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Research Article

Bioassay-guided Fractionation, Antihyperglycemic and Antioxidant Properties of the Methanol Leaf Extract of *Helianthus annuus*

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Abstract
The aim of the study was to separate the crude methanol extract of *Helianthus annuus* into fractions and to evaluate the antidiabetic, antioxidant and preliminary chemical properties of the fractions. The bioassay-guided separation of *H. annuus* extract was done using column and thin layer chromatographic techniques. The extract yielded thirteen fractions. Bioactivity screening of the fractions (60 mg/kg) using alloxan-induced hyperglycemic rats showed that fraction 8, 9, 10 and 13 caused various degrees of reduction in FBG in time-dependent manner. The activities of the fractions were compared to the crude extract (600 mg/kg). The crude extract (HAE), glibenclamide, fractions 8, 9, 10 and 13 caused 66.74%, 57.43%, 61.36%, 59.80%, 70.63% and 78.03% reductions in FBG, respectively, at 6 h. *In vitro* anti-oxidant tests at the concentration of 400 µg/ml showed that HAE, fraction 8, 9,10 and 13 gave 89.00%, 30.42%, 47.90%, 88.03% and 92.72% anti-oxidant activity, respectively, using DPPH model and 3.69, 0.95, 0.23, 0.67 and 0.28 µM anti-oxidant potential, respectively, in FRAP model. Ascorbic acid used as control has antioxidant potential of 2.00 µM at concentration of 1000 µg/ml using FRAP model and 99.31% at concentration of 400 µg/ml using DPPH model. Phytochemical spot tests of the HAE showed the presence of saponins, alkaloids, flavonoids, terpenes, glycosides and tannins while fraction 8 and 10 contain mainly saponins, glycosides and sterols and fraction 13 contains mainly tannins.The study validates the use of the preparations from *H. annuus* leaves in folklore diabetic therapy.

Keywords: Alloxan monohydrate, diabetes mellitus, antioxidant, triterpenoid saponins, *Helianthus annuus*

INTRODUCTION

Herbal medicine has been used by many people in the developing countries for the treatment of various ailments[1]. This is because herbal medicines are cheaper, less toxic and easily accessed than most orthodox medication. Some orthodox medicines have reduced efficacy due to drug resistance, hypersensitivity reactions and other side effects[2]. In recent times, plants with medicinal values have provided interesting area of research, as source of novel drug for the treatment of plethora of disease. One of such disease, which from ancient times has posed threat to both humans and animals, is diabetes mellitus.

Diabetes mellitus is chronic metabolic disorder characterized by hyperglycemia with impaired metabolism of carbohydrate, fat and protein resulting from defect in insulin secretion, insulin action or a combination of both factors [3]. World Health Organization (WHO) reported that about 171 million people worldwide suffer from diabetes mellitus, which is about 2.8% of the world population [4]. Worldwide projection suggest that more than 300 million people will have diabetes by the year 2025 and the global cost of treating diabetes and its complications could reach US $1 trillion annually[5]. It is treated or managed through exogenous insulin administration, lifestyle change and use of oral hypoglycemic agents. Several hypoglycemic agents have been developed to be employed in the management of diabetes mellitus and its complications but none has met the qualities of an ideal hypoglycemic agent[6]. On the other hand, many plant species are known in folk medicine of different cultures for the treatment of diabetes mellitus[7]. In spite of this, few of these plants have been given proper scientific screening for hypoglycemic properties. The World Health Organization has recommended that this area warrant further scientific interest[6].

Helianthus annuus (Asteraceae) is an annual plant native to America. It possesses large inflorescence; it has a rough hairy stem and broad coarsely toothed leaves. It has a cosmopolitan distribution and grows up to 1.5 – 3.5 metres in height. It has been reported to possess a number of therapeutic values in folk medicines in managing of a wide range disease such as diabetes mellitus, respiratory disorder, stomach problems, diuresis and inflammatory diseases[8]. Currently, there is scientific evidence of the antimicrobial[9], anti-inflammatory[10], antidiabetic and antioxidant[6] properties of the crude extract of the leaves

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Table 1: Yield of the fractions of HAE

Fractions	Yield (mg)	Percent yield (% w/w)
Fraction 1	26.4	0.264
Fraction 2	396.9	3.969
Fraction 3	95.4	0.954
Fraction 4	28.3	0.283
Fraction 5	125.3	1.253
Fraction 6	314.2	3.142
Fraction 7	239.0	2.390
Fraction 8	97.7	0.977
Fraction 9	47.6	0.476
Fraction 10	90.7	0.907
Fraction 11	299.6	2.996
Fraction 12	2881.5	28.815
Fraction 13	2030.0	20.300

of *H. annuus*. The present study is undertaken to separate the crude methanol extract of *H. annuus* into fractions and to evaluate the antidiabetic, antioxidant and preliminary chemical properties of the fractions.

MATERIALS AND METHODS

Animals

Forty five male Albino Wistar rats (120-170 g) obtained from the Laboratory Animal Unit of the Department of Veterinary Physiology and Pharmacology, University Nigeria, Nsukka, were used for this study. They were housed in wire mesh cage at 6 rats per cage and were fed *ad libitum* with standard commercial pelleted feed (Vital feed®, Nigeria) with free access to clean drinking water. They were kept at environmental temperature and natural cycle of light and darkness. They were maintained in accordance with the recommendation of the *Guide for the Care and Use of Laboratory Animals*[11]. They were allowed two weeks to acclimatize before the commencement of the experiment. The animal experiment protocol was approved by University of Nigeria, Nsukka animal ethics committee.

Extraction and Separation of the Plant Material

The fresh green leaves of *H. annuus*, were collected from the premises of University of Nigeria, Nsukka in Enugu State, South Eastern Nigeria in June 2012 and were confirmed as *H. annuus* by a plant taxonomist and was catalogued UNN/VPP/2012/02 in the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka herbarium. The leaves were dried on top of laboratory bench at room temperature and pulverized into a coarse powder using a laboratory hammer mill. Four hundred gram of the plant material was extracted by cold maceration method using 80% methanol (Sigma-Aldrich, Germany) with intermittent shaking for 48 h. The extract was filtered with Whatmann No. 1 filter papers and concentrated *in vacuo* to dryness using rotary evaporator (Buchi Labotechnik, Switzerland) and was referred to as *H. annuus* extract (HAE).

The HAE was separated into fractions using column chromatography[12]. Briefly silica gel 60 G for column chromatography (Vicker, West York England) was used as the stationary phase and 10 g of HAE adsorbed to sea sand was loaded on top of the column. The column was eluted with n-hexane, chloroform, ethyl acetate and methanol in ascending order of polarity. Four hundred and thirty nine aliquots 10 ml of column fractions were collected and spotted on pre-coated silica gel (F_{254}) aluminium plate for thin layer chromatography (TLC) (Merck, Germany) and eluted with chloroform-ethyl acetate-methanol (11:1:1) in a small chromatographic tank to further separate the various fractions based on their relative mobility on TLC plate and colour reactions with ultra violet light[13]. This procedure yielded 13 fractions which were concentrated to dryness using rotary evaporator at reduced pressure and 40°C and was referred to as *H. annuus* extract fractions (HAEF). The percentage of the crude HAE and fraction were calculated using the

Table 2: Percentage reduction of fasting blood glucose level in alloxan-induced hyperglycemic rats treated with crude and fractions of HAE and glibenclamide (mean ± SEM %).

Treatment	Dose (mg/kg)	1 h	3 h	6 h
Glibenclamide	2	19.34 ± 14.06	24.68 ± 12.26	57.43 ± 4.53
Crude extract	600	51.48 ± 14.42	60.74 ± 9.79*	66.74 ± 12.96
Fraction 1	60	42.01 ± 4.04	49.05 ± 0.95	53.53 ± 0.90
Fraction 2	60	9.11 ± 8.08a	29.64 ± 1.23	54.34 ± 23.09
Fraction 3	60	35.06 ± 21.63	40.05 ± 22.15	42.52 ± 23.62
Fraction 4	60	44.89 ± 7.68	47.09 ± 2.91	53.91 ± 5.07
Fraction 5	60	19.30 ± 11.22	42.61 ± 25.57	55.75 ± 17.63
Fraction 6	60	2.12 ± 6.97a	19.09 ±8.18a	43.33 ± 10.00
Fraction 7	60	14.75 ± 2.62	24.05 ± 2.24a	16.79 ± 19.83*a
Fraction 8	60	21.21 ± 5.89	38.09 ± 4.90	61.36 ± 11.83
Fraction 9	60	55.22 ± 8.65	54.38 ± 7.80	59.80 ± 9.11
Fraction 10	60	36.74 ± 6.40	63.76 ± 9.84	70.63 ± 6.90
Fraction 11	60	50.05 ± 4.30	52.23 ± 7.55	54.41 ± 9.72
Fraction 12	60	41.85 ± 6.14	47.43 ± 4.57	55.68 ± 0.32
Fraction 13	60	60.11 ± 19.26*	68.11 ± 15.30*	78.03 ± 2.68

*= $p < 0.05$ are statistically different vertically when compared to glibenclamide

a= $p < 0.05$ are statistically different vertically when compared to crude extract of HAE

formular:
Weight of extract or fraction /Weight of starting material × 100

Induction of experimental diabetes mellitus

The rats were fasted overnight, weighed using a weighing balance and the fasting blood glucose (normal FBG) level was determined using (ACCU-Check Advantage® II) glucometer test kit with blood collected via a snip on the tail vein. Diabetes was then induced with alloxan monohydrate at the dose of 160 mg/kg body weight injected intraperitoneally. The diabetic state was checked in treated rats by measuring their FBG levels every other day. Diabetes was established on day seven post treatment. Rats with FBG level greater than 126 mg/dl were considered diabetic and used for the study[14].

Bioassay screening of the fractions of HAE for bioactivity in alloxan-induced hyperglycemic rats

Following induction of hyperglycemia as previously described, forty five hyperglycemic male rats were randomly assigned into fifteen (A - O) groups of three animals per group. The animals were fasted overnight but were allowed free access to water. The groups A and B received crude HAE (600 mg/kg) and glibenclamide (2 mg/kg), respectively while groups C-O received 60 mg/kg of fractions 1-13, respectively. The extract and fractions were diluted with 5% tween 20. Blood samples were collected from the tail vein to determine the FBG level at 0 min. Fasting blood glucose level were determined at 1, 3 and 6 hours post treatment. Percentage reductions in FBG level in experimental animals were calculated using the formula given below:

Percentage reduction in FBG level =
(FBG before treatment - FBG after treatment) × 100
FBG before treatment

Phytochemical spot test

The crude and fraction of HAE were subjected to

Table 3: Result of phytochemical spot test of crude extract and fractions of HAE

Fraction	saponins	alkaloids	flavonoids	terpenes/ sterols	glycosides	tannins
Crude HAE	+	+	+	+	+	+
Fraction 1	-	-	-	-	+	-
Fraction 2	-	-	-	+	+	-
Fraction 3	-	-	-	+	+	-
Fraction 4	-	-	-	+	+	-
Fraction 5	+	-	-	+	+	-
Fraction 6	+	-	-	+	+	-
Fraction 7	+	-	-	+	+	-
Fraction 8	+	-	-	+	+	-
Fraction 9	+	-	-	+	+	-
Fraction 10	+	-	-	+	+	-
Fraction 11	+	-	-	+	+	+
Fraction 12	+	+	-	+	+	+
Fraction 13	+	+	+	+	+	+

Table 4: Antioxidant effects of the crude and fractions of HAE using DPPH photometric assay (Mean ± SEM, %).

Concentration (µg/ml)	25	50	100	200	400
Ascorbic acid	92.15 ± 0.36*	94.08 ± 0.80*	94.78 ± 0.68*	96.38 ± 4.31*	99.33 ± 0.40*
Crude HAE	68.93 ± 4.51*	65.37 ± 0.81*	90.94 ± 0.16*	91.75 ± 0.49*	89.00 ± 3.50*
Fraction 1	1.41 ± 0.35	-0.71 ± 0.51	-0.56 ± 0.50	3.06 ± 0.45	6.58 ± 0.59
Fraction 2	-0.33 ± 1.32	2.91 ± 0.69	8.23 ± 3.22	11.28 ± 1.07	16.12 ± 0.73
Fraction 3	1.41 ± 0.71	1.31 ± 4.72	2.49 ± 1.11	2.96 ± 0.86	4.42 ± 0.76
Fraction 4	-1.32 ± 1.32	6.35 ± 2.43	4.94 ± 0.14	6.82 ± 1.20	11.57 ± 0.16
Fraction 5	-15.42 ± 5.18	-6.56 ± 7.55	-9.59 ± 0.99	-6.39 ± 3.09	7.01 ± 1.06
Fraction 6	1.64 ± 1.95	-1.36 ± 4.82	5.36 ±2.24	11.42 ± 1.45	23.84 ± 3.19
Fraction 7	11.75 ± 5.57	7.52 ± 0.46	14.10 ± 0.86	19.23 ± 2.27	28.35 ± 1.23
Fraction 8	-6.63 ± 3.66	-3.72 ± 0.90	13.27 ± 2.18	27.67 ± 0.74	30.42 ± 2.27
Fraction 9	-7.61 ± 6.98	28.64 ±3.16	41.75 ± 51.62	39.32 ± 4.40	47.90 ± 0.32
Fraction 10	-7.12 ± 5.09	2.27 ± 3.34	46.12 ± 0.28	64.89 ± 2.14*	88.03 ± 0.86*
Fraction 11	-22.82 ± 23.99	42.07 ± 2.54	98.38 ± 1.29*	93.04 ± 0.43*	91.59 ± 1.38*
Fraction 12	-9.71 ± 0.74	29.29 ± 3.00	66.02 ± 6.46*	80.26 ± 0.58*	89.00 ± 1.54*
Fraction 13	29.94 ± 3.25	76.54 ± 5.09*	80.10 ± 5.35*	83.82 ± 0.86*	92.72 ± 1.84*

* = significant activity

preliminary phytochemical spot test using the method of Treas & Evans [15]. The presence of alkaloids, saponins, tannins, glycosides, terpenes/sterols and flavonoids were tested.

Determination of in vitro antioxidant activities

(1) 2, 2-diphenyl-1-picrylhydrazyl (DPPH) photometric Assay

The free radical scavenging activity of the extract and fractions was analyzed by the DPPH Assay using spectrophotometer[16]. The test sample (2 ml in methanol) at different concentration (25, 50, 100, 200 and 400 µg/ml) was mixed with 0.5 mM DPPH (in 1 ml of methanol) in a cuvette. The absorbance at 517 nm was taken after 30 min of incubation in the dark at room temperature using spectrophotometer (Spectrumlabs, USA). It was done in triplicate and the percentage antioxidant activity was calculated as follows.

% antioxidant activity (AA) = 100-[{(absorbance of sample - absorbance of blank) ×100}/ absorbance of control]

One mililiter of methanol plus 2.0 ml of the sample was used as the blank while 1.0 ml of the 0.5 mM DPPH solution plus 2.0 ml of methanol was used as the negative control. Ascorbic acid (vitamin C) was used as reference standard[17].

(2) Ferric reducing antioxidant power (FRAP)

The method described by Benzie and Strain[18] was employed in the determination of the ferric reducing antioxidant power of HAE and fractions.

The working solution was freshly prepared. Three mililiter of FRAP reagent and 100 µl of fraction in methanol at concentrations of 25, 50, 100, 200 and 400 µg/ml was mixed. It was done in triplicate. The reaction was monitored up to 4 min at 593 nm using spectrophotometer (Spectrumlabs, USA) at 37^0C. The ascorbic acid used as the standard drug was tested using the same procedure. Calculations were made by using a standard curve.

$$\text{FRAP value of sample (µM)} = \frac{\text{Changes in absorbance (0- 4 min)}}{\text{Changes in ABS of STD (0- 4 min)}} \times \text{FRAP value of standard (1000}$$

Statistical analysis

Data obtained were analyzed using one-way analysis of variance (ANOVA) and the variant mean were separated by least significant difference (LSD) of the different groups. Significance was accepted at the level of $p < 0.05$.

RESULTS

Separation of HAE

The chromatographic separation of HAE yielded 439 eluted 10 ml aliquot fractions, which were further pooled into 13 fractions. The yields of the fractions are presented in Table 1.

Antihyperglycemic effects of the fractions of HAE on alloxan-induced hyperglycemic rats

The results of the antihyperglycemic effects of the fractions of HAE on alloxan-induced hyperglycemic rats were presented in Table 2. The percentage reduction in FBG levels of group of rats treated with fractions (60 mg/kg) and crude extract (600 mg/kg) were compared with group of rats treated with glibenclamide (2 mg/kg). The fractions (60 mg/kg) showed a time dependent increase in percentage reduction in FBG level. At 1 h post treatment, the crude extract, fractions 9, 11 and 13 caused more than 50% reduction of FBG. The values were significantly ($p < 0.05$) higher than the value for the group of rat that received glibenclamide (2 mg/kg). Also at 3 h post treatment, the crude extract, fractions 9, 10, 11 and 13 caused more than 50% reduction in FBG, which were significantly ($p < 0.05$) higher than the rats that received glibenclamide. At 6 h after treatment, the crude extract and all fractions except fractions 3, 6 and 7 caused more than 50% reduction in FBG. However, only fractions10, 13 and crude extract was significantly ($p < 0.05$) higher than the rats that received glibenclamide (2 mg/kg). Based on time dependent effects, fractions 10 and 13 may be considered to be the most active fractions.

Phytochemical spot test of crude extract and fractions of HAE

The result of the phytochemical spot test of the crude and fractions of HAE were presented in Table 3. The crude of HAE and fraction 13 showed the presence of saponins, alkaloids, flavonoids, terpenes/sterols, glycosides and tannins. Fraction 1 showed the presence of only glycosides while fractions 2 - 4 contained terpene/sterols and glycosides. Fractions 5 – 10 contained saponins, terpene/sterols and glycosides. Fraction 11 showed the presence of saponins, terpenes, glycosides and tannins while fraction 12 contained alkaloids, terpene/sterols, saponins, glycosides and tannins.

Antioxidant effects of the crude and fractions of HAE using DPPH photometric assay

The result of the antioxidant activity of the crude extract and fractions of HAE using DPPH photometric assay are presented in Table 4. Fraction 1-9 did not produce any significant ($p > 0.05$) antioxidant effects in all the concentration used. At concentration of 25 µg/ml, only ascorbic acid and the crude extract showed 92% and 69% antioxidant effects, respectively, while at 50 µg/ml ascorbic acid, crude extract and fractions 13 showed antioxidant effects of 94%, 65%, and 77% respectively. As the concentration increased from 100 - 400 µg/ml, more fractions like fractions 10, 11, 12 and 13 showed progressive antioxidant effect like ascorbic acid and crude extract of HAE.

Antioxidant effects of the crude and fractions of HAE using ferric reducing antioxidant power (FRAP)

The results of the antioxidant effects of the crude and fractions of HAE using ferric reducing antioxidant power (FRAP) at concentration of 25, 50, 100, 200 and 400 µg/ml are presented in Table 5. The result showed that at 400 µg/ml concentration only the crude extract showed high antioxidant effect of 3.69 µM while fractions 8, 10, 11 and 12 showed weak antioxidant activity of 0.95, 0.67, 0.78 and 0.64 µM, respectively.

DISCUSSION

Table 5: Antioxidant effects of the crude and fractions of HAE using ferric reducing antioxidant power (FRAP) (Mean ± SEM, µMol/L).

Concentration (µg/ml)	25	50	100	200	400
Crude extract	0.13 ± 0.01	0.12 ± 0.00	0.10 ± 0.21	3.34 ± 0.11	3.69 ± 0.11
Fraction 1	0.02 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	0.07 ± 0.02	0.24 ± 0.01
Fraction 2	0.01 ± 0.01	0 ± 0.02	0.36 ± 0.04	0.22 ± 0.04	0.20 ± 0.03
Fraction 3	0.08 ± 0.02	0.24 ± 0.04	0.18 ± 0.04	0.12 ± 0.06	0.27 ± 0.05
Fraction 4	0.12 ± 0.01	0.06 ± 0.00	0.06 ± 0.02	0.53 ± 0.05*	0.23 ± 0.04
Fraction 5	0.08 ± 0.02	0.08 ± 0.04	0.28 ± 0.05	0.10 ± 0.22	0.27 ± 0.02
Fraction 6	0.31 ± 0.10	0.10 ± 0.01	0.13 ± 0.01	0.33 ± 0.09	0.39 ± 0.07
Fraction 7	0.50 ± 0.19 *	0.45 ± 0.21	0.27 ± 0.00	0.34 ± 0.02	0.43 ± 0.18
Fraction 8	0.22 ± 0.07	0.23 ± 0.08	0.37 ± 0.01	0.59 ± 0.01*	0.95 ± 0.25*
Fraction 9	0.10 ± 0.02	0.18 ± 0.09	0.03 ± 0.00	0.21 ± 0.03	0.23 ± 0.11
Fraction 10	0.41 ± 0.17	0.15 ± 0.03	0.39 ± 0.10	0.51 ± 0.04*	0.67 ± 0.08*
Fraction 11	0.17 ± 0.00	0.29 ± 0.02	0.28 ± 0.03	0.49 ± 0.03	0.78 ± 0.10*
Fraction 12	0.06 ± 0.01	0.18 ± 0.02	0.14 ± 0.01	0.23 ± 0.03	0.64 ± 0.02*
Fraction 13	0.09 ± 0.07	0.44 ± 0.04	0.17 ± 0.01	0.28 ± 0.23	0.28 ± 0.02

* = significant activity

The cold maceration method of extraction of *H. annuus* leaves for 48 h yielded 10.04% w/w of dark greenish extract. The extract was pasty & oily in consistency, bitter in taste and has aromatic smell. The crude extract was separated using bioassay-guided column and thin layer chromatography technique into 439 eluted aliquot fractions. They were pooled based on their colour reaction under UV lamp and relative mobility in Thin Layer Chromatographic (TLC) plate into 13 fractions. The percentage yield of the fractions was determined using standard method. Thin layer chromatography is a basic method suitable for rapid detection of drug substances. It is a method for separating individual components of a sample by using thin layer of silica gel as a stationary phase. TLC technique is advantageous in that it is selective, specific and rapid in identifying drug substances than the simple characterization method using chemical reagent, which reveal substance by colour and precipitation reaction test[19]. Chemical test should however, complement TLC findings. There is no interference by excipient in TLC and the method can be used for identification, purity test and semi-quantitative estimation of the active ingredient in the dosage form[20].

The choice of the dose of crude extract (600 mg/kg) was based on the findings of the previous work[6] and was employed here to track the antidiabetic potential of the fractions. The antidiabetic screening of the fractions was done using 60 mg/kg of each fraction, which is one tenth of the highest dose (600 mg/kg) used in crude extract experiments. Generally chromatographic separation of the fractions concentrates the bioactive principles, with associated increase in bioactivity. Secondly, the yield of the fractions is drastically decreased, hence the decision to use one tenth of 60 mg/kg HAE. The screening of the fractions for antihyperglycemic effect in alloxan-induced hyperglycemic rats showed that the fractions exhibited time-dependent increase in percent reduction of FBG in varied degrees. Fractions 9, 10, 11 and 13 were considered to be the active fractions because they showed time-dependent increase in percent reduction of FBG (Table 3). At 6 h post administration, fractions 10 and 13 significantly ($p < 0.05$) increased the percent reduction in FBG when compared with glibenclamide (2 mg/kg). However, the antidiabetic effect of fraction 10 and 13 were not significant ($p > 0.05$) when compared with the crude extract (HAE, 600 mg/kg).

Phytochemical spot test is a simple characterization method using chemical reagent, which reveals substance by colour and precipitation reaction tests[19]. The phytochemical spot test result grouped the crude extract and fractions of HAE into five groups based on their phytochemical constituents (Table 4). It showed that the most active fractions (9 and 10) were composed mainly of triterpenoid-saponins. Triterpenoid-saponins are the most common saponins and mainly found in dicotyledoneous angiosperms and possess some important pharmacological properties like antifungal, anti-inflammatory, anti-ulcer, expectorant and antioxidant effects[21].

The crude extract and fractions of HAE demonstrated appreciable antioxidant activity as revealed in DPPH and FRAP photometric assay. The crude extract and fractions of HAE produced a concentration dependent increase in antioxidant activity. The DPPH assay showed that 400 µg/ml of the crude and fractions 10 and 13 of HAE produced (89.00, 88.03 and 92.72% respectively) antioxidant activity which were significantly lower compared with ascorbic acid (99.33%) antioxidant activity. Unlike the DPPH assay, FRAP assay of fractions 8, 9, 10 and 13 at 400 µg/ml showed weak antioxidant activities of 0.95, 0.25, 0.67 and 0.28 µM, respectively when compared with the crude HAE (3.69 µM at 400 µg/ml) and ascorbic acid (2.0 µM at 1000 µg/ml). From this observation, the crude HAE maintained high antioxidant potential using both *in vitro* antioxidant models which may be responsible for the potent

antihyperglycemic effects (Table 3), while fractions 10 and 13 also maintained high antioxidant activity using DPPH *in vitro* model. Therefore, antioxidant effect of the crude HAE, fractions 10 and 13 may be responsible for antihyperglycemic effect. Since fraction 13 is the last run with 100% methanol in the separation, it contained the same phytochemical components as the crude HAE and may not be considered as a real fraction. Therefore the most active fraction is fraction 10 which contains saponins, terpenes and glycosides.

Insulin deficiency which characterizes diabetic state causes impairment of glucose utilisation leading to an increased generation of oxygen free radicals and the development of diabetic complications[22]. The *in vitro* antioxidant effect of crude extract and fraction of HAE demonstrated in this our study, will help to mop up the reactive oxygen species associated with diabetes; preventing the development and progression of diabetes complications.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a dark-coloured crystalline powder composed of stable free radical molecules. In laboratory, it is used to monitor chemical reactions involving radical most notably antioxidant assay[23]. The DPPH radical has a strong absorption band centered at about 517-520 nm. Its radicals have a deep violet colour in solution, and become colourless or pale yellow when neutralized[24]. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at 520 nm or in the electron paramagnetic resonance of the DPPH[24,25].

Ferric reducing antioxidant power assay as described by Benzie and strain (1999) is based on the principles that the presence of reductant (i.e. antioxidants) in the sample would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be determined by measuring the formation of Pearl's Prussian blue at 593 nm. Increase in absorbance indicates an increase in reductive ability. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant effect through breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Generally, FRAP is inferior to DPPH as FRAP measures only the reduction of Fe^{3+} to Fe^{2+} [18] while DPPH measures the reduction of many other oxidants [19]. This may therefore be the cause of the weak FRAP effect of the fractions as observed in this study.

CONCLUSION

The crude extract and fractions of HAE showed promising antihyperglycemic effect, which may be mediated by triterpenoid-saponins through either pancreatic, extra-pancreatic and/or antioxidant mechanisms. Purification of the crude extract improved the antihyperglycemic and antioxidant activities of the fractions. The antihyperglycemic and antioxidant effects of the crude and the fractions justified the folkloric use of this medicinal plant by the natives in clinical management of diabetes mellitus. Further work is required for the chronic antidiabetic and antioxidant effects, chronic toxicity and further purification and characterization of the active principles.

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CONFLICT OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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