20	Y = 0.019x + 24.33	1351
F 21	Y = 0.0134x + 8.29	3112

** showing very high inhibition (LC_{50}) < than 100 and * showing moderate (LC_{50}) between 100 and 500 µg/mL

Results of GC-MS analysis of the hexane fraction (F1) and hexane/ ethyl-acetate (8:2) fraction (F4) of the crude extract

Fractions F1 and F4 were subjected to GC-MS analysis and a total of twenty-one (F1) and twenty-three (F4) compounds were identified. The names of the compounds identified, their abundance and retention time are shown in Tables 6 and 7 (arranged in descending order of abundance). The structures of the compounds identified are shown in Figs. 1 and 2. The most abundant compounds in F4 were hexacosane (16.2%), (8 S,8aS)-4-(1-methylethylidene)-3,8-dimethyl-1,2,6,7,8,8a-hexahydroazulen-5-one (14.1%), octacosane (14.0%), heptacosane (12.8%) and 1-Iodooctadecane with (6.3%). The compounds in F1 with highest abundance were 8-amino-2,6-dimethoxylepidine (19.24%), *1 H*-imidazole, 4,5-dihydro-2-methyl-(12.69%), 3-hexene, 3-ethyl-2,5-dimethyl (11.50%), 1,8-cineole (1,8-eucalyptol) (10.60%), Linoleic acid (7.69%) and methyl hexadecanoate (6.01%). The 2-methyl-4,5-dihydro-1 *H*-imidazole was a compound present in both F1 and E4 with an abundance of 12.7 and 4.5% respectively. These 2 fractions had a combination of several classes of compounds ranging from hydrocarbons, fatty acid derivatives, monoterpenes, alcohols alkaloids and phenol derivatives.

Discussion

Bioactivity-guided fractionation is considered to be an effective approach which isolates active chemical constituents and pure compounds from medicinal plants [4]. *Rosmarinus. officinalis* plant has diverse chemical composition and bio-activities with various pharmacological perspective making it draw attention that has attracted many researcher studies [32–35]. Spectrophotometric and chromatographic methods coupled with good extraction techniques have contributed to getting lead compounds for drug discovery from natural products. The choice of the extraction solvent system and technique depends on the goals of the research at hand [36–38]. Fractionation of *R. officinalis* extract (in 50% methanol in dichloromethane) aimed at separating compounds based on their polarity. The study was able to isolate and

Table 4 The effect in the blood glucose in Wistar rats after a single dose oral administration of the glibenclamide, the crude and 10 fractions with mean of < 30% alpha –amylase inhibition

	0 min	60 min	3 h.	6 h.	24 h.	difference	*P*-value
+vie control	32.9 ± 2.4	31.2 ± 1.1	27.7 ± 1.3	24.4 ± 1.1	19.3 ± 0.8	13.6 ± 1.1↓	0.001*
-ve control	23.3 ± 0.5	24.8 ± 0.9	24.9 ± 2.1	27.8 ± 0.8	28.1 ± 1.1	-4.8 ± 0.4↑	0.023**
crude	16.8 ± 0.7	17.5 ± 1.2	16.1 ± 0.6	15.1 ± 3.2	14.1 ± 3.3	2.7 ± 2.2↓	0.019*
F1	28.1 ± 0.8	28.0 ± 0.9	26.0 ± 1.1	18.8 ± 0.8	15.6 ± 0.3	12.5 ± 0.2↓	0.003*
F 4	21.5 ± 2.1	20.93 ± 1.6	21.3 ± 0.5	18.1 ± 1.2	10.1 ± 0.8	11.3 ± 3.1 ↓	0.006*
F 9	19.5 ± 0.7	19.1 ± 1.3	18.9 ± 2.1	15.6 ± 0.8	14.6 ± 0.6	4.9 ± 0.4↓	0.001*
F 11	15.50 ± 0.8	16.4 ± 1,3	17.5 ± 0.6	17.2 ± 1.5	20.5 ± 1.5	-5.5 ± 0.2↑	0.01**
F12	16.8±	19.3 ± 0.8	21.6 ± 0.4	22.0 ± 2.1	23.4 ± 0.7	-6.6 ± 0.3↑	0.00*
F13	20.5 ± 0.9	21.0 ± 1.2	21.2 ± 1,3	19.4 ± 0.8	15.1 ± 0.6	3.4 ± 0.5↓	0.03*
F16	20.0 ± 1.2	19.5 ± 0.9	17.0 ± 0.5	16.6 ± 1.1	16.8 ± 1.7	3.3 ± 0.1↓	0.03*
F18	19.7 ± 0.1	21.1 ± 0.5	18.2 ± 1.0	16.5 ± 0.8	16.0 ± 1.2	3.7 ± 0.1↓	0.04*
F 19	18.1 ± 0.1	191 ± 0.5	17.6 ± 0.5	16.3 ± 0.7	14.2 ± 3.1	3.9 ± 0.7↓	0.00*
F 21	15.9 ± 0.7	17.1 ± 0.6	16.1 ± 0.9	15.0 ± 1.2	15.5 ± 1.3	0.4 ± 0.2↓	0.122

*Indicates a significant reduction (↓); ** significant increase (↑) of blood glucose in the STZ diabetic induced Wistar rats from 0 min to 24 h, $p \leq 0.05$

Table 5 The percentage glucose reduction effect of extracts of crude and chromatographic fractions of *R. Officinalis* in STZ diabetic induced Wistar rats compared with the standard drug, glibenclamide, after oral administration at 0 min, 1, 3, 6 and 24 h testing intervals

	% redu 0–1 h	% redu 1–3 h	% redu 3–6 h.	% redu 6–24 h.	% redu Overall
Positive control	5.2 ± 0.1 *	11.1 ± 0.5 *	12.2 ± 0.4*	55.7 ± 0.5*	41.2 ± 0.1*
Negative control	-6.4 ± 0.3	-0.4 ± 0.5	-11.9 ± 0.6	-17.4 ± 0.4	-20.8 ± 0*
crude	-4.4 ± 0.4	8.2 ± 0.5 *	6.4 ± 1.1*	18.1 ± 0.6*	16.3 ± 0.2*
F 1	0.6 ± 0.7	7.2 ± 0.6*	27.7 ± 1.3*	66.8 ± 0.7*	44.5 ± 0.4*
F 4	2.5 ± 0.6	-1.8 ± 0.5	14.9 ± 0.6*	62.5 ± 0.4*	52.8 ± 1.3*
F9	1.9 ± 0.8	1.4 ± 0.7	17.5 ± 0.4*	31.5 ± 0.6*	25.1 ± 0.8*
F11	-5.6 ± 0.1	-7.1 ± 1.4	1.7 ± 1.1	-29.0 ± 0.6	-32.3 ± 0.7
F 12	-14.9 ± 0.4	-12.1 ± 0.7	-2.8 ± 1.6	-29.7 ± 0.8	-39.4 ± 1.4
F 13	-2.4 ± 1.4	-1.1 ± 0.9	8.6 ± 0.8*	27.9 ± 1.5*	26.4 ± 0.0*
F16	2.7 ± 0.7	12.8 ± 1.3*	2.5 ± 1.1	19.7 ± 0.7*	16.3 ± 0.9*
F18	-7.3 ± 0.6	13.7 ± 0.6*	9.3 ± 0.1*	22.4 ± 0.9*	18.8 ± 0.2*
F19	-5.1 ± 0.1	7.5 ± 2.1*	7.6 ± 0.7*	23.9 ± 1.1*	21.5 ± 0.3*
F21	-7.3 ± 0.5	5.5 ± 0.4 *	7.0 ± 1.3*	2.9 ± 0.2	2.7 ± 0.2

Abbreviations - red (reduction), hr (hour). * indicates significant blood glucose reduction by the standard drug, crude and chromatographic fractions of *R. officinalis* ($p \leq 0.05$) level in the tested interval along the column

Table 6 The 21 GC-MS identified compounds of fraction 1 (F1) showing their abundance and retention time

Abundance (%)	Library/ID F1	Retention time
19.2	8-amino-2,6-dimethoxylepidine	34.3
12.7	2-mmethyl-4,5-dihydro-*1 H*-imidazole	9.3
11.5	3-ethyl-2,5-dimethyl-3-hexene,	10.0
10.6	1,8-cineole (1,8-eucalyptol)	11.7
7.7	linoleic acid	25.2
6.0	methyl hexadecanoate	23.5
5.2	bornyl acetate	15.9
4.5	1-cyclopropyldodecanone	8.5
4.0	2-methyl-2-(1-methylethyl)oxirane	4.7
4.0	2,3-dimethylhexane,	9.5
3.4	Trans, trans-2-ethylbicyclo[4.4.0]decane	10.4
2.3	4-methyl-2-pentanol	6.5
1.9	octahydro-1,3-methano-5bH-cyclobuta[cd] pentalen-5b-ol (tetracyclo[5.3.0.0^{2,6}.0^{3,9}]decan-2-ol)	7.3
1.6	DL-glutamic acid	13.0
1.6	bis-1,1′-(6-hydroxy-2,5-benzofurandiyl)ethanone	33.6
1.1	*N1*-(4-dimethylaminobenzylideno)-*N2*-(5-nitrofurfuryli-deno)hydrazine	7.2
1.1	phenylephrine	24.2
0.6	2-(adamantan-1-yl)-*N*-(1-adamantan-1-ylethyl) acetamide	26.8
0.6	2-allylaminomethylene-5,5-dimethylcyclohexane-1,3-dione	25.9
0.3	1,5-heptadien-3-yne	21.1
0.1	*N*-(methylthio)carbonyloxamide	26.3

Table 7 The 23 GC-MS identified compounds of fraction 1 (F4) showing their abundance and retention time

Abundance %	Name Library/ID F4	Retention time
16.2	hexacosane	29.88
14.1	(*8 S,8aS*)-4-(1-methylethylidene)-3,8-dimethyl-1,2,6,7,8,8a-hexahydroazulen-5-one	34.29
14.0	octacosane	31.68
12.8	heptacosane	30.71
6.3	1-iodooctadecane	31.30
5.0	9-(2-propenyl)anthracene,	33.66
4.8	*cis*-1-ethyl-2-methylcyclohexane,	9.98
4.5	2-methyl-4,5-dihydro-1*H*-imidazole	9.31
4.0	11-methylnonacosane	32.52
3.9	2-(acetoxymethyl)-3-(methoxycarbonyl)biphenylene	29.13
3.4	*(Z)*-N-benzyloxy-1-(2,3-dihydrobenzofuran-5-yl) ethanimine or *(Z)*-1-(2,3-dihydro-5-benzofuryl)ethanone *O*-benzyl oxime	35.48
2.3	4-methyl-1-heptene,	9.55
2.3	3-methyl-4-methylenehexane	8.48
2.3	3-methylheneicosane,	28.39
2.2	pyrroliphene	8.94
0.6	2-hexanol	6.49
0.5	2-heptyl-4-phenyl-1,3-dioxolane,	14.23
0.3	2,2-dimethylpropanal oxime	14.98
0.1	*N*-(1-methylpropyl)acetamide,	18.14
0.1	*N*-ethyl-*N*-cyclopentylacetamide,	22.22
0.1	N-methyl-3-piperidinecarboxamide	16.72
0.1	N-methyl-1,3-Propanediamine, -	22.99
0.1	(Z)-1,1-dimethoxy-9-octadecene	25.88

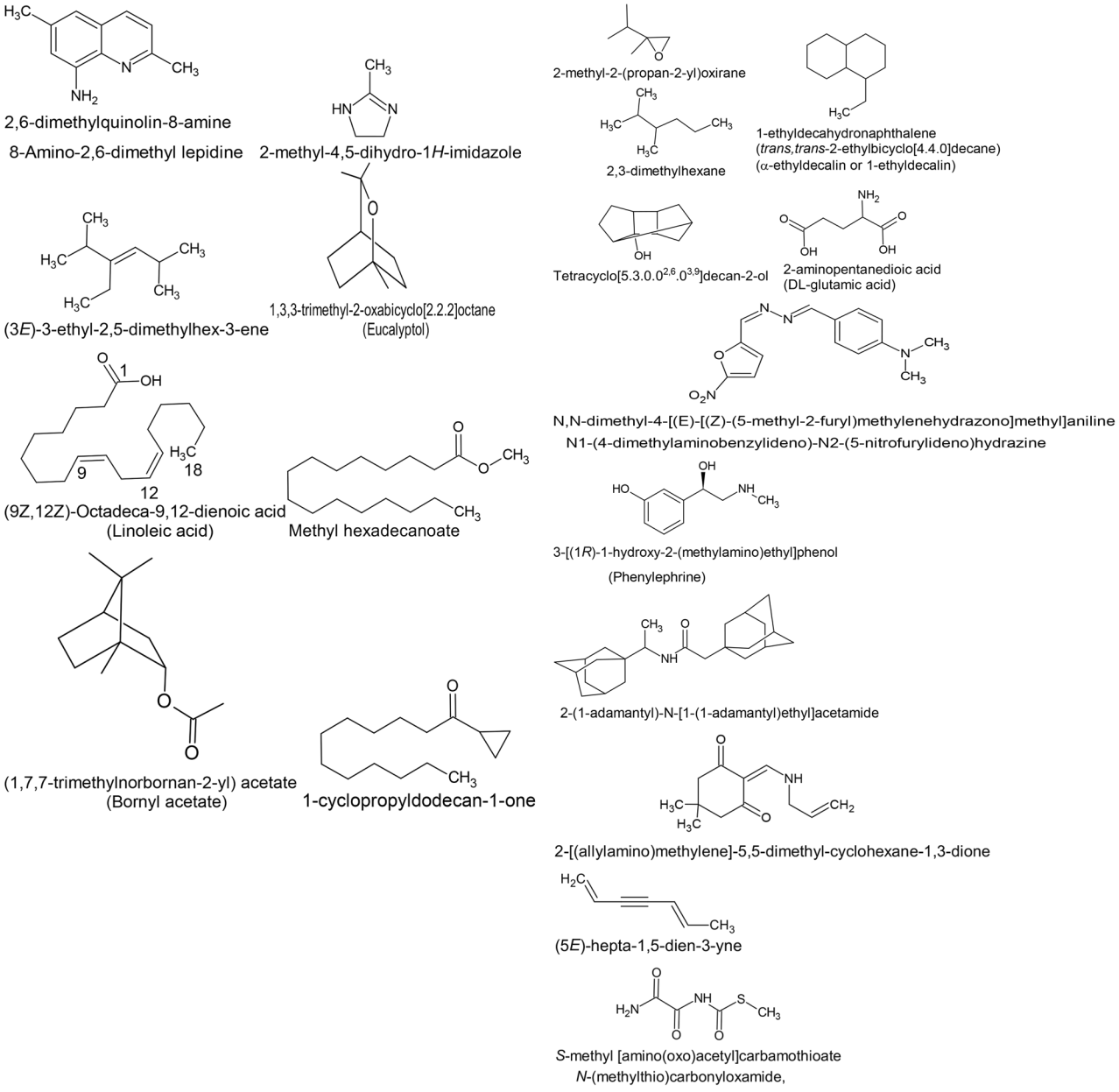

Fig. 1 The structures from the hexane fraction (F1) of of *R. officinalis* leaves crude extract obtained from 50% methanol in dichloromethane

identify compounds from 2 fractions that showed both high amylase inhibition and high hypoglycemic activity.

Plants secondary metabolites are found to be diverse in nature and employing various solvent systems optimizes the output. Earlier phytochemical analysis revealed that the extracts of *R. officinalis* using different solvents were rich in many phytochemicals [39]. Maceration of the dry *R. officinalis* leaves was found to give considerable yield of crude extract (19.2% w/w). Successive column elution with increasing polarity of organic solvents provided various fractions with some having a high amylase inhibition activity. Bioassay guided isolation (in vitro and in vivo) is an isolation method which is gaining popularity because it is able to provide prediction on how an expected bioactive component would effect a biological system [40]. This process involves repeated fractionation and bioassay testing in determined biological systems until a pure bioactive compound is achieved.

Earlier study had reported high alpha-amylase inhibition of fractions of *R. officinalis* essential oil; ethyl acetate, methanol and the crude with LC_{50} of 28.36, 34.11 and 30.39 µg/mL respectively using the 2-chloro-4-nitrophenol-D-maltotrioside method [41]. Another study had shown that *R. officinalis* crude extract possesses an

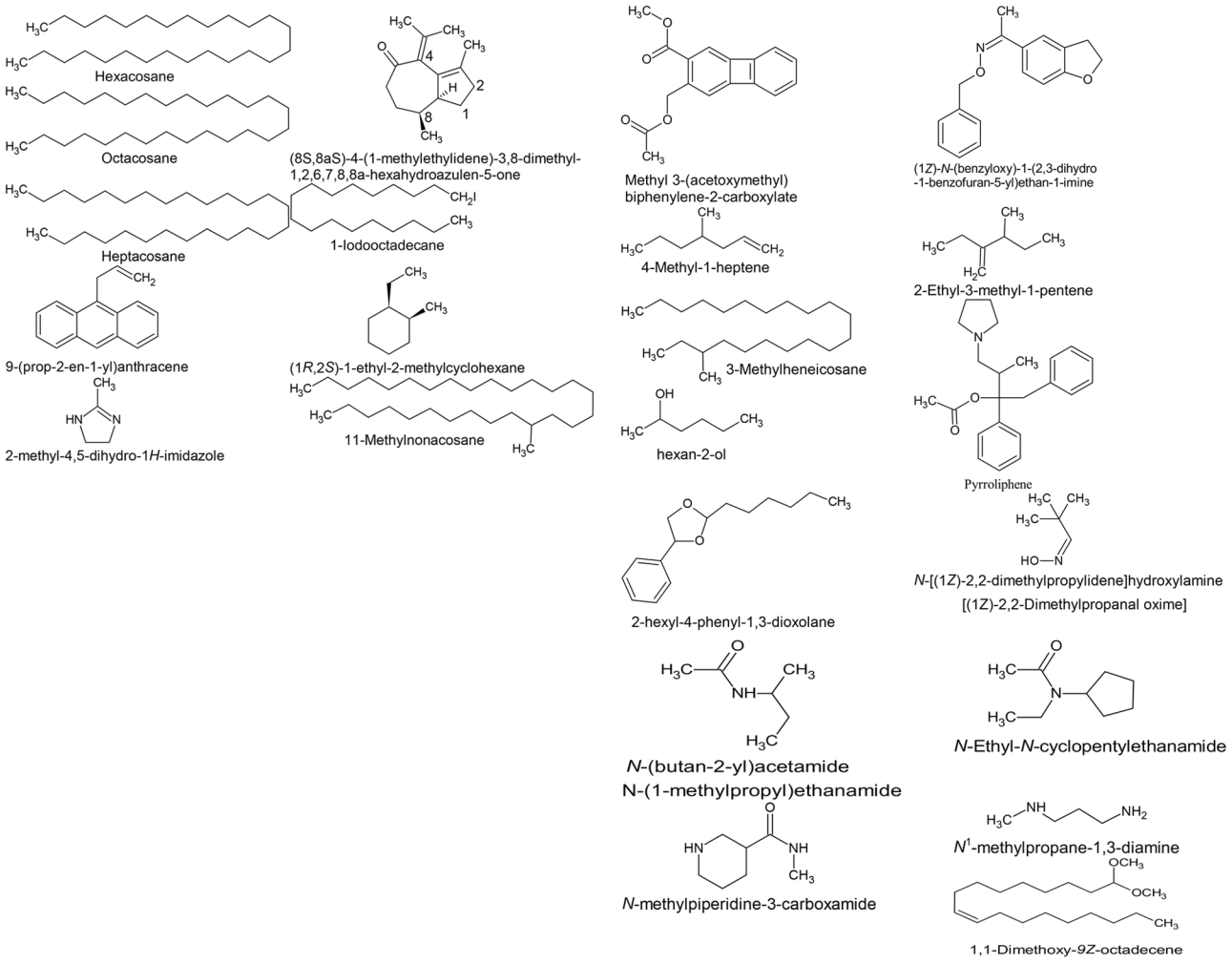

Fig. 2 The structures from the hexane/ethyl acetate (8:2) fraction (F4) of*R. officinalis* leaves crude extract obtained from 50% methanol in dichloromethane

alpha-amylase inhibitory of between 45–75%in agreement with the current study because they closely relate [42]. A high to moderate potency inhibition of the alpha-amylase of the fractions and the crude (LC_{50} of 483.8 µg/m) was realized in our current study. The following fractions: F1 (10/ 0 hex), F4 (8/2 hex: EtoAC), F8 (4/6 hex: EtoAC), F16 (8/2 EtoAC: MeOH), F18 (5/5 EtoAC: MeOH) and F19 (3/7 EtoAC: MeOH) all gave alpha-amylase inhibition LC_{50} of less than 500 µg/mL which is considered moderate. The most potent was F4 with 2.8 µg/mL which closely compared to the standard drug, glibenclamide (2.5 µg/mL). This study suggests that the alpha-amylase inhibition activity found was due to the presence of bioactive metabolites from the crude and the fractions of *R. officinalis*. The intestinal alpha-amylase together with alpha-glucosidase enzymes are known to delay the digestion of carbohydrates which eventually causes the lowered postprandial hyperglycemia [10]. Polyphenolic compounds such tannins, flavonoids and phenolic acids have been reported to have alpha-amylase inhibitory potential [43]. The alpha-amylase enzyme is known to break polysaccharides into disaccharides and oligo-saccharides which eventually are broken into glucose to enhance carbohydrate digestivity [44]. A number of anti-diabetic drugs utilize the mechanism of inhibiting this enzyme to bring about an anti-hyperglycemic process. Acarbose is one of the prescribed anti-diabetic drug whose action involves the reduction of the rate of glucose absorption through inhibition of alpha-amylase and alpha-glucosidase activity [45]. The study suggests that *R. officinalis* can be utilized in the isolation of natural alpha-amylase enzyme for carbohydrate digestion. There was also an implied correlation between the alpha-amylase inhibition and an anti-hypoglycemia activity. Previous studies of *R. officinalis* anti-diabetic potential in human participants showed a reduction of fasting blood glucose of up to 18.25% [46], comparative with the current results of 16.5% for the crude extract. The blood sugar reduction (BSR)) was three times higher in F4 (52.8% BSR) and more than two times in F1 (44.5% BSR) than the crude

extrac (16.5% BSR) suggesting that fractionation may yield more potent metabolites. The two fractions F4 and F1 showed a glucose reduction higher than that of glibenclamide (41.2% BSR), the standard drug. This study projects on attaining pure anti-diabetic molecules after further purification and these could be leads for drug discovery. The fractions (F12) and (F13) showed an increase of blood glucose more than the diabetic control suggesting their cytotoxic nature.

Many phytochemicals such alkaloids and phenols are known to regenerate the damaged β-cells caused by the cytotoxic chemicals such as STZ, thus reducing the oxidative stress in the diabetic animals. Alkaloids were also found to increase insulin secretion to body tissues thus increasing glucose absorption [47]. Carnosol, which is reported to be the major phenolic compound in *R. officinalis*, has been found to show significant antidiabetic effect [48]. It is therefore suggested that the secondary metabolites found in *R. officinalis* crude extracts and the fractions are responsible for the observed anti-hyperglycemic activity in the STZ diabetic Wistar rats. Previous profiling of hexane fraction of *R. officinalis* by GC-MS identified 47 metabolites. The 47 compounds were from different classes with phenolic diterpenes representing the most abundant class [49]. Separable phytoconstitutents of which included phenolic derivatives. Monoterpenes, fatty acid derivatives and long chain hydrocarbons were identified in this current study. The compound, 2-methyl-4,5 dihydro-1*H*-imidazole, found in both fractions (F1 and F4) is a derivative of 1,3-diazole which has been a useful for derivatization of potent biologically active compounds including those with anti-diabetic bioactivity **(51).** The most abundant compound in F4 was a long chain hydrocarbon hexacosane (16.2%). and F1 a phenolic derivative, -amino-2,6-dimethoxylepidine (19. 2%).

This study hopes to isolate, purify and characterize the molecule bringing about the anti-hypoglycemic activity.

Conclusion and recommendations

This study has shown that 50% dichloromethane: methanol *R. officinalis* crude extract possesses significant alpha-amylase inhibitory activity and anti-hyperglycemic potency. The chromatographic fractions F1 and F4 showed best bioactivity than the rest of the fractions with proven strong anti-diabetic activity qualifying them for GC-MS analysis to determine their chemical composition. This study has established the basis of study in isolation of the antidiabetic compounds from *R. officinalis* and is promising in finding compounds which can lead to future studies with respect to getting new, cheaper and safe anti-diabetic agents from *R. officinalis.* This study has validated the traditional use for *R. officinalis* as an anti-diabetic therapy.

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Author contributions
ZNK, JMM, MMP, JMW and ENN conceptualized and designed the study. ZNK performed the experiments, analyzed and interpreted data of the experiments. ENN and JMW assisted in data analysis and interpretation. JMM, MMP, JMW and ENN supervised the research work. ZNK drafted the manuscript, which was revised by JMM, MMP, JMW and ENN. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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