Journal of Advances in Medical and Pharmaceutical Sciences
2(3): 75-88, 2015, Article no.JAMPS.2015.011

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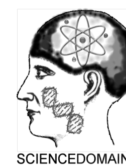

Isolation and Structural Characterization of the Most Active Antidiabetic Fraction of *Corchorus olitorius* Seed Extract

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Authors' contributions

This work was carried out in collaboration among all authors. Author MOE designed the study, collected all data, performed the statistical analysis, and wrote the first draft of the manuscript. The other authors took part in the study design, supervised the work, read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMPS/2015/14855
Editor(s):
(1) Faiyaz Shakeel, Department of Pharmaceutics, King Saud University, Saudi Arabia.
Reviewers:
(1) Anonymous, KSA.
(2) Rahul Gupta, AMITY Institute of Biotechnology, AMITY University Uttar Pradesh, India.
(3) Anonymous, Serbia.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=887&id=36&aid=7356

Original Research Article

Received 25th October 2014
Accepted 22nd November 2014
Published 16th December 2014

ABSTRACT

Aims: This study was carried out as a step further in our investigation of the antidiabetic properties of ethanolic seed extract of *Corchorus olitorius* (ewedu) in the bid to Isolate and Structurally Elucidate the Most Active Antidiabetic Fraction of *Corchorus olitorius* Seed Extract. Previous studies with the crude extract of the seed had shown positive results.
Study Design: Whole animal experimental research.
Place and Duration of Study: Department of Pharmacology, Usmanu Danfodio University, Sokoto, Nigeria between Sept 2008 and July 2013.
Methodology: The chloroform fraction of the liquid-liquid partition of ethanolic seed extract of *Corchorus olitorius* was subjected through column chromatography (cc) using a thin layer chromatography (TLC) to isolate pure compounds tagged C1, C2 and C3. The antidiabetic effects

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of the three isolates were tested in alloxan induced diabetic albino rats. Glibenclamide at a dose of 0.2mg/kg and normal saline were used as positive and negative controls respectively
Results: The results showed that there was no statistical significance difference (p >0.05) between the FBS of the treated and the positive control groups. The percentage reduction in fasting blood sugar levels of the diabetic rats produced by the pure compound C2 , C3, and glibenclamide were 17%, 10%, and 18% at the 3rd hour of treatment respectively, and 9.7%, 8.2%, and 14.4% at the 2nd hour respectively. The most active pure compound (C2) which produced a near comparatively similar result with glibenclamide was sent for Nuclear magnetic resonance (NMR) spectroscopy for structural elucidation and confirmed with gas chromatography and mass spectroscopy (GC MS) to be stearic acid ethyl ester.
Conclusion: The percentage reduction in fasting blood sugar level of diabetic rats produced by the C2 column chromatography (cc) fraction of ethanolic seed extract of *Corchorus olitorius* portrayed a lot of potentials though more evaluations is still needed.

Keywords: Thin Layer Chromatography (TLC); Nuclear Magnetic Resonance (NMR); structural elucidation; Gas Chromatography and Mass Spectroscopy (GC MS); column chromatography and Corchorus olitorius.

1. INTRODUCTION

Medicinal herbs have consistently been considered the leading source of pharmaceuticals, employed in the treatment of various human diseases due to their high chemical diversity and broad biological functionality [1]. Herbal medicine is mostly compounded from natural products therefore there is a likelihood of them being accepted by the body than synthetic substances [2]. Metformin, an important drug used in treatment of diabetes is a derivative of plant-derived compound guanidine from *Galega officinalis* [3]. Several indigenous medicinal plants are employed in the traditional management of diabetes mellitus but there is need to conduct pharmacognostic and pharmacological studies to ascertain/authenticate their therapeutic values.

The plant of interest in this study, *Corchorus olitorius* (C.O) grows in grassland and does well on abandoned fields, often close to marshes, rivers and lakes, range from warm temperate through tropical desert to wet forest life zones, at up to 1250(–1750) m altitude [4]. It thrives best under hot and humid conditions [4]. The geographical origin of *Corchorus olitorius* is often disputed; it is rather pan tropical in distribution [4]. In the savanna and Sahel zone, it grows best during the hot rainy season [4]. It is cultivated where annual rainfall averages 600–2000 mm [4]. The optimal temperature is 25–32ºC and growth stops below 15ºC [4]. In Nigeria a day length of 12.5 hours caused a much stronger vegetative growth expressed in weight of roots, stems and leaves than a day length of 11.5 hours, but the fruit and seed production was higher at a photoperiod of 11.5 hours [4]. The plant grows best in sandy loam soils rich in organic matter and grows poorly on heavy clay [4]. It is a leading leaf vegetable in Côte d'Ivoire, Benin, Nigeria, Cameroon, Sudan, Kenya, Uganda and Zimbabwe [4]. It is also cultivated as a leaf vegetable in the Caribbean, Brazil, India, Bangladesh, China, Japan, Egypt and the Middle East [4]. *Corchorus* is genus of about 40-100 species of flowering plants in the family *malvaceae* [4]. The specie of interest is *Corchorus olitorius.* Others are *Corchorus capsularis, Corchorus tridens, Corchorus walcottii, etc* [4].

Corchorus olitorius (C.O) is an annual, much-branched herb 90-120 cm tall with glabrous stems, leaves 6-10 cm long and 3.5-5 cm broad, with pale yellow flowers and black trigonous seeds [5]. The leaves of C.O was reported to have hypoglycaemic effect [6] and high antibacterial activity [7]. The seed protein enriched diet was found to increase rats body weight [8]. There was a failure to produce adverse effects in young chicken, with levels of seeds (C.O) up to 5% of the diet [9]. The seeds were found to contain reasonable percentage of biologically active cardiac principals [10]. The plant stem is a source of jute fibre, and folkloric uses includes, seeds for purgative, leaves for dysentery, fever, gonorrhea and demulcent [11]. Its local name in Nigeria includes; ewedu (Yoruba name), oyoyo (Hausa name) and arira (Igbo name). Other vernacular names include Jew's mallow, krinkrin, tossa jute, bush okra, West African sorrel to mention few. It is used as a leafy mucilaginous vegetable for sauce/soup [4].

The part of the plant targeted in this study is the seed reported to have a hypoglycaemic effect [12]. The safety of the crude seed ethanolic extract of C.O was evaluated in white albino rats as a prelude to the assessment of its hypoglycemic effect and found to be 5000 mg/kg [13]. In the bid to further evaluate the ethanolic seed extract it was fractionated using different solvents (hexane, ethyl acetate, chloroform and saturated butanol) and tested for antidiabetic effect in white albino rats. The chloroform fraction of the liquid liquid fractionation was reported to have antidiabetic effect following testing in normoglycaemic, oral glucose challenged, and alloxan induced diabetic albino rats [14]. The present study is the final stage in the evaluation of the effect of the ethanolic seed extract following liquid liquid partition fractionation and column chromatography fractionation in order to isolate the pure compound possibly responsible for the antidiabetic effect and its structural elucidation. Therefore the rational of this study was to identify the compound responsible for the antidiabetic effect of the ewedu seed extract.

2. MATERIALS AND METHODS

2.1 Laboratory Animals

White albino Rats (Sprague Dawley rats) of both sexes from the Biological Department of Usman Dan Fodiyo University (UDUS) were used for the study. The rats were housed in metal cages in the laboratory at temperature between 35-37ºC; 12hr/12hr light/dark cycle and maintained with free access to standard rat feeds and water, for 7 days before experimentation. 12hrs before experimentation, food was withdrawn but water available *ad libitum*.

2.2 Extraction and Fractionation Procedure/Column Chromatography Procedure

Extraction and fractionation was according to Gandhi et al. [15] and Leila et al. [16] with some modification in the choice of primary solvent (water) and partitioning solvents (hexane, chloroform, ethyl acetate and butanol). The most potent fraction, chloroform, was selected and subjected to fractionation in column chromatography using silica gel.

A glass chromatography column was set up. The column was then filled with hexane. 20 g of silica gel (60 mesh) was placed in a flask and hexane poured into the flask, the slurry was then packed into the column while tapping the glass. After packing, the excess solvent was drained until it just reached the top level of the silica gel. A thin layer of cotton was placed on top of the column to prevent it from being disturbed when fresh solvent was added. The sample (5 g of chloroform fraction) was dissolved in a very small amount of chloroform and silica gel then loaded dried to the top of the column. A small amount of the eluting solvent (hexane, hexane- ethyl acetate) was added and allowed to drain in until the mixture was a little way into the adsorbent, then the column was filled to the top with eluting solvent (hexane). The solvent system started with 100% hexane and 0% ethyl acetate then subsequently increasing the polarity by 1%. The eluent were collected in numbered test tubes (15 mls) for TLC monitoring. The procedure was stopped on eluent of the last pure compound. The solvent level was never allowed to drop below the top of the adsorbent. Thin layer chromatography (TLC) was used to monitor the effectiveness of this separation. The process was discontinued when the compound(s) desired were off the column. The eluent with the same pure compounds were pooled and finally separated using a preperative TLC plate (Fig. 1) with concentration zone in a glass developing chamber, after collecting compound in the rotary evaporator. The obtained pure compound(s) were isolated and identified through thin layer chromatography (TLC). The pure compounds were then bioassayed in alloxan induced diabetic rats. The dose administered was calculated thus; in 5 gram of chloroform fraction, 2.3 gram of total pure compound was extracted following column chromatography. And the dose employed on bioassay of fractions was 500 mg/kg. Therefore that dose (500 mg/kg) contained about 230mg of active compound. Hence the dose for the bioassay was chosen to be 230 mg/kg. This calculation took cognizance of previous work and dose of its local use. The most active pure compound (C2) was sent for Nuclear magnetic resonance (NMR) spectroscopy for structural elucidation.

2.3 Nuclear Magnetic Resonance (NMR) Spectroscopy for the Elucidation of the Stereochemistry of the Most Active C.O Seed Fraction

NMR data were recorded at 25ºC for samples of 1–20 mg of compound dissolved in CDCl3 using Topspin 300 MHz, Bruker Germany at University of Pretoria, South Africa. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with TMS as internal

standard reference which was added to the sample before recording, operating at 300 and 75.5 MHz respectively. The proton magnetic resonance (^{1}H-NMR) was recorded telling of the the number of signals (which tells how many different "kinds" of protons there are in a molecule), the position of the signals (which tells about the electronic environment of each kind of proton), the intensities of the signals (which tells how many protons of each kind there are), the splitting of a signal into several peaks (which tells about the environment of a proton with respect to other, nearby protons). The ^{13}C NMR Spectrum or CMR spectrum was generated in the fundamental way as the proton NMR spectrum. Recorded were, the number of signals (telling how many different carbons or different sets of equivalent carbons there are in a molecule), the splitting of a signal (telling how many hydrogens are attached to each carbon), the chemical shift (telling the hybridization of each carbon) and another chemical shift (telling about the electronic environment of each carbon with respect to other, nearby carbons or functional groups). The results are presented as 1dimensional (1D) i.e. 1D ^{1}H and ^{13}C NMR and 2dimensional (2D) i.e. *2D ^{1}H,^{1}H-COSY* (Correlated Spectroscopy), 2D Nuclear Overhauser Enhancement Spectroscopy (NOESY), HMQC (Heteronuclear Multiple Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation). After the structure was proposed, the named structure was put in the Chem Draw Ultra Version 8 software for simulation to compare with NMR generated ppm (parts per million).

Fig. 1. A preperative TLC plate with concentration zone

2.4 Gas Chromatography/mass Spectrometry (GC MS) of the most Active C.O Seed Fraction

The sample (less than 1ng) was run in a GC MS Agilent Technologies (7890A) at the Department of chemistry, University of Pretoria, South Africa. Gas chromatography and mass spectrometry are, in many ways, highly compatible techniques. In both techniques, the sample is in the vapor phase. The compound exiting the gas chromatograph is a trace component in the GC's carrier gas at a pressure of about 760 torr, but the mass spectrometer operates at a vacuum of about 10^{-6} to 10^{-5} torr. This is a difference in pressure of 8 to 9 orders of magnitude. GC can separate volatile and semi volatile compounds with great resolution, but it cannot identify them. MS can provide detailed structural information on most compounds such that they can be exactly identified, but it cannot readily separate them [17]. MS detection enables the determination of molecular masses of target compounds and the elucidation of their molecular structure. The molecular structure can be deduced from the specific fragmentation patterns that organic compounds show upon bombardment with fast electrons in an MS ion source. This mode is called electron ionisation (EI) under which sample molecules lose an electron resulting in a molecular ion (M+., radical cation). Due to the high amount of energy (70 eV) impacted to the molecular ion it usually fragments producing further smaller ("daughter") ions with characteristic relative abundances that provide a "fingerprint" for that molecular structure. Using searchable data bases, this information helps to identify compounds of interest and supports structural elucidation of unknown components [18]. The result therefore comes out showing peaks of the unknown compound compared with peaks of known compounds in the computer data base (incorporated in the GCMS) suggesting the structure as well.

2.5 Antidiabetic Studies

2.5.1 Induction of diabetes in rats

The rats were induced diabetes by peritoneal injection with alloxan monohydrate dissolved in sterile normal saline solutions at a dose of 150 mg/kg body weight after overnight fast [19,20,21]. Animals with moderate hyperglycemias, blood glucose of 150 mg/dl and above were considered diabetic and employed in the study 3 days after alloxan induction. The

normal control was injected intraperitoneally with sterile normal saline (2 ml/kg).

2.5.2 Hypoglycemic activity in alloxan induced diabetic rats

In this experiment the groups of rats were

Group C1	Consisted of alloxan induced diabetic rats to which 230 mg/kg C1 fraction was administered.
Group C2	Consisted of alloxan induced diabetic rats to which 230 mg/kg C2 fraction was administered.
Group C3	Consisted of alloxan induced diabetic rats to which 230 mg/kg C3 fraction was administered.
Group 4	Consisted of alloxan induced diabetic rats to which glibenclamide (0.2 mg/kg) was administered.
Group 5	Consisted of alloxan induced diabetic rats to which vehicle (normal saline) was administered. Glucose levels were measured just prior to and 1, 2 and 3 hours after extract/ drug were administered (adm) (t=0min) [21]. And each group consisted of 5 rats each.

2.6 Statistical Methods

Results were expressed as Mean+ S.D. The instat statistical software was employed. ANOVA was used to compare means using turkey Kramer test. Significances is P ≤ 0.05.

3. RESULTS AND DISCUSSION

3.1 Column Chromatography (cc) of Chloroform Fraction

The pure compounds were pooled according to likes (similar compounds) following each cc as in Table 1. The pure compounds C1, C2 and C3 were identified in Figs. 2a, 2b and 2c TLC plates.

3.2 Bioassay of Pure Compounds Isolated from Chloroform Fraction of C.O (C1, C2 and C3) in Alloxan Induced Diabetic Rats

There was no statistical significance seen using the FBS change (changes of FBS post treatment) (Table 2) but the percentage reduction in blood sugar (untreated FBS-treated FBS /untreated FBS X 100) showed pure compounds C2, C3, and glibenclamide with blood sugar reduction of 17%, 10%, and 18% respectively at the 3rd hour of treatment (Table 3) and 9.7%, 8.2%, and 14.4% respectively at the 2nd hour.

3.3 Structural analysis of C2 using NMR

The NMR spectra are as seen in the1D ^{1}H NMR (Fig. 3), 1D ^{13}C NMR (Fig. 4), 1D ^{13}C DEPT (Fig. 5), 2D ^{1}H NOESY (Fig. 6), 2D ^{1}H COSY (Fig. 7) and 2D ^{1}H- ^{13}C HMBC (Fig. 8). And the ^{1}H and ^{13}C NMR (Chem draw ultra version 8) simulated NMR (Fig. 9) confirmed the structure proposed. The structure proposed is stearic acid ethyl ester (Fig. 10). Its other names (synonyms) are Ethyl Stearate; Eighteen ethyl; Stearic acid ethyl and Ethyl Octadecanoate. Presented also are the ^{1}H and ^{13}C NMR chemical shift table with reference known like compounds (Tables 4 and 5).

3.4 Structural analysis of C2 using GC MS

The result of the GC MS came out showing peaks of the unknown compound (Fig. 11a) compared with peaks of known compounds (Fig. 11b) in the computer data base (incorporated in the GCMS) with a suggested structure, confirming the NMR structure elucidated earlier as Stearic acid ethyl ester (Fig. 10) with molecular weight of 312.

4. DISCUSSION

The chromatographic and preparative TLC separation showed the chloroform fraction to have C1, C2 and C3 compounds. The bioassay in diabetic rats showed the C2 to be most active with 17% reduction and glibenclamide having 18% at the 3rd hour post treatment. C3 only had a 10% reduction in blood sugar at the 3rd hour. The efficacy of the C2 compound is therefore likened to glibenclamide used in the treatment of diabetes. This finding further authenticates the leaves hypoglycaemic effect [6,24], traditional practitioners' usage of and antidiabetis reports of crude ethanolic extract [12] / chloroform fractions effects of *Corchorus olitorius* seed [14].

The compound C2, which is the most active pure compound of the chloroform fraction, following NMR and GC MS structural elucidation was found to be stearic acid ethyl ester with molecular weight of 312.53, molecular fomula $C_{20}H_{40}O_2$, an esterified product of Stearic Acid. Treatment of cells with fatty acid ethyl esters has

demonstrated the ability to induce apoptosis and phases [25] which supports/validate the folkloric report of *Corchorus olitorius* use in the treatment of tumours [26]. Stearic Acid ethyl ester (a saturated fatty acid), have been reported to be essential for biological activities of lipopolysaccharides [27]. Furthermore, it displays the capacity to induce NFκB (nuclear factor κB) activation and Cox-2 expression [28] which validate *Corchorus olitorius* folkloric use in the treatment of pains and fever in human [26] and reports of anti inflammatory and anti pyretic activity in rats [29]. In this study stearic acid ethyl ester has been found to have hypoglycaemic effect likened in percentage of blood sugar interfere with the cell cycle in the G_2/M and S reduction to glibenclamide. And to the best of our knowledge, this is the 1st time stearic acid ethyl ester of *Corchorus olitorius* plant derivative is reported of hypoglycaemic activity. This finding buttresses the fact that Medicinal herbs have consistently been considered the leading source of pharmaceuticals, employed in the treatment of various human diseases due to their high chemical diversity and broad biological functionality [1]. Another plant-derived compound is guanidine from *Galega officinalis,* from which Metformin an important drug used in treatment of diabetes was derived [3].

Table 1. Pooling of eluents of column chromatography (cc)

CC	C1	C2	C3	C1 with 2	C2 with 3
1	11-12	25-32	35-45	13-24	32-34
2	8-11	27-32	42-60	12-26	33-41
3	28-34	61-85	100-110	35-60	86-99
4	21-27	44-60	66-83	29-43	61-83
5	6-8	14-27	36-60	9-13	28-35
6	6-11	26-46	58-62	12-25	47-57
7	5-9	19-29	45-69	10-18	30-44

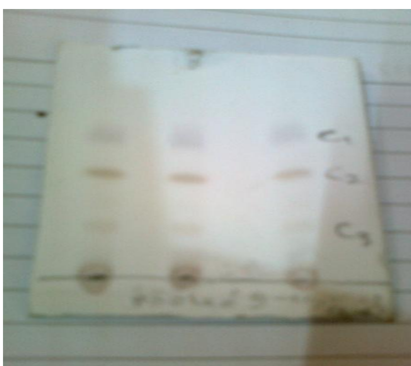


Fig. 2a. Separated and visualized spots of chloroform fraction

Fig. 2b. TLC of Pure compound C1 and C3 confirmed

Fig. 2c. TLC of Pure compound C2 confirmed

Table 2. FBS change of pure compounds of chloroform fraction in diabetic rats

FBS	C1	C2	C3	Glibenclamide	Control
0hrs	364.20±164.14	302.00±122.76	340.60±127.09	344.40±158.60	302.40±116.86
30 minutes	424.80±163.3	310.80±152.34	363.40±86.38	360.00±151.16	308.00±120.29
1hr	407.20±164.18	303.80±163.32	349.80±91.25	322.40±126.76	299.20±115.09
2hr	384.20±167.14	272.60±146.33	312.60±93.70	294.80±148.35	295.0±111.36
3hr	356.40±133.95	250.60±138.03	306.40±85.69	279.80±143.65	294.80±113.55

*Values are mean ± SD (n=5). *significant difference (p<0.05)*

Table 3. Pure compounds percentage reduction in blood sugar in diabetic rats

FBS	C1	C2	C3	Glibenclamide	Control
30 minutes	-16.6%	-2.9%	-0.1%	-4.5%	-1.9%
1hr	-11.8%	-0.6%	-2.7%	-6.4%	1.1%
2hr	-5.5%	9.7%	8.2%	14.4%	2.4%
3hr	2.1%	17.0%	10.0%	18.8%	2.5%

Percentage reduction in blood sugar%= untreated FBS-treated FBS /untreated FBS X100

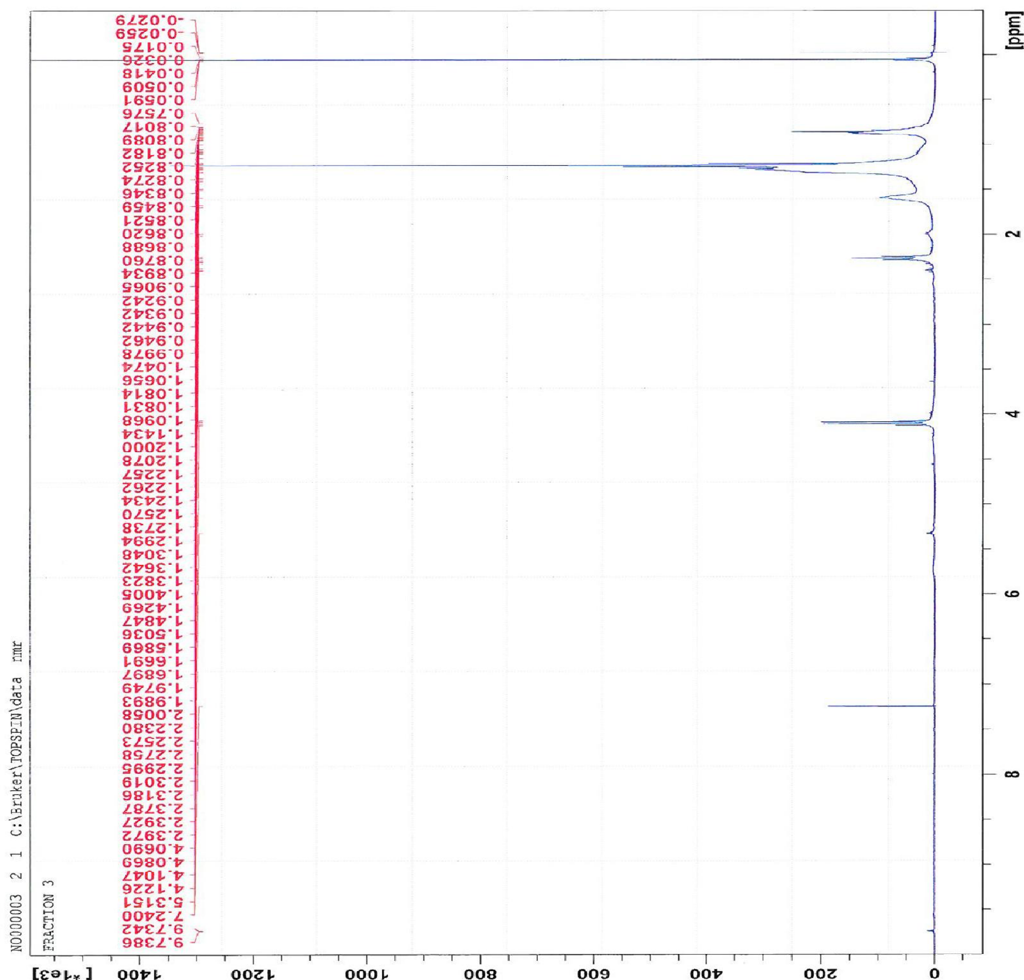


Fig. 3. The 1D ^{1}H NMR with chemical shift ppm shows protons signals in the molecule with the chemical shift

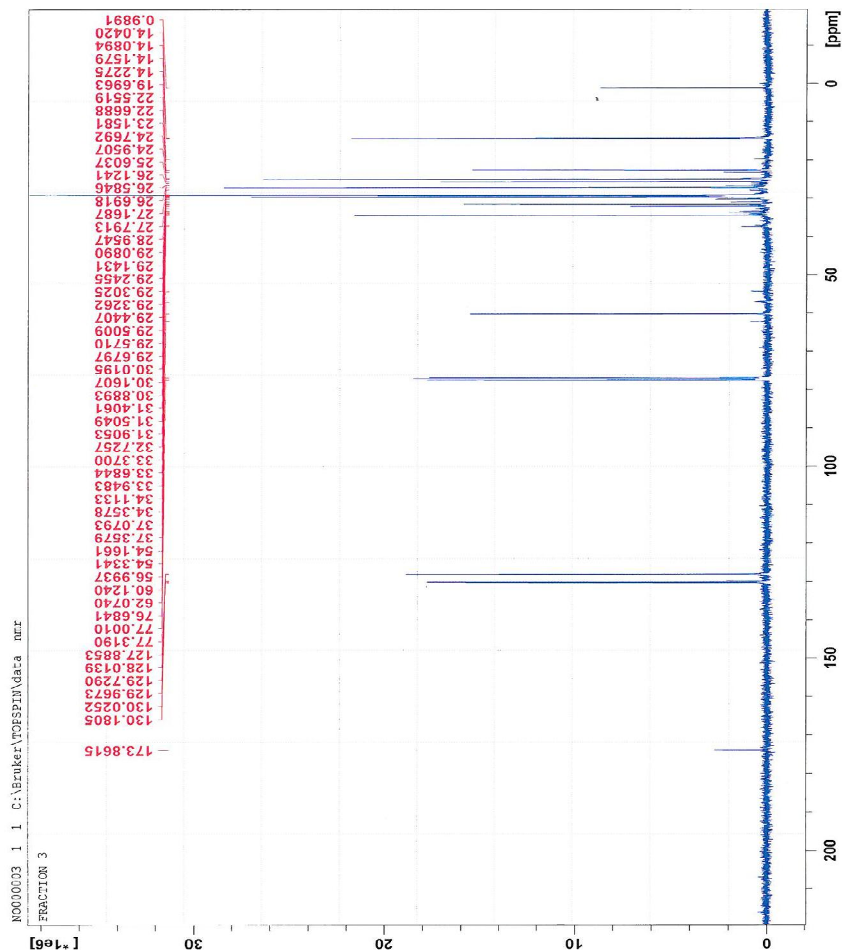


Fig. 4. 1D ^{13}C NMR with chemical shift ppm Shows ^{13}C signals with its chemical shift

Table 4. The ^{1}H chemical shift compared with reference known like compounds

c/p no	δ_H (ppm) of C2	δ_H (ppm) computer	δ_H (ppm)ref1	δ_H (ppm)ref2
1				
2	2.26	2.25	2.35	1.25
3	1.67	1.68	1.65	Nc
4	1.30	1.29	1.3	Nc
5	1.30	1.29		Nc
6	1.28	1.29		Nc
7	1.24	1.29		Nc
8	1.23	1.29		Nc
9	1.23	1.29		Nc
10	1.21	1.29		Nc
11	1.20	1.29		Nc
12	1.14	1.29		Nc
13	1.10	1.29		Nc
14	1.10	1.29		Nc
15	1.10	1.29		Nc
16	1.10	1.29	1.4	Nc
17	1.36	1.33		Nc
18	0.95	0.96	0.88	

Nc (not cited); c/p no (carbon position number); ref (reference)Ref 1 [22]; Ref 2 [23]

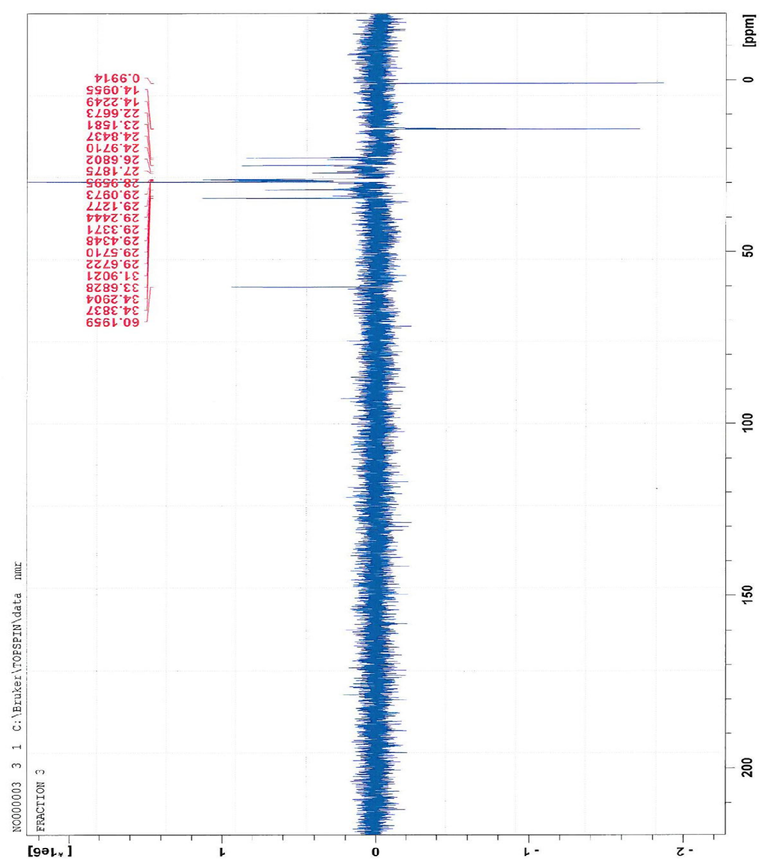


Fig. 5. 1D ^{13}C DEPT showing shift of CH_2 and CH_3 positions

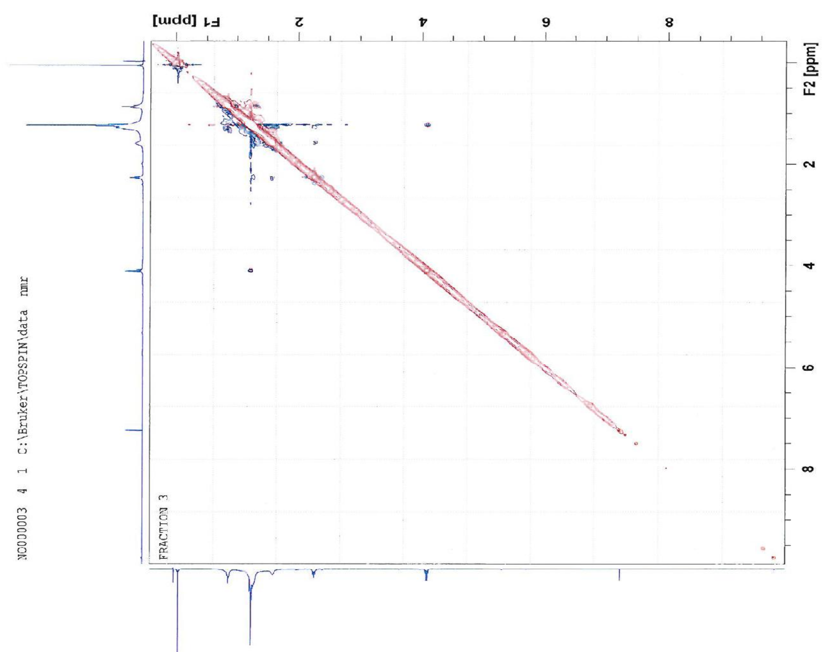


Fig. 6. 2D ^1H NOESY Showing the ^1H ^1H through space coupling information

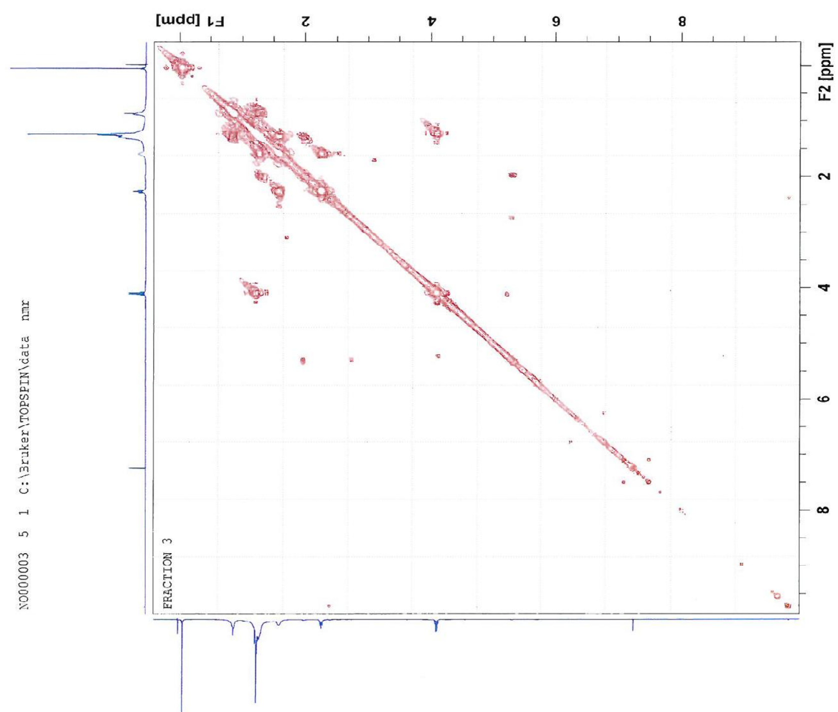


Fig. 7. 2D ^{1}H ^{1}H COSY Showing the ^{1}H ^{1}H through bond coupling information

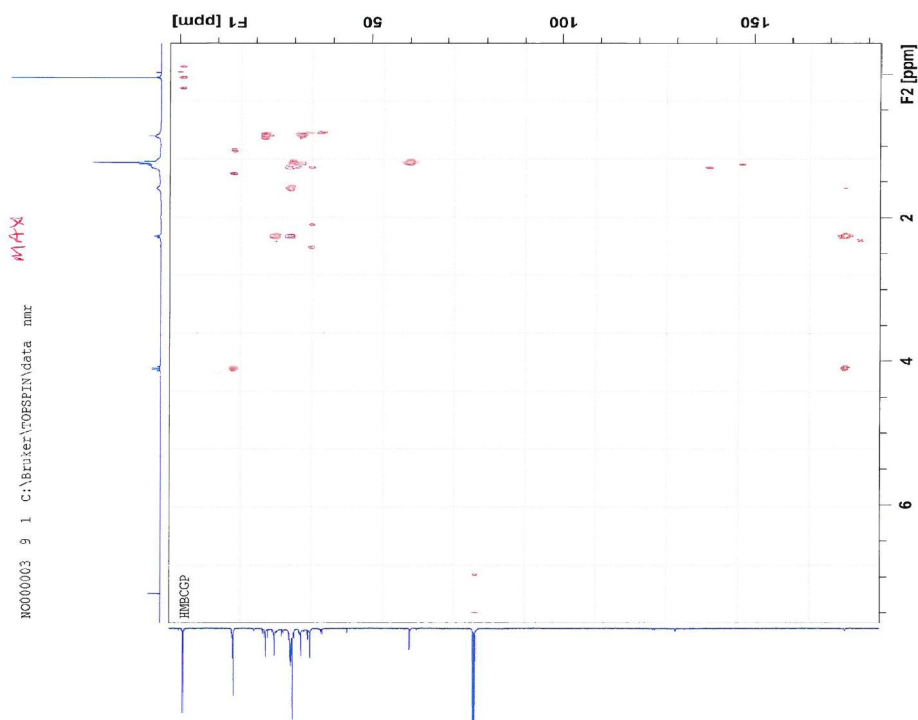
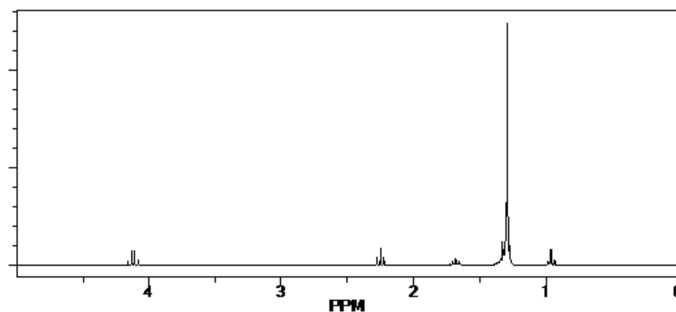


Fig. 8. 2D 1H- ^{13}C HMBC Shows crosspeaks for protons and carbons separated by 2 bonds

^{1}H



^{13}C

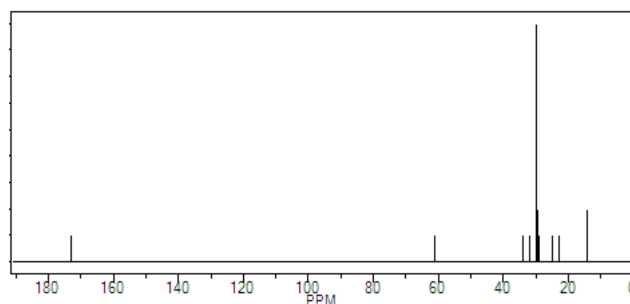


Fig. 9. Computer simulated ^{1}H and ^{13}C NMR

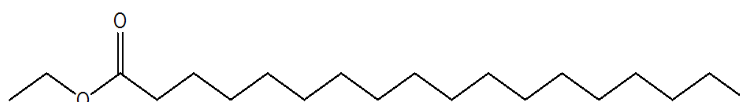


Fig. 10. Stearic acid ethyl ester (ethyl octadecanoate) structure molecular formula: $C_{20}H_{40}O_2$

Table 5. ^{13}C NMR chemical shift compared with reference known like compounds

c/p no	δ_c (ppm) of C2	δ_c(ppm) Computer	δ_c (ppm)ref1	δ_c (ppm)ref2
1	173.9	173.1	173.4	173.8
2	33.7	33.9	39.5	Nc
3	25.0	25.1	33.9	Nc
4	29.1	29.1	27.3	Nc
5	29.4	29.4	Nc	Nc
6	29.7	29.7	Nc	Nc
7	29.6	29.7	Nc	Nc
8	29.3	29.7	Nc	Nc
9	29.2	29.7	Nc	Nc
10	29.0	29.7	Nc	Nc
11	27.2	29.7	Nc	Nc
12	26.7	29.7	Nc	Nc
13	26.7	29.7	Nc	Nc
14	24.8	29.7	Nc	Nc
15	25.0	29.4	Nc	Nc
16	31.9	31.9	Nc	Nc
17	22.7	22.8	Nc	Nc
18	14.2	14.1	17.1	14.0
19	60.2	61.3	Nc	60.1
20	14.1	14.1	17.1	14.0

Nc (not cited); c/p no (carbon position number); ref (reference) Ref 1[22]; Ref 2[23]

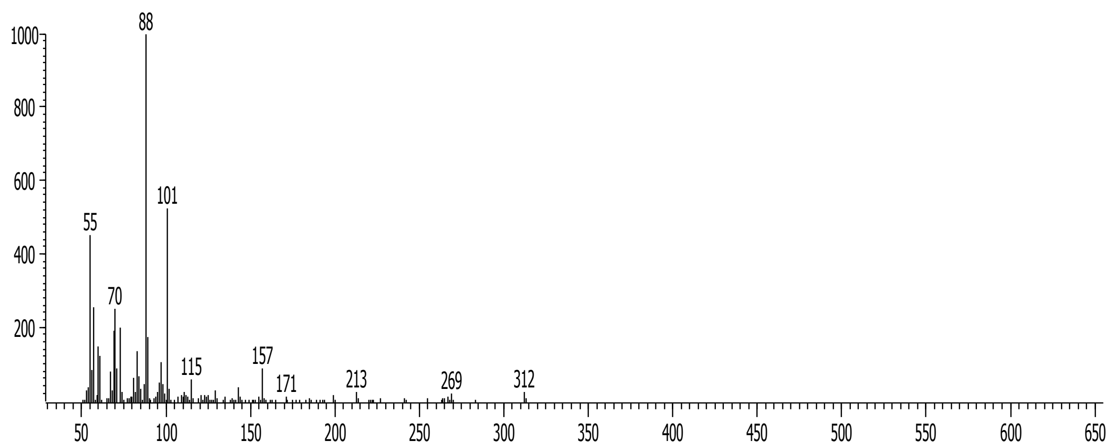


Fig. 11a. Compound C2 GC MS data showing peaks

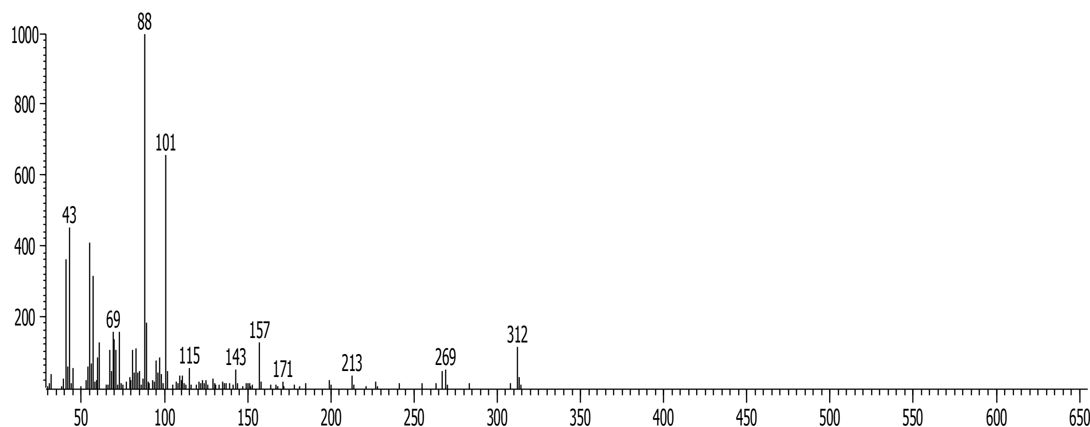


Fig. 11b. GC MS data base for like compound (Octadecanoic acid ethyl ester)

The percentage reduction in fasting blood sugar level of diabetic rats produced by the C2 column chromatography (cc) fraction (stearic acid ethyl ester) of ethanolic seed extract of *Corchorus olitorius* portrayed a lot of potentials though more evaluations is still needed. Therefore there exist a great potential for the development of a novel anti diabetic agent/agents in the seed of *Corchorus olitorius* as elucidated in this research. Furthermore, the toxicity of the crude ethanolic extract in previous study [13] cannot be exactly inferred on the fractions or the isolated pure compounds (stearic acid ethyl ester particularly). Therefore, further researches in the following areas are recommended.

1. Assessment of safety profiles of the isolated fractions in whole animal and stem cells preparations.
2. Column chromatography separation of other fractions into pure compounds be undertaken and anti diabetic bioassay of pure compounds obtained.
3. Toxicity study of potential pure compounds should be undertaken.

5. CONCLUSION

In conclusion, the most active anti diabetic fraction of the crude ethanolic extract of *Corchorus olitorius* seed is the isolated pure compounds stearic acid ethyl ester though,

further research is needed in the development of the compound for pharmaceutical use

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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