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Full Length Research Paper

Phytochemical and pharmacological studies *of Citharexylum quadrangulare* Jacq. leaves

Marwa Hassan Hussaen Mohammed, Ashraf Nageeb El-Sayed Hamed*, Hany Ezzat Khalil and Mohamed Salah Kamel

Department of Pharmacognosy, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt.

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Chromatographic investigation of the *Citharexylum quadrangulare* leaves led to isolation of nine compounds viz., stigmasterol (1), *β*-sitosterol (2), oleanolic acid (3), duranterectoside B (4), durantoside I (5), cirsimaritin 4'-O-*β*-D-glucopyranoside (6), 5-deoxypulchelloside (7), lamiide (8) and cirsimaritin 4'-O-*β*-D-glucopyranoside 4"-sodium sulphate (9). The compounds (1, 3 and 4) were isolated for the first time from genus *Citharexylum*. The structures of the isolated compounds were determined by interpretation of their spectroscopical data and comparison with published literature. The aqueous fraction exhibited the most significant anti-inflammatory activity. Its effect was more potent than the reference drug. While, the most significant anti-pyretic sample was the total methanol extract. Also, it showed a gastroprotective activity with preventive index (78%). Finally, the ethyl acetate fraction demonstrated a significant decrease in blood glucose level on hyperglycemic alloxaned rats.

Key words: *Citharexylum quadrangulare*, Verbenaceae, anti-inflammatory, anti-pyretic, gastroprotective, anti-diabetic.

INTRODUCTION

Verbenaceae is a large family, which contanis 100 genera and around 3000 species (Datta, 1988; Dahiya, 1979; Datta, 1970). It is considered as a potential source of natural products particularly, flavonoids, essential oils (Rizk and Al-Nowaihi, 1989; Rizk, 1986), iridoids, anthocyanins, quinones and caffeic acid derivatives, while alkaloids are rare (Kenner and Requena, 1996; Evans, 1996; Hall, 1976). One of these genera is *Citharexylum*. It includes 115 species and is distributed in South Florida, Guyana, Suriname and Venezuela (Wagner et al., 1999; Datta, 1988; Dahiya, 1979; Datta, 1970). One of these plants is *Citharexylum quadrangulare* Jacq., which is known as fiddlewood (Wagner et al., 1999). It has a synonym; *C. spinosum* L. (Bedevian, 1994). It was reported in the folkloric medicine as anti-arthritic, anti-pyretic, diuretic and liver disorders (Wagner and Wolf, 1977). The previous phytochemical study of *C. quadrangulare* resulted in the isolation of flavonoids viz., cirsiliol 4'-O-*β*-D-galactopyranoside, cirsimaritin 4'-O-*β*-D-gluco-pyranoside and cirsimaritin 4'-O-*β*-D-glucopyranoside 4"-sodium sulfate (Shalaby and Bahgat, 2003). Further investigation on *C. spinosum* and *C. quadrangulare* led to isolation of iridoids such as lamiidoside, lamiide, duranterectoside C, durantoside I,

*Corresponding author. E-mail: ashrafnag@mu.edu.eg, ashrafnag@yahoo.com, Tel:++20862347759. Fax:++20862369075

citharone, phlomiol, 5-deoxypulchelloside I (Balazs et al., 2006; Shalaby and Bahgat, 2003; Khalifa et al., 2002).

The available biological literature showed that *C. quadrangulare* was evaluated for many activities as antihypertensive, gastrointestinal tract disorders, immunomodulatory, hepatoprotective and anti *Schistosoma mansoni* cercariae (Bahgat et al., 2005; Shalaby and Bahgat, 2003; Khalifa et al., 2002). On these bases and computer survey employing different data bases including Scifinder, few literature have been reported. This provoked us to carry out further phytochemical and pharmacological investigations on this plant.

METHODOLOGY

General

The NMR spectra were measured using a Jeol JNM-LA 400 and 600 MHz. FT NMR spectrometer (Japan), using tetramethylsilane (TMS) as internal standard and chemical shifts were expressed in δ ppm. Column chromatography (CC) was performed by using Normal Phase-Silica gel (NP-Silica, 70-230 mesh, FLUCA, Germany), Sephadex LH-20 (GE Health Care, Sweden) and Diaion HP-20 (Mitsubishi Chemical Corp., Japan). Fractions were monitored by using thin layer chromatography (TLC) Silica gel 60 precoated plates F_{254} (Merck, Germany) and spots were visualized by heating Silica gel plates and sprayed with 10% H_2SO_4 in MeOH (v/v). The TLC plates were allowed to dry at room temperature and then heated at 110°C till the colors develop and reach their maxima (Stahl, 1970).

Plant material

The leaves of *C. quadrangulare* Jacq. were collected (June 2007) from El-Orman Garden (Giza, Egypt). It was identified by Agricultural Engineer/Trease Labeeb (El-Orman Garden). A voucher specimen has been deposited at Pharmacognosy Department, Faculty of Pharmacy, Minia University under the registration number (Mn-Ph-Cog-005).

Preliminary phytochemical screening

The air-dried powdered leaves of *C. quadrangulare* Jacq. were macerated in 70% methanol (MeOH). The total methanolic extract of the leaves (TMEL) was subjected to preliminary phytochemical screening for its constituents (Sofowora, 1993; Trease and Evans, 1985; Harborne, 1973; Schmidt, 1964; Clause, 1961).

Extraction and isolation

The powdered leaves (1.8 kg) were exhaustively extracted with 70% MeOH (3x, 5 L each) and yielded (250.0 g of TMEL). Physical partitioning of TMEL with petroleum ether and water, followed by chloroform ($CHCl_3$) and water and finally ethyl acetate (EtOAc) and water, was performed and yielded four main fractions viz., petroleum ether (30.0 g), $CHCl_3$ (29.6 g), EtOAc (45.8 g) and aqueous (99.6 g).

The petroleum ether fraction was fractionated on NP-Silica column (Φ=7, L=200 cm, 900 g), employing gradient technique, increasing polarity from petroleum ether to EtOAc and 42 fractions were collected (500 ml each). The similar fractions were pooled together. Sedimentation from fraction 18 (0.5 g, eluted by petroleum ether-EtOAc, 80:20) was a mixture of two compounds (**1** and **2**), which was further crystallized by MeOH (78 mg, colorless needles, R_f=0.59, system; petroleum ether-EtOAc, 7:3). Fractions 19-23 (2.6 g, eluted by petroleum ether-EtOAc, 80:20) was further purified on NP-Silica gel for column (Φ=2, L=100 cm, 100.0 g), employing gradient technique with increasing polarity from petroleum ether to EtOAc and 40 fractions were collected (10 ml each). Compound **3** (11.8 mg, white amorphous powder, R_f=0.45, system; petroleum ether-EtOAc, 7:3) was eluted in fractions (8-12) by (petroleum ether-EtOAc, 65:35).

The EtOAc fraction was purified on NP-Silica gel column (Φ=10, L=200 cm, 1.4 kg), employing gradient technique, increasing polarity from $CHCl_3$ to MeOH and 36 fractions were collected (500 ml each). The similar fractions were collected together.

Fractions 17-20 (5.94 g, eluted by $CHCl_3$-MeOH, 80:20) were purified on Diaion HP-20 (Φ=4, L=100 cm) and eluted with gradient elution from H_2O to MeOH (H_2O, 20% MeOH, 50% MeOH, 80% MeOH and MeOH, 1.5 L each). The 80% MeOH fraction (3.3 g) was left for 2 h, then filtered. The filtrate was concentrated under reduced pressure to yield (0.7 g). It was purified on NP-Silica (Φ=2, L=80 cm, 28.0 g) employing gradient elution from $CHCl_3$ to MeOH and 120 fractions were collected (10 ml each). The similar fractions were pooled together. The fractions 39-42 (350 mg, eluted by $CHCl_3$-MeOH, 95:5) were further purified on Sephadex LH-20 (Φ=2, L=60 cm), eluted with MeOH and led to a mixture of two compounds **4** and **5** (141 mg, faint yellow residue, R_f=0.34, system; $CHCl_3$-MeOH, 90:10). Compound **6** (90.3 mg, yellow amorphous powder, R_f=0.76, system; $CHCl_3$-MeOH-H_2O, 15:6:1) was precipitated from the MeOH fraction (1.4 g).

Fractions 21-24 (8.5 g, eluted by $CHCl_3$-MeOH, 75:25) were fractionated on Diaion HP-20 column (Φ=4, L=150 cm) using gradient elution with H_2O-MeOH (H_2O, 20% MeOH, 50% MeOH, 80% MeOH and MeOH, 1.5 L each). The 20% MeOH fraction (2.4 g) was purified on NP-Silica (Φ=2, L=80 cm, 100.0 g), using gradient elution from $CHCl_3$ to MeOH and 70 fractions were collected (10 ml, each). The fraction 18 (410 mg, eluted by $CHCl_3$-MeOH, 80:20) was further purified on Sephadex LH-20 column (Φ=2, L=80 cm), eluted with MeOH and led to compound **7** (90.9 mg, faint yellow residue, R_f=0.48, system; $CHCl_3$-MeOH-H_2O, 80:20:1). Moreover, fraction 26 (380 mg, eluted by $CHCl_3$-MeOH, 70:30) was further purified on Sephadex LH-20 column (Φ=2, L=60 cm), eluted with MeOH and led to compound **8** (47.6 mg, faint yellow residue, R_f=0.31, system; $CHCl_3$-MeOH-H_2O, 80:20:1). Finally, 50% MeOH fraction (3.2 g) was purified on NP-Silica column (Φ=2, L=150 cm, 130.0 g), isocratically eluted with $CHCl_3$-MeOH-H_2O (80:20:1) and 200 fractions were collected (10 ml each). The fractions (105-144, 130 mg) were purified on Sephadex LH-20 column (Φ=2, L=60 cm), eluted with MeOH and led to compound **9** (3 mg, yellow amorphous powder, R_f=0.42, system; $CHCl_3$-MeOH, 80:20).

Animals

Adult male Sprague-Dawley albino rats (220-250 g) were used. They were purchased from the animal house, Faculty of Agriculture, Minia University. The animals were kept under identical environmental circumstances, fed with standard nutrition and water *ad libitum* and left to adapt to the environment for at least 7 days prior to the experiments at 22±2 °C under a 12/12 h light/dark cycle. They were handled only at the time of experiments and during cage washing. All conditions were ensured to reduce animal distress. The care and procedures involving animals were conducted in conformity with the institutional guidelines of the Pharmacology and Toxicology Department, Faculty of Pharmacy, Minia University and in agreement with the provisions of the Declaration of laboratory

Table 1. Important signals in ^{1}H-NMR spectral data of compounds **1**-**4**.

No.	Compounds; δ_H (Integration, Multiplicity, *J* in Hz)			
	(1)	(2)	(3)	(4)
1	----	----	----	5.77 (1H, br.s)
3	3.54 (1H, m)	3.54 (1H, m)	3.20 (1H, br.d, 10.4)	7.42 (1H, s)
4	2.24 (2H, m)	2.24 (2H, m)	----	----
6	5.34 (1H, m)	5.34 (1H, m)	----	2.29 (2H, m)
9	----	----	----	2.81 (1H, s)
10	----	----	----	1.10 (3H, s)
12	----	----	5.20 (1H, br.s)	----
18	0.68 (3H, s)	0.68 (3H, s)	----	----
19	0.99 (3H, s)	0.99 (3H, s)	----	----
22/23	4.98,5.71 (2H, m)	----	----	----
23	----	----	1.10 (3H, s)	----
24	----	----	0.91 (3H, s)	----
25	----	----	0.88 (3H, s)	----
26	----	----	0.76 (3H, s)	----
27	----	----	0.97 (3H, s)	----
29	----	----	0.73 (3H, s)	----
30	----	----	0.90 (3H, s)	----
Me gps	0.77-0.97 (m)	0.77-0.97 (m)	----	----
COOMe	----	----	----	3.70 (3H, s)
1'	----	----	----	4.61 (1H, d, 7.8)
7''	----	----	----	7.00 (1H, d, 12.4)
8''	----	----	----	6.07 (1H, d, 12.4)

Compounds **1**-**3** were measured in $CDCl_3$; compound **4** was measured in MeOD. All compounds were measured by using 400 MHz.

animals of the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Determination of acute toxicity (LD_{50})

The acute toxicity of the TMEL of *C. quadrangulare* was determined by the following experimental model described by Schapoval et al. (1998). This was carried out by measuring the lethal dose for 50% of the laboratory animals (LD_{50} method). Different single oral doses (0.5, 1, 2, 4 and 8 g/kg, per oral) of TMEL of the plant [suspended in 0.5% carboxymethylcellulose solution (CMC)] were administered to 6 groups (5 animals each) of rats (240±10 g). The control group received an equivalent dose of TMEL vehicle (0.5% CMC solution, 10 ml/kg, per oral). Both the test and control groups were observed for 48 h under normal environmental conditions, with free access to food and water.

Pharmacological activities

All groups were orally given the tested samples by using gavage. In all experiments, rats were randomly divided into 7 groups (5 animals each) as follows:

1- The 1st group (negative control) non-treated one, administered 10 ml/kg (0.5% CMC).
2- The 2nd group (positive control) treated with the standard drugs (indomethacin or acetylsalicylic acid or ranitidine or glibenclamide).
3- The 3rd group treated with TMEL.
4- The 4th group treated with petroleum ether fraction.
5- The 5th group treated with $CHCl_3$ fraction.
6- The 6th group treated with EtOAc fraction.
7- The 7th group treated with aqueous fraction.

The groups from 3rd to 7th were orally given doses of 100 mg/kg (the extract and the different fractions were suspended in 0.5% CMC).

Anti-inflammatory activity

The TMEL and its fractions were evaluated by the carrageenan-induced paw edema method in the rats (Winter et al., 1962). The tested samples were suspended in 0.5% CMC solution and administered orally to the rats (230±10 g), one hour prior to the subcutaneous injection of 0.1 ml carrageenan suspension (1% w/v in normal saline solution) into the sub-plantar area of the right hind paw (Vogel and Vogel, 1997). The 2nd group was given indomethacin at a dose of 8 mg/kg (Sawadogo et al., 2006; Petrovic et al., 2003). Edema measurements were determined in mm with a plethysmometer (Ferreira, 1979) after 0, 1, 2, 3 and 4 h carrageenan injection. The results are listed in (Table 5).

Anti-pyretic activity

Anti-pyretic effect was evaluated by employing yeast-induced fever

Table 2. Important ^{1}H-NMR spectral data of compounds (**5-9**).

No.	Compounds; δ_H (Integration, Multiplicity, *J* in Hz)				
	(5)	**(6)**	**(7)**	**(8)**	**(9)**
1	5.80 (1H, br.s)	----	5.48 (1H, d, 2.3)	5.81(1H, s)	----
3	7.35 (1H, s)	6.80 (1H, s)	7.38 (1H, s)	7.43 (1H, s)	6.94 (1H, s)
5	----	12.78 (1H, s)	2.87 (1H, d, 9.2)	----	12.60 (1H, s)
6	2.29 (2H, m)	----	2.26 (1H, m)	2.37 (1H, dd, 15.1, 5.2) 2.25 (1H, dd, 15.1, 3.4)	----
OMe-6	----	3.72 (3H, s)	----	----	3.73 (3H, s)
7	----	----	3.49 (1H, m)	3.52 (1H, m)	----
OMe-7	----	3.87 (3H, s)	----	----	3.93 (3H, s)
8	----	6.81 (1H, s)	2.26 (1H, m)	----	6.98 (1H, s)
9	2.92 (1H, s)	----	2.80 (1H, m)	2.78 (1H, s)	----
10	1.13 (3H, s)	----	1.05 (3H, d, 7.3)	1.00 (3H, s)	----
COOMe	3.70 (3H, s)	----	3.67 (3H, s)	3.73 (3H, s)	----
1'	4.61 (1H, d, 7.8)	----	4.55 (1H, d, 7.9)	4.59 (1H, d, 7.9)	----
2',6'	----	7.99 (2H, d, 8.6)	----	----	8.08 (1H, d, 8.9)
3',5'	----	7.19 (2H, d, 8.6)	----	----	7.21 (1H, d, 8.9)
1''	----	5.06 (1H, d, 7.0)	----	----	5.10 (1H, d, 7.7)
7''	7.72 (1H, d, 16.1)	----	----	----	----
8''	6.55 (1H, d, 16.1)	----	----	----	----

Compounds (**5**, **7** and **8**) were measured in MeOD. Compounds (**6** and **9**) were measured in DMSO-d_6. All compounds were measured by using 400 MHz, except compound 8 was measured by using 600 MHz.

according to Teotino et al. (1963). It was performed on rats (225±5 g) by subcutaneous injection (in the back, below the nape of the neck) of 20% aqueous suspension of yeast in a dose of 10 ml/kg to induce pyrexia (Kang et al., 2008). The rectal temperature of each animal was recorded, using digital thermometer, which was inserted 2 cm into the rectum, before and 18 h after the yeast injection, when the temperature was at the peak (Panthong et al., 2003). The animals that did not show a minimum increase of 0.5 °C in temperature after 18 h were discarded. The rectal temperature of each animal was recorded again at 30 min interval for 3 h following the drug administration. The positive control group was given acetylsalicylic acid (100 mg/kg) (Gege-Adebayo et al., 2013). The results are displayed in (Table 6).

Gastroprotective activity

The procedure of gastroprotective activity was started by feeding the rats (235±10 g) with a standard diet of commercial rat chow and tap water. They were left to acclimatize to the environment for at least one week prior the experiment. Rats fasted for 24 h prior to the experiment in mesh-bottomed cages to decrease coprophagia, but the animals had free access to water except for the last hour before the experiment (Inas et al., 2011). All the experiments were performed during the same time of the day to avoid variations due to diurnal rhythms of putative regulators of gastric functions (Garrik et al., 1986).

All these treatments were given one hour before the induction of gastric ulceration by a large oral dose of indomethacin (40 mg/kg) (Choudhary et al., 2014; Raji et al., 2011; Mishra et al., 2009).

The reference drug (ranitidine, 50 mg/kg) was administered (Choudhary et al., 2014). After one hour of administration of the drugs, the rats were sacrificed by cervical dislocation. Their stomachs were removed and opened along the greater curvature and then washed with serum physiological solution and macroscopic gross mucosal lesion were counted and scored to determine the severity of these lesions (Inas et al., 2011). The protective effect of TMEL and its fractions were compared with indomethacin and ranitidine groups.

The assessment of gastric mucosal lesions in each stomach was measured. Moreover, the ulcer score for each stomach was expressed as the total length of gastric lesions in that stomach. Afterwards, the mean ulcer score for each group was calculated. The preventive index (P.I.) of a given drug was calculated from the following equation (Hano et al., 1976). The results are shown in Table 7.

$$\text{P. I.} = \frac{(\text{U. I. of ulcerated group}) - (\text{U. I. of treated group})}{(\text{U. I. of ulcerated group})} \text{ x } 100$$

Where, U.I. is the ulcer index.

Anti-diabetic activity

The rats weighing 230±10 g were allowed to fast for 24 h prior to experiment and diabetic rats were obtained by the administration of a single dose of intraperitoneal injection of alloxan 120 mg/kg body weight (Vogel and Gang, 2002). After 72 h of alloxan injection, diabetes was confirmed by testing blood sugar level by using Accu-Chek® Glucometer to monitor the blood sample from the tail vein. When, blood glucose level (BGL) above 200 mg/dl was considered diabetic. The 2nd group treated with standard drug (glibenclamide, 0.5 mg/kg) (Hazra et al., 2011). The blood samples were taken and measured. They were taken at zero, 1, 2, 3 and 4 h following drug

Table 3. ^{13}C-NMR spectral data of compounds (**1**-**3**).

No.	Compounds (δ_C, Multiplicity)			No.	Compounds (δ_C, Multiplicity)		
	(1)	(2)	(3)		(1)	(2)	(3)
1	37.3,t	37.3,t	38.5,t	16	28.8,t	28.2,t	23.0[d],t
2	31.7[a],t	31.7[a],t	27.2[a],t	17	56.1[b],d	56.0[b],t	46.5,s
3	71.8,d	71.8,d	79.0,d	18	12.0[c],q	12.0[c],q	41.0,d
4	42.4,t	42.4,t	38.8[b],s	19	19.4[d],q	19.4[d],q	45.9,t
5	140.8,s	140.8,s	55.3,d	20	40.4,d	36.1,d	30.7,s
6	121.7,d	121.7,d	18.3,t	21	21.2,q	18.7,q	33.8,t
7	31.9[a],t	31.9[a],t	32.7[c],t	22	138.2,d	34.0,t	32.5[c],t
8	31.9[a],d	31.9[a],d	39.3[b],s	23	129.3,d	26.2,t	28.1,q
9	50.2,d	50.2,d	47.7,d	24	51.2,d	45.9,d	15.5,q
10	36.5,s	36.5,s	37.1,s	25	31.9,d	29.2,d	15.3,q
11	21.1,t	21.1,t	23.4[d],t	26	19.0[d],q	19.8[d],q	17.2,q
12	39.7,t	39.8,t	122.7,d	27	21.2,q	19.1[d],q	25.9,q
13	42.4,s	42.4,s	143.6,s	28	25.3,t	23.1,t	183.1,s
14	56.9[b],d	56.8[b],d	41.6,s	29	12.0[c],q	12.0[c],q	33.1,q
15	24.4,t	24.3,t	27.7[a],t	30	----	----	23.6,q

[a, b, c, d]values may be interchangeable within the same compound ($CDCl_3$, 100 MHz).

treatment. The results are demonstrated in (Table 8).

Statistical analyses

The statistical analyses of the obtained results were done using GraphPad Prism 5 (Graphpad Software, San Diego California, USA). The obtained outcomes were expressed in terms of mean±SEM. Differences between the mean values for individual groups were assessed by one-way analysis of variance. In all analyses, (**P<0.05*) or (***P<0.01*) or (****P<0.001*) were taken to indicate statistical significance.

RESULTS AND DISCUSSION

The preliminary phytochemical screening of *C. quadrangulare* TMEL showed the presence of various metabolites such as carbohydrates and/or glycosides, flavonoids, unsaturated sterols and/or triterpenes, saponins and tannins. While, it was free from many constituents such as crystalline sublimate substances, volatile oils, alkaloids and/or nitrogenous substances, cardenolides and anthraquinones.

Furthermore, the petroleum ether and EtOAc fractions of TMEL were purified by employing diversity of chromatographic techniques; including open columns of Silica gel, Diaion HP-20 and Sephadex LH-20 to afford nine compounds (Figure 1) viz., compounds (**1**-**3**) from petroleum ether fraction and compounds (**4**-**9**) from EtOAc fraction. They belong to different classes; two steroloidal aglycones: stigmasterol (**1**) (Maima et al., 2008) and *β*-sitosterol (**2**) (Maima et al., 2008); triterpenes: oleanolic acid (**3**) (Chang et al., 2009); four iridoids: duranterectoside B (**4**) (Takeda et al., 1995), durantoside I (**5**) (Takeda et al., 1995), 5-deoxypulchelloside (**7**) (Shalaby and Bahgat, 2003; Khalifa et al., 2002) and lamiide (**8**) (Yalcin et al., 2007; Khalifa et al., 2002) and finally two flavonoids: cirsimaritin 4'-O-*β*-D-glucopyranoside (**6**) (Shalaby and Bahgat, 2003) and cirsimaritin 4'-O-*β*-D-glucopyranoside 4''-sodium sulphate (**9**) (Shalaby and Bahgat, 2003). The structures of the isolated compounds were elucidated by comparing spectroscopical data (Tables 1 to 4) with the reported literature and also comparing their physical properties with the authentic samples. Three of them (compounds **1, 3** and **4**) were isolated for the first time from genus *Citharexylum*. This is very important in the chemotaxonomical study of the plant.

The TMEL of *C. quadrangulare* was evaluated against the lethality effect on rats up to 8 g/kg (exceeded ten times of the therapeutic dose 100 mg/kg) during the first 48 h. Moreover, no toxic manifestation has been observed such as paw-licking, stretching, respiratory distress, diarrhea (Arthur et al., 2011). Thus, *C. quadrangulare* has a wide margin of safety.

TMEL and its fractions exhibited anti-inflammatory activities due to its inhibition of the carrageenan induced edema. The significant decrease of paw edema has been obtained with the aqueous and petroleum ether fractions, after 4 h, comparing with indomethacin as positive control (Table 5). The phytochemical investigation of *C. quadrangulare* indicated that it has considerable contents of sterols, triterpenes and flavonoids. Most of these compounds are responsible for the anti-inflammatory effects (Lalitha and Gayathiri, 2013; Perez, 2001). Moreover, the previous studies indicated that flavonoids such as rutin, quercetin, luteolin, biflavonoids, steroids

Table 4. ^{13}C-NMR spectral data of compounds (**4-9**).

No.	Compounds (δ_C, Multiplicity)						No.	Compounds (δ_C, Multiplicity)					
	(4)[1]	(5)[1]	(6)[2]	(7)[1]	(8)[1]	(9)[2]		(4)[1]	(5)[1]	(6)[2]	(7)[1]	(8)[1]	(9)[2]
1	94.0,d	94.1,d	----	96.0,d	94.5,d	----	5'	77.3,d	77.3,d	116.6,d	78.1[b],d	78.4,d	116.6,d
2	----	----	163.3,s	----	----	163.3,s	6'	62.7,t	62.7,t	128.2,d	62.7,t	62.8,d	128.1,d
3	152.2,d	152.2,d	103.6,d	153.8,d	152.5,d	103.6,d	1''	136.2,s	135.7,s	100.0,d	----	----	99.2,d
4	115.5,s	115.5,s	182.1,s	111.0,s	115.4,s	182.2,s	2''	----	----	73.2,d	----	----	73.2,d
5	69.0,s	68.9,s	152.0[a],s	38.7[a],d	69.2,s	152.0[a],s	2'',6''	129.1,d	129.2,d	----	----	----	----
6	45.3,t	45.6,t	132.0,s	77.5[b],d	46.7,t	131.9,s	3''	----	----	76.6[b],d	----	----	75.0[b],d
7	80.4,d	80.7,d	158.6,s	79.9,d	77.8[a],d	158.7,s	3'',5''	131.4[b],d	131.0[b],d	----	----	----	----
8	78.7,s	78.7,s	91.4,d	39.0[a],d	79.1,s	91.7,s	4''	130.1[b],d	130.0[b],d	69.8,d	----	----	75.1[b],d
9	58.1,d	58.3,d	152.5[a],s	40.2,d	58.1,d	152.6[a],d	5''	----	----	77.2[b],d	----	----	75.3[b],d
10	21.3,q	21.3,q	105.1, s	13.9,q	21.3,q	105.1,s	6''	----	----	60.7,t	----	----	60.7,t
11	167.9[a],s	167.9[a],s	----	169.1,s	168.0,s	----	7''	144.7,d	146.4,d	----	----	----	----
							8''	120.6,d	119.1,d	----	----	----	----
1'	99.6,d	99.6,d	124.0,s	99.7,d	99.6,d	123.9,s	9''	167.1[a],s	168.1[a],s	----	----	----	----
2'	74.4,d	74.4,d	128.1,d	74.5,d	74.4,d)	128.1,d	OMe-6	----	----	60.0,q	----	----	60.0,q
3'	78.2,d	78.2,d	116.6,d	77.8[b],d	77.4[a],d	116.6,d	OMe-7	----	----	56.3,q	----	----	56.4,q
4'	71.6,d	71.6,d	160.3,s	71.5,d	71.7,d	160.2,s	COOMe	51.8,q	51.8,q	----	51.8,q	51.7,q	----

[a, b] values may be interchangeable within the same compound. [1](MeOD) and [2](DMSO-d_6). All compounds were measured by using 100 MHz, except compound (**8**) was measured by using 150 MHz NMR.

Table 5. Anti-inflammatory activities of *C. quadrangulare*.

Group	Thickness of the paw (mm)/h				
	0	1	2	3	4
Control	5.55±0.12	5.87±0.17	5.03±0.23	4.87±0.10	4.90±0.12
Indomethacin	5.19±0.17	4.32±0.10***	4.33±0.15*	3.99±0.11**	3.94±0.14**
TMEL	5.42±0.19	4.91±0.26**	4.29±0.24*	4.29±0.23*	3.90±0.23**
Petroleum ether fr.	5.18±0.23	4.70±0.22***	4.42±0.25	4.16±0.95**	3.80±0.18***
$CHCl_3$ fr.	5.30±0.28	4.60±0.25***	4.23±0.20*	4.24±0.16*	3.90±0.16**
EtOAc fr.	5.39±0.23	4.80±0.10**	4.41±0.21	4.02±0.16**	4.23±0.16*
Aqueous fr.	4.95±0.22	4.60±0.14***	4.04±0.18*	3.80±0.18***	3.66±0.20***

Values represent Mean±SEM, (n=5). Significant difference (*$P<0.05$, **$P<0.01$ and ***$P<0.001$). Indomethacin (8 mg/kg) and the other drugs (100 mg/kg).

and triterpenoids produced significant anti-inflammatory activities (Silva et al., 2005). The obtained anti-inflammatory results are in line with other previous studies in family Verbenaceae (Shukla et al., 2011; Amir et al., 2011; Krishnaraju et al., 2009; Monteiro et al., 2007; Penido et al.,

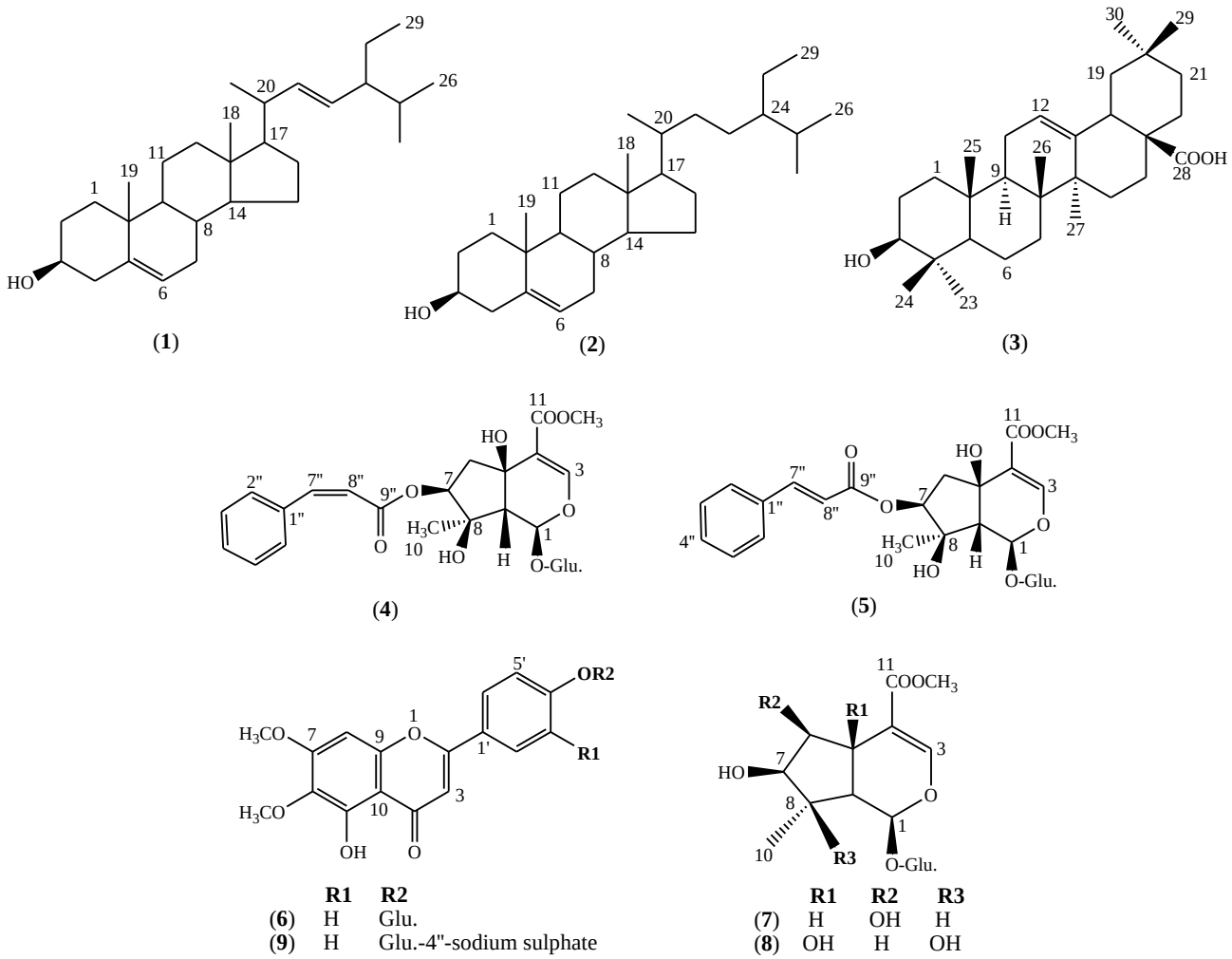

Figure 1. Structures of the isolated compounds **1**-**9**.

2006; Abena et al., 2003).

The anti-pyretic activity of TMEL and its fractions have been evaluated by causing reduction in yeast-induced pyrexia (Table 6). The rectal temperature reached normal level after 1.5, 2, 1, 3 and 3 h with TMEL, petroleum ether, $CHCl_3$, EtOAc and aqueous fractions, respectively. The $CHCl_3$ fraction showed a weak anti-pyretic effect, based on decreasing the rectal temperature without reaching the normal level after 3 h. Aforementioned information about the chemical profile of the *C. quadrangulare* showed the presence of the major constituents as sterols, triterpenes and flavonoids, can explain the anti-pyretic activity (Hossain et al., 2011; Boakye-Gyasi et al., 2011; Achuta et al., 2011). This deduction is concentrated in TMEL. Our findings are in line with previous studies in family Verbenaceae (Rohit et al., 2012; Shukla et al., 2011; Abena et al., 2003).

Table 7 shows that the TMEL has a significant gastroprotective effect, while the petroleum ether and $CHCl_3$ fractions have a moderate gastro-protective activity. On the other hand, the EtOAc and aqueous fractions displayed a weak gastro-protective activity. The preliminary phytochemical investigation of TMEL of *C. quadrangulare* exhi-bited a positive result with Liebermann-Burchard's test (Schmidt, 1964) indicating the presence of sterols. Moreover, from petroleum ether fraction, sterols and triterpenes compounds were isolated; hence gastroprotective activity of these fractions may be due to presence of terpenoidal or steroidal compounds (Tovey et al., 2011; Subhadhirasakul and Pechpongs, 2005). The aforementioned gastroprotective results are in line with other previous studies in family Verbenaceae (Tajik et al., 2015; Chellappan and Pemiah 2014; Jothi et al., 2010; Monteiro et al., 2007; Penido et al., 2006).

The anti-diabetic activity was concentrated in the EtOAc fraction. This clearly appeared in decreasing the blood glucose level, while the TMEL and $CHCl_3$ fraction have moderate effects. But, the petroleum ether fraction increases the blood glucose level (13%). The previous

Table 6. Anti-pyretic activities of *C. quadrangulare*.

Group	Rectal temperature (°C)/min							
	Pre-yeast	Pre-drug	30	60	90	120	150	180
Control	36.67±0.23	38.03±0.33	37.47±0.53	37.37±0.23	37.90±0.20	37.60±0.70	38.33±0.17	37.83±0.07
Acetylsalicylic acid	35.00±0.39	38.40±0.49	37.13±0.38	37.57±0.28	35.47±0.23***	35.40±0.15**	35.33±0.24***	35.30±0.32***
TMEL	35.83±0.13	38.40±0.15	36.53±0.12	35.60±0.17***	35.80±0.10***	36.03±0.24*	35.90±0.10***	35.80±0.17***
Petroleum ether fr.	36.00±0.28	38.47±0.28	37.10±0.10	36.20±0.15**	35.97±0.18***	36.00±0.40*	36.27±0.20***	36.03±0.15***
$CHCl_3$ fr.	35.67±0.36	38.17±0.37	37.80±0.51	36.50±0.23*	36.33±0.29***	36.20±0.36*	36.03±0.41***	36.27±0.49**
EtOAc fr.	35.87±0.29	38.13±0.29	37.23±0.12	36.13±0.24**	36.17±0.12***	36.33±0.29*	36.40±0.23***	35.97±0.35***
Aqueous fr.	35.90±0.40	39.13±0.47	37.30±0.50	36.20±0.46**	36.57±0.35***	36.07±0.52*	36.07±0.33***	35.90±0.36***

Values represent Mean±SEM, (n=5). Significant difference (**P<0.05, **P<0.01* and ****P<0.001*). All drugs (100 mg/kg).

Table 7. Gastroprotective activities of *C. quadrangulare*.

Group	Mean ulcer score (mm)	P.I. (%)
Control	25.0±8.38	----
Ranitidine	3.0±1.08	88
TMEL	5.5±1.85	78
Petroleum ether fr.	8.5±1.55	66
$CHCl_3$ fr.	9.5±4.66	62
EtOAc fr.	15.8±5.66	38
Aqueous fr.	22.5±2.66	10

Values represent Mean±SEM, (n=5). Ranitidine (50 mg/kg) and the other drugs (100 mg/kg).

studies demonstrated that various flavonoids especially quercetin possesses anti-diabetic activities (Vessal et al., 2003; Hif and Howell, 1985). Therefore, the highest anti-diabetic activity, which was shown by the EtOAc (58%) fraction could be attributed to its content of flavonoids. All results are listed in Table 8. Our findings are in line with previous studies in family Verbenaceae (Rohit et al., 2012; Zanatta et al., 2007; Villasenor and Lamadrid, 2006).

Conclusion

Investigation of TMEL of *C. quadrangulare* afforded three compounds, which were reported for the first time in the genus in addition to six known compounds. The TMEL and its fractions showed important pharmacological activities such as anti-inflammatory, anti-pyretic, gastroprotective and anti-diabetic. Therefore, it could have a supportive role in the pharmaceutical field towards the development of new drugs.

Conflict of interests

The authors have not declared any conflict of

Table 8. Anti-diabetic activities of *C. quadrangulare*.

Group	Blood glucose level (mg/dl)/h				
	0	1	2	3	4
Control	205±1.0	205±0.7	201±5.0	201±5.3	199±7.3
%	100.00	100.00	98.04	98.04	97.07
Glibenclamide	231±5.6	220±15.0	195±13.6	179±12.3	171±11.4**
%	100.00	95.23	84.41	77.49	74.03
TMEL	221±13.3	256±13.9	359±20.6	316±20.8	187±12.3*
%	100.00	115.84	162.44	142.99	84.62
Petroleum ether fr.	414±30.0	541±4.7	508±7.7	485±25.4	470±20.9
%	100.00	130.68	122.71	117.15	113.53
$CHCl_3$ fr.	235±10.7	304±17.2	295±9.1	257±11.7	203±13.2*
%	100.00	129.36	125.53	109.36	86.38
EtOAc fr.	512±6.6	546±15.7	426±22.3	333±19.0	219±9.7***
%	100.00	106.64	83.20	65.04	42.77
Aqueous fr.	209±4.1	219±8.0	222±8.7	205±6.3	202±6.1
%	100.00	100.00	106.22	98.09	96.65

Values represent Mean±SEM, (n=5). Significant difference (**P<0.05, **P<0.01* and ****P<0.001*). Glibenclamide (0.5 mg/kg) and the other drugs (100 mg/kg).

interest.

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Full Length Research Paper

Inhibitory activity of fractions of *Senna nigricans* toward protein tyrosine phosphatase 1B and dipeptidyl peptidase IV

Yusuf Saidu, Suleiman Alhaji Muhammad*, Lawal Suleiman Bilbis and Babangida Muhammad Sani

Department of Biochemistry, Usmanu Danfodiyo University, Sokoto, Nigeria.

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Protein tyrosine phosphatase (PTP) 1B and dipeptidyl peptidase (DPP) IV are important in down regulation and secretion of insulin, respectively. While PTP 1B regulates insulin binding to its receptor, DPP IV hydrolyses incretin, which is an important regulator of postprandial insulin secretion. The study evaluated the *in vitro* inhibitory effect of different fractions of *Senna nigricans* against PTP IB and DPP IV and the results were compared with standard inhibitors, sumarin and P32/98. The methanolic extract was further fractionated using Soxhlet apparatus and then the most potent fraction eluted through column on silica gel. The result indicated that the methanolic fraction had the highest inhibition percentage of 56.43±3.98 against PTP 1B. The inhibition of PTP 1B by methanolic fraction was significantly (P<0.05) higher than that of the standard inhibitor, sumarin. The PTP 1B inhibition by ethyl acetate fraction (31.34±5.40%) was not significantly (P>0.05) different from that of sumarin, while hexane fraction had the inhibition of 19.03±4.24% which was significantly (P<0.05) decreased as compared with sumarin. The result of DPP IV inhibition indicated that the methanolic, hexane and ethyl acetate fractions were not significantly different, but all the fractions were significantly less active than the standard inhibitor, P32/98 which recorded 63.1±4.67% inhibition of DPP IV. Because of *S. nigricans* as sources of PTP 1B inhibitors most especially and to some extent DPP IV inhibitors, the plant may be a potential source for the discovery of lead compounds as PTP 1B and DPP IV inhibitors to treat type 2 diabetes mellitus.

Key words: Protein tyrosine phosphatase, dipeptidyl peptidase, inhibitors, *Senna nigricans*.

INTRODUCTION

Diabetes mellitus is a metabolic disorder which is due to a defect in insulin secretion, insulin action, or both. A consequence of this is chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism. Insulin deficiency or resistance led to various metabolic alterations in diabetic subjects or animals which increased blood glucose and caused dyslipidaemia (Himma et al., 2014). Type 2 diabetes is the most

*Corresponding author. E-mail: smbinna98@gmail.com. Tel: +2348063779088.

Abbreviations: TLC, thin layer chromatography; **PTP,** protein tyrosine phosphatase; **DTT,** dithiothreitol

common form, accounting for about 85 to 90% of all diabetic cases, and is due to a combination of impaired insulin secretion and insulin resistance (Mlinar et al., 2007). The prevalence of type 2 diabetes mellitus is rapidly increasing worldwide, and it is estimated that more than 430 million people will be affected by 2030 (Shaw et al., 2010). Therefore, a continuous search for new therapeutic options is necessary due to limitations of the current available treatment. The medicinal properties of plant species have made an outstanding impact in the origin and evolution of many traditional herbal therapies. Traditional medicine has gained popularity world over owing to high cost of orthodox medicine (Hudaib et al., 2008). Natural products have been shown to play a significant role in the development of novel drugs for the treatment and prevention of diseases (Gilani and Rahman, 2005).

Senna (Cassia) nigricans is a very important plant of Ayurvedic medicines. The plant is a variable, branching, erect shrub and it is highly drought resistant and suitable for desert. The leaves of the plant have been reported to contain pharmacologically bioactive substances. Studies have shown that this plant possesses anti-inflammatory (Chidume et al., 2001), antioxidant (Kumaran and Karunakaran, 2007), hypoglycaemic (Jalalpure et al., 2004), and anticancer activities (Yadav et al., 2010).

Protein tyrosine phosphatase (PTP) 1B has been implicated in the negative regulation of insulin signaling by dephosphorylating the insulin receptor as well as insulin receptor substrate and selective inhibitions of this enzyme have emerged as a new drug target for the treatment of type 2 diabetes mellitus (Teng et al, 2011). Dipeptidyl peptidase (DPP) IV is a serine protease which causes breakdown of incretin hormones; glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are released into the intestine in response to nutrient ingestion and stimulate insulin secretion induced by glucose (Mentlein et al., 1993). Both peptides, GLP-1 and GLP are rapidly degraded by DPP IV into an inactive form (Vilsboll et al., 2003). Thus, inhibition of DPP IV activity has the potential to be a novel therapeutic strategy to treat type 2 diabetes mellitus (Ahren, 2005). Current antidiabetic drugs in use for long-term therapy are found to be associated with various toxic effects owing to which the developmental process in antidiabetic drug research has shifted its emphasis to natural plant sources having minimal side effects (Nayak et al., 2009; Veerapur et al., 2010). Therefore, the study is aimed at isolating PTP 1B and DPP IV inhibitors from *S. nigricans*

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used are of analytical grade. PTP IB and DPP IV drug discovery assay kits were purchased from Enzo® Life Sciences.

Plant

In the current study, *S. nigricans* was collected from Sokoto and the plant was identified by a taxonomist from Botany Unit, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria.

Fractionation of the extract

The plant sample was shade dried and ground to powder using laboratory pestle and mortar. Ten grams of the powdered sample was extracted in 100 ml of methanol for 72 h at room temperature. At the end of the 72nd hour, the extract was filtered using Whatman No 1 filter paper. The filtrate was concentrated using rotary evaporator. The obtained residue was left to dry in a drying cabinet and stored in an air tight labeled container at 4°C until required. Further extraction of the plant material was carried out in a soxhlet apparatus successively 5 h each with hexane, ethyl acetate and methanol. The percentage yield of crude methanol was 29.8%, while 12.3, 9.6 and 8.5% were recorded for hexane, ethyl acetate and methanol, respectively after soxhlet extraction.

Column chromatographic separation of the *S. nigricans*

The methanol soxhlet fraction of the *S. nigricans* was further fractionated using column chromatography on silica gel. The methanol fraction was fractionated with gradient column chromatography with silica gel 60 (Merck, Germany) as the stationary phase and hexane:ethyl acetate and ethyl acetate:methanol mixture as the mobile phase starting with the least polar and gradually increasing the polarity. The fractions were initially collected from column in 10 ml volumes, rerun on TLC and the fractions with same RF values were merged for bioassay of PTP 1B and DPP IV inhibitory activities.

DPP IV inhibition assay

The fractions were screened for DPP IV inhibition at 100 µg/ml in a total volume of 100 µl using DPP IV drug discovery assay kits, a product of Enzo Life Sciences. The inhibitor (P32/98) was diluted in the assay 1 in 10 µl of assay buffer (50 mM Tris, pH 7.5). The substrate, H-Gly-Pro-pNA was diluted in 1 in 50 µl of assay buffer. The plant samples were reconstituted in buffer (50 mM Tris, pH 7.5) to give 1 µg/µl. The DPP IV (BML-SE434-9090) was reconstituted 1 in 50 µl of the assay buffer. The assay mixture was prepared in a 96 well plate to contain 10 µg per 100 µl assay mixture of the sample, 15 µl of DPP IV (17.3 µU/µl) and 50 µl of the substrate and the volume made up to 100 µl with the assay buffer. The inhibitor well contained 10 µl of the inhibitor (P32/98) in place of the extract. The control tube had neither inhibitor nor the extract and the blank was prepared using the substrate and the buffer only. The plate was read continuously at 405 nm, in a microplate reader at 1 min intervals for 10 min.

PTP 1B inhibition assay

The kit components were thawed and held on an ice bath except BIOMOL RED™ that was stored at room temperature. The substrate, insulin receptor β residues 1142-1153, pY-1146 (IR5) was reconstituted to 1.5 mM by assay buffer and dH_2O. The asssay buffer, 100 mM MES, pH 6.0 containing 300 mM NaCl, 2 mM EDTA, 2 mM DTT and 0.1% NP-40 was diluted with equal volume of dH_2O and kept on ice untill required. The enzyme, PTP 1B (human recombinant) was prepared in x1 cold assay buffer. Stock

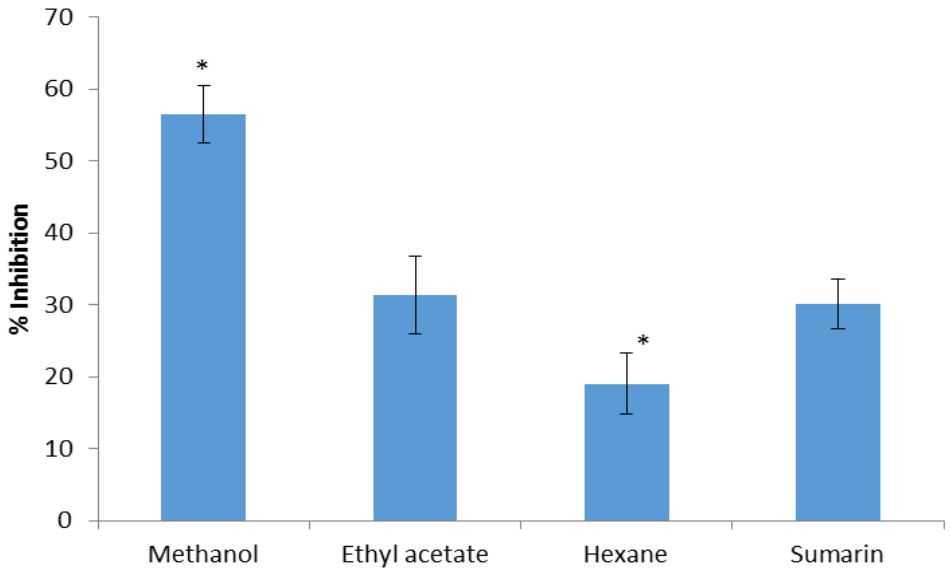

Figure 1. PTP 1B Inhibition of Soxhlet Fractions of *S. nigricans*. Data are expressed as Mean ± SD, n = 3 replicate, *P< 0.05 when compared with sumarin.

of 10 mM of the inhibitor, suramin was prepared in assay buffer. The assay mixture was prepared in a 96 well plate to contain 10 µg per 100 µl assay mixture of the sample. Other components of the the assay mixture were the buffer, the enzyme and the substrate. The substrate was added last. This was done strictly according to the manufacturers specifications.

Data analysis

The data are expressed as mean ± standard deviation (SD) of 3 replicate. The values were expressed as percentage inhibition. SPSS (version 17.0) was used for data analysis and P value of <0.05 was considered significant.

RESULTS

The percentage PTP 1B and DPP IV inhibitions of Soxhlet fractions of *S. nigricans* are presented in Figures 1 and 2, respectively. The results show that methanolic fraction had the highest percent inhibition of 56.43±3.98% for PTP 1B (Figure 1) as against the standard inhibitor, sumarin (30.12±3.46%). The percent PTP 1B inhibition of ethyl acetate fraction (31.34±5.40%) was not significantly (P>0.05) different from that of sumarin, while hexane fraction had the inhibition of 19.03±4.24% which was significantly (P<0.05) lowered as compared to sumarin (Figure 1).

DPP IV inhibition (Figure 2) indicated that the methanolic fraction (32.6± 4.33%), hexane fraction (28.2±5.36%) and ethyl acetate fraction (27.3±6.06%) were not significantly different, but all the fractions were significantly less active than the standard inhibitor, P32/98 which recorded 63.1± 4.67% inhibition of DPP IV.

The column chromatographic fractions of the methanolic fraction (Soxhlet) were merged based on the results of the thin layer chromatography into 19 fractions. The results of the PTP 1B and DPP IV inhibition studies are presented in Table 1. The results indicated further that fraction 12, which was eluted from the column using 1:3 ethyl acetate in hexane, had the highest PTP 1 B inhibition of 56.43% as compared to 30.12% for the standard inhibitor, suramin. The effect of the fractions on DPP IV indicated that the fractions that were active were significantly less active than the standard inhibitor.

Extracts with NI showed no inhibition, and actually activated the activities of the respective enzymes.

DISCUSSION

Type 2 diabetes mellitus which accounts for about 85 to 95% of diabetes cases is associated with considerable morbidity and mortality. In this study, the effects of fractions of *S. nigricans* on the activities of PTP I and DPP IV were investigated. The effect of methanolic fraction against PTP 1B was significantly higher than that of the standard inhibitor, sumarin. The PTP 1B and DPP IV inhibition assays were used in this study to elucidate the inhibitory effect of the fractions of *S. nigricans.* The methanolic extracts of plants such as *Psidium guajava* (Oh et al., 2005), *Salvia miltiorrhiza* (Han et al., 2005) and *Centratherum anthelminticum* (Arya et al., 2013) have been reported to exhibit significant effect against PTP 1B activities. Consistently, our findings indicated methanolic fraction displayed the highest percentage inhibition of 56.43%. This observation suggests that

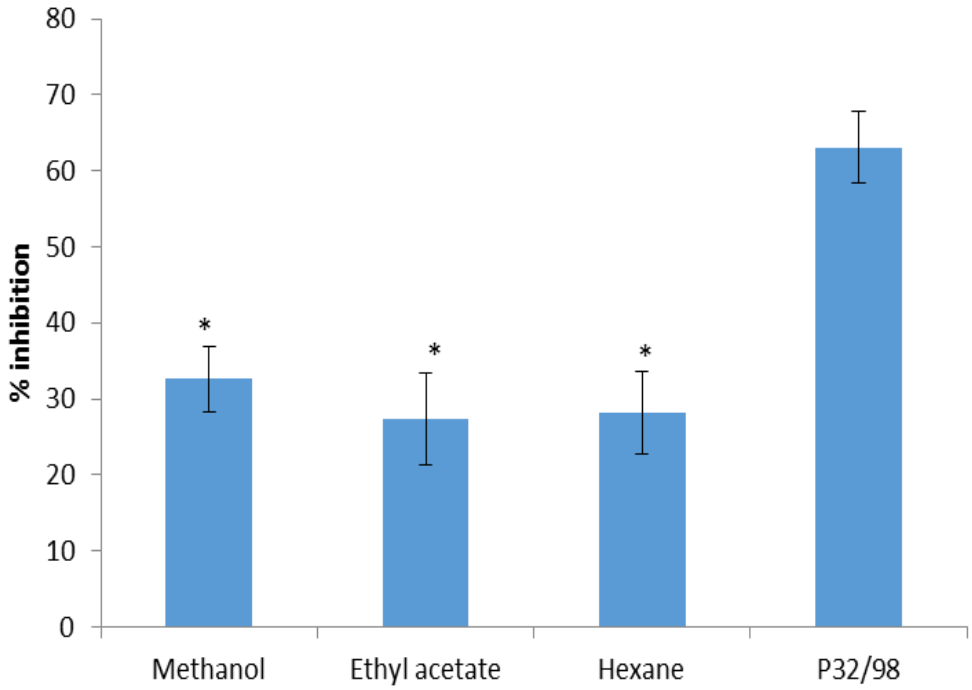

Figure 2. DPP IV Inhibition of Soxhlet fractions of *S. nigricans*. Data are expressed as Mean ± SD, n = 3 replicate, *P<0.05 when compared with P32/98.

Table 1. Inhibition of PTP 1B and DPP IV of the column chromatographic fractions of the methanolic fraction.

Fraction**	Percentage Inhibition	
	PTP 1 B	DPP IV
1	20.48	9.12
2	10.95	NI
3	NI	NI
4	NI	NI
5	37.38	NI
6	28.38	12.11
7	8.64	NI
8	28.78	NI
9	NI	NI
10	31.34	NI
11	44.48	7.23
12	56.43	12.12
13	13.51	NI
14	17.58	NI
15	NI	NI
16	17.93	NI
17	NI	6.23
18	NI	NI
19	NI	NI
20	23.48	NI
Inhibitor*	30.12	63.1

*Suramin for PTP IB and P32/98, MW=260.4 for DPP IV, **Fractions were initial collected from column in 10 mL volumes, rerun on TLC and the fractions with same RF values were merged for bioassay.

methanol may be the best solvent for extraction of PTP 1B inhibitors from *S. nigricans*. The DPP IV inhibitory activity of the three fractions were not significantly different, but were significantly lower than that of the control, P32/98. Many other studies have reported inhibition of DPP IV enzymatic activity by plant extract; the aqueous leaves extract of *Cistus incanus* L. (Lendeckel et al., 2002), methanolic leaves extract of *Mangifera indica* (Yogisha and Raveesha, 2010), hexane extract of *Annona squamosa* (Davis et al., 2012), ethanolic extract of *Urena lobata* (Yudi et al., 2015) and leaf extract of *Ocimum sactum* and fruit extract of *Momordica charantia* (Singh et al., 2014). Therefore, since prevalence of diabetes mellitus has escalated, a multi-model therapeutic strategy is urgently required for the treatment of the disorder and to large extent; *S. nigricans* and other plants that have been reported to be potential source(s) of inhibitors of PTP 1B and DPP IV could be isolated to treat type 2 diabetes mellitus. Fractionation using soxhlet apparatus followed by column chromatography and then thin layer chromatography indicated the fractionation of the PTP 1 B inhibitor(s) into fraction 12 of the column fractions. Unfortunately, it appears that the DPP IV inhibitory activity of *S. nigricans* has been lost upon the fractionation. This may be attributed to the fact that most of the bioactive components of plants act in synergy and this could be responsible for loss in the effect of *S. nigricans* against DPP IV inhibition.

Based on the findings in this study, it was hypothesized that crude extract which contains varieties of bioactive components may be more beneficial to multifactorial diseases like diabetes than any single bioactive compound. Although, further studies such as *in vivo* inhibitory and kinetic studies of PTP IB and DPP IV using crude fractions and pure isolate from *S. nigricans* are required to validate this hypothesis.

Conflict of Interests

The authors have not declared any conflict of interests.

Conclusion

The results of the current study demonstrated the potential of *S. nigricans* as a source of lead compound(s) in the development of inhibitors(s) of PTP 1B and DPP IV in the management of type 2 diabetes mellitus.

ACKNOWLEDGEMENT

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