ISSN: 2321-3132

Research Article

International Journal of Chemistry and Pharmaceutical Sciences

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Alternative Therapy in Type 1 Diabetes: The effect of *Croton Membranaceeus* extracts

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Received: 8 April 2014, Accepted: 27 May 2014, Published Online: 27 June 2014

Abstract

Backgroud: *Croton membranaceus* is a shrub which grows in most African countries mainly the coastal belt. It is an important medicinal plant used by Ghanaian Traditional Medical Practitioners in the treatment of benign prostatic hyperplasia (BPH) and diabetes. The present study aims to investigate the effects of 96% ethanol and aqueous extracts of *C. membranaceus*. **Methods:** The powdered root (3 kg) was separately cold macerated using 96% ethanol and aqueous. The doses for the study were fixed based on the Irwin test. The hypoglycaemic effect of both extracts (with doses 100 mg/kg, 600 mg/kg) was studied in streptozotocin-induced diabetic rats. **Results:** The extracts caused reduction of glucose levels after 6 hours and 28 days of administration in the streptozotocin-induced diabetic rats. Compound N[N-(2-methylbutanoyl) glutaminoyl]-2-phenylethylamine was isolated from *C. membranaceus* and may be one of the active constituents responsible for the observed activity. **Conclusion:** The findings demonstrate the potential of both ethanolic and aqueous extract of *C. membranaceus* on type 1 diabetes mellitus, justifying the traditional use of the plant in the management of diabetes by traditional healers in Ghana.

Keywords: *Croton membranaceus*, N [N-(2-methylbutanoyl) glutaminoyl]-2 phenyl ethylamine, streptozotocin, diabetes

Contents

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PAPER-QR CODE

1. Introduction

Diabetes mellitus is a major health problem affecting approximately 6% of Ghanaians and projected to increase in sub-Saharan Africa by 2020 (Amoah *et al*., 2002; Hall *et al*., 2011; Mbanya *et al*., 2010). Although there are conventional medical treatments to manage diabetes with its associated complications, patients in Ghana with

diabetes mellitus like any other chronic illnesses tend to have higher propensities to use complementary and alternative medicines (CAM) (Kretchy *et al*., 2014). The tendency to use CAM is based on concerns about the unwanted effects of the orthodox medicines and issues of medication affordability as well as the effectiveness of some of these CAM remedies (Kretchy *et al*., 2014). The majority of unorthodox anti-diabetic regimens are from plant origins with documented reports of indigenous plants possessing hypoglycaemic activities. For example extracts of *Murraya koenigii* (Rutaceae), *Capparis sepiaria* (Capparaceae), *Barleria lupulina* (Acanthaceae) and *Butea monosperma* (Fabaceae) have been shown to possess hypoglycaemic effects (Suba *et al*., 2004; Somani *et al*., 2006; Lawal *et al*., 2008; Selvamani *et al*., 2008). Most of the extracts from these afore-mentioned and other medicinal plants have been found to contain substances like glycosides, alkaloids, terpenoids, and flavonoids, (Joshi and Chauhan, 2013; Chowdhury Saikat *et al*., 2013) which may be responsible for the hypoglycaemic effects. In some cases, the active constituents have been isolated, characterized and their effects have been shown to be comparable to the standard hypoglycaemic agents like glibenclamide and metformin. An example is galegine, which was isolated as an active hypoglycaemic agent from *Galega officinalis* (Fabaceae) and served as a template for the synthesis of metformin (Snearder, 1985). Also, the cyclopropamide amino acids, hypoglycine A and B isolated from *Blighia sapida* (Sapindaceae)(Yusuf *et al*., 1994), and the novel terpenoid-type quinones SP-18904 and SP-18905, isolated from *Pycnanthus angolensis* (Myristicaceae) (Luo *et al*., 199) are some examples of active hypoglycaemic constituents from medicinal plants. In Ghana, while several reputable traditional health practitioners continue to make claims for medicinal plants in the management of diabetes, the use of medicinal plants in various ethno-botanical literatures has been reported (Irvine, 1961; Mshana *et al*., 2000; Abbiw, 1990). Until recently, *C. membranaceus*, which is one of the main ingredients among herbal medicines dispensed at the Centre for Plant Medicine Research (CPMR), Mampong Akuapem in Ghana for the treatment of benign prostatic hyperplasia (BPH) (Mshana *et al*., 2000), has been found to possess antihyperglycaemic properties (Sarkodie *et al*., 2014). Preliminary phytochemical screening indicates the presence of alkaloids and terpenoids in all parts of the plant. The leaves contain cyanogenic glycosides. One of its chemical constituents has been isolated and identified as julocrotine (1), a peptidyl alkaloid previously isolated from *C humilis* L (Aboagye et al., 2000; Kutney et al., 1971).

This work seeks to provide more scientific knowledge of hypoglycaemic activity on the 96% ethanol extract of the roots of *C. membranaceus* which is used in treatment of BPH and the aqueous extract of the roots of the plant. This work may also form the basis for the formulation of herbal hypoglycaemic products for clinical use after their safety has been ascertained. Additional scientific basis to support the traditional healers who use *C. membranaceus* to treat diabetes mellitus will also be provided. Again the results have the potential of supporting the development of the herbal medicine industry, providing employment that will lead to the socioeconomic empowerment of both skilled and unskilled labour.

2. Materials and Methods

Plant material

The roots of *C. membranaceus* were harvested at the Krobo-Gyakiti area in the Eastern Region of Ghana in January, 2013 and identified at the herbarium of the CPMR by Mr. Blagogee, a taxonomist. Voucher specimen (CPMR/2013/S-01) has been deposited at the CPMR Herbarium.

Extraction

The roots of *C. membranaceus* were chopped into pieces, air-dried over a period of fourteen days at room temperature and milled into a coarse powder. The pulverised material (3.0 kg) was extracted three times by cold maceration with 26 litres of 96%v/v ethanol. The extract was decanted, filtered with filter paper and concentrated under reduced pressure using a rotary evaporator. It was then freeze dried to give a brown gelatinous material (EECM), which has a yield (7.3 w/w %). Another pulverized material of *C. membranaceus* (3.0 g) was boiled in 6.0 liters of water for 60 mins and cooled. The resultant extract was filtered through cotton wool, pre-frozen and lyophilized into powder using a freeze dryer. The dried powdery extract (AECM) was weighed to determine the yield (8.7 % w/w) and stored in a desiccator at room temperature.

Phytochemical Screening:

A portion of the freeze dried extract of the *C. membranaceus* root was screened phytochemically using standard methods (Harbone, 1998).

Animal husbandry used in the experiments

Adult male Sprague-Dawley rats aged approximately 4 months and weighing 210 - 220 g were used for the study. They were housed under standard conditions at ambient temperature and supplied with standard pellet food and tap water ad libitum at the Department of Pharmacology's animal house. All rats were treated in accordance with the National Institute of Health Guidelines for the care and use of laboratory animals (NIH, Department of Health and Human Services publication Number 85, revised 1985). The Ethics Committee of CPMR approved the research protocol.

Induction of diabetes in rats using streptozotocin (STZ)

The bioassay model employed was according to the description of Sarkodie *et al*., 2013 with some modification. Streptozotocin (0.588 g) was dissolved in freshly prepared 0.1M citrate buffer of pH-4.5. The normal blood glucose level of the rats was taken and was found to be in the range of (3.9-4.7 mmol/l). Rats were injected intraperitoneally with a single dose of 60 mg STZ per kg body weight after an overnight fast. After injection, rats were given free access to food and water. After a rest period of 48 hours, diabetes was confirmed by determing the fasting blood glucose levels. Rats with blood glucose level above 10.0 mmol/l were selected for the experiment.

Test for hypoglycaemic activities of *C. membranaceus* extracts

The diabetic rats were randomly divided into six groups of five animals, Groups A, B, C, D, E and F. Groups A, B were given oral doses of 100 and 600 mg/kg body weight of aqueous extract of C. membraneceus (AECM) and Groups C and D received 100 and 600 mg/kg body weight of 96% of ethanol extract of *C. membraneceus* (EECM) respectively. Group E was given 2 units/kg body weight of reference drug, insulin, to serve as positive control and Group E received 5 ml/kg body weight of distilled water (normal control group).

Statistical analysis

All the data provided in this study represents means ± S.E.M. The results were analysed by one-way ANOVA followed by Dunnett's Multiple Comparison Test to establish significance ($p < 0.001$) between the treated and the vehicle groups.

Reagents

Aluminum Oxide for chromatography, type 507C-neutral, 100–125 mesh was obtained from Fluka Chemie AG, CH –9470 Buchs, Switzerland. Chloroform, Petroleum ether (boiling range 40–60^{o}C), Ethanol, Hydrochloric acid, Methanol, Diethyl ether and silica gel G6F254 for thin layer chromatography were obtained from BDH Chemicals Ltd, Poole, England.

Isolation of constituents

Ten kilograms of pulverized roots of *C. membranaceus* was macerated in 5% HCl overnight. After filtration, the extract was basified with ammonia to pH 9-10 and extracted with chloroform, dried with anhydrous sodium sulphate and evaporated under reduced pressure to obtain 5.30g semisolid residue. *C. membranaceus* extract was adsorbed onto Aluminum oxide, type 507C, neutral, Grade II and eluted successively with combinations of petroleum ether, diethyl ether, chloroform and methanol. Elution with petroleum ether/diethyl ether (1:9) yielded a crystalline substance provisionally referred to as CM-3. The constituent CM-3 isolated were identified based on H-NMR, 13C-NMR, HSQC, HMBC and ESI-MS data.

3. Results and Discussion

Results:

Hypoglycaemic effect of AECM and EECM after 6 hours and 28 days Treatment

The blood glucose levels of the vehicle (normal diabetic control) after 6 hours increased by 20.45 ± 0.95 %. The blood glucose levels of diabetic rats treated with AECM (100, 600 mg/kg body weight) decreased by 39.72 ± 2.122, 32.64 ±2.38 % respectively after the 6th hour (Figure 1). Those treated with EECM (100, 600 mg/kg) had their blood glucose levels lowered by 47.51 ± 2.06 and 34.75 ± 2.38 % respectively. All the doses of EECM showed relatively high hypoglycaemic effect than those of AECM, 6 hours after drug administration, at the selected dose levels. Insulin, the standard drug (2 u/kg) lowered the blood glucose levels by 86.64 ± 0.10 %. The extracts at the selected doses and insulin showed significant hypoglycaemic activities ($p < 0.001$) as compared to the vehicle group 6 hours after treatment. On the 28th day, the vehicle (diabetic control) increased by 19.04 ± 0.90 % in blood sugar level. AECM at 100 and 600 mg/kg body weight decreased the blood glucose levels by 78.69 ± 0.14 and 77.30 ± 0.11 % respectively (Figure 2).

On the other hand, EECM at 100 and 600 mg/kg body weight decreased the blood glucose levels by 61.41 ± 2.26 and 58.46 ± 1.14 % respectively. Insulin, 2 u/kg body weight lowered the blood glucose by 87.45 ± 0.07 %. Throughout the study, EECM at 100 mg/kg body weight exhibited the greatest hypoglycaemic activity compared to those of AECM. The hypoglycaemic activity of AECM at 100 mg/kg body weight was comparable to that of the standard drug. All the extracts (AECM and EECM) and the standard drug demonstrated statistically significant hypoglycaemic activity ($p < 0.001$) when compared to the vehicle control group.

Table 1. Results of Phytochemical Screening of powdered *C. membranaceus*

Plant Secondary Metabolites	*C. membranaceus*
Alkaloids	+
Terpenoids	+
Tannis	-
Reducing sugars	+

+ = present; - = absent

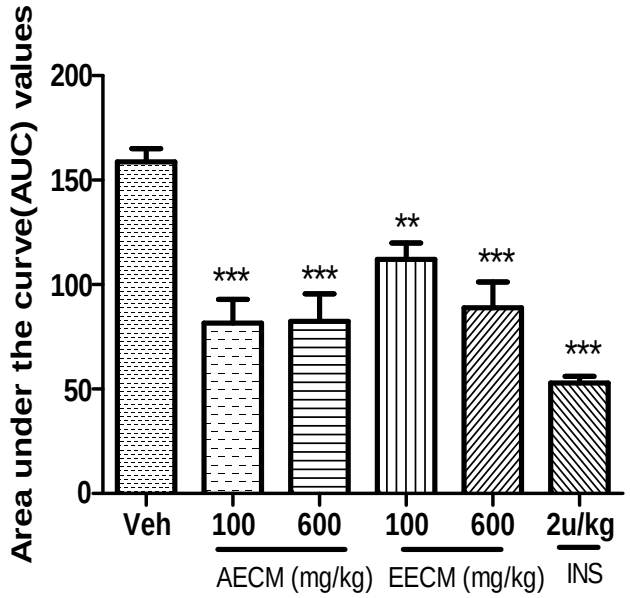

Figure 1. Area under the curve of AECM and EECM on blood glucose level in diabetic rats, 6 hours after treatment

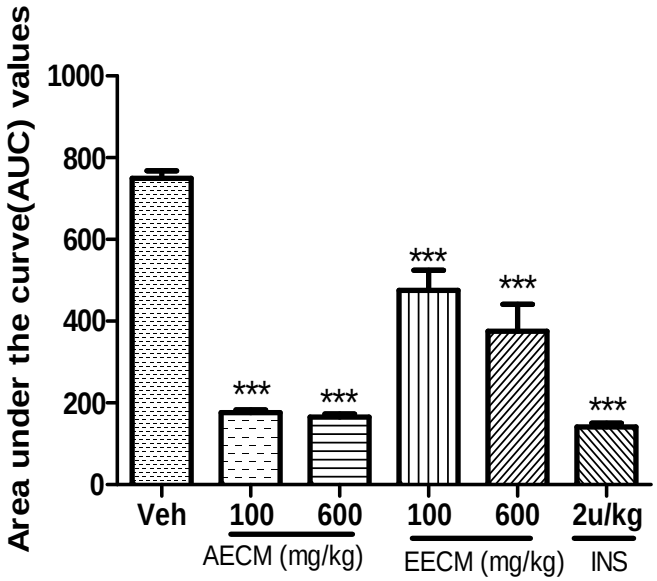

Figure 2. Area under the curve of AECM and EECM on blood glucose level in diabetic rats, 28 days after treatment

Table 2. NMR assignments and HMBC connectivities for CM-3 isolated from *C. membranaceus*

Position	NMR assignments		HMBC	
	^{13}C	^{1}H	^{2}J	^{3}J
1	68.54	-	-	-
2&6ax	38.42	1.29	22.23	30.84, 47.99, 38.42
2&6eq	38.42	1.63	22.23, 68.54	38.42, 47.99
3&5ax	22.23	1.32	38.42	22.23
3&5eq	22.23	1.55	38.42, 47.99	22.23, 68.54
4	47.99	1.15	22.23, 72.48	26.54, 38.42
7	30.84	1.13	68.54	38.42
8	72.48	-	-	-
9&10	26.54	1.10	72.48	47.99, 26.54

Characterisation of CM-3

ESI-MS data obtained for CM-3 are as follows: (Positive mode) m/z (rel int): 195.2 [(M + Na)$^+$ 100%], 196.08 (10.68%), 197.3(0.81%); ESI-MS (Negative mode) m/z (rel int): 281.27 [(M + 5Na – 6H) 16.5%], 255 (62.7%), 241.3 (19.2%), 227.3 (11.7%), 171.2 [(M – H)$^-$ 100%], 155.2 [(M – OH)$^-$ 55.1%]. ^{1}H-NMR (500MHz, $CDCl_3$ + CD_3OD) and ^{13}C – NMR (125.8MHz, $CDCl_3$ + CD3OD) are presented in table 2.

Again, ESI – MS data for CM – 3 are as follows: (Positive Mode): m/z: 372.3 [(M+K)$^+$ 15.26%], 356.4 [(M + Na)$^+$ 100%]. ESI-MS (Negative Mode): m/z: 333.03 [(M)$^-$ 15.93%], 332.43 [(M – H)$^-$ 100%] MS/MS of 332.4 (Negative Mode): m/z: 228.2 [{(M-H) – $C_6H_5CH_2CH_2$}$^-$ 100%], MS/MS of 356.4 (Positive Mode): m/z: 339.3 [{(M +Na) – OH}$^+$ 100%]; ^{1}H-NMR (500MHz, $CDCl_3$ + CD_3OD) and ^{13}C-NMR (125MHz, $CDCl_3$ + CD_3OD) are presented in table 3.

Table 3. NMR assignments and HMBC connectivities of N[N-(2-methylbutanoyl)glutaminoyl]-2-phenylethylamine (3) isolated from *C. membranaceus*

Position	NMR assignments (ppm)		HMBC	
	^{13}C	^{1}H	^{2}J	^{3}J
1	128.26	7.27 *m*	138.99, 128.65	128.26
2	128.65	7.18 *m*	126.12, 128.26	-
3	126.12	7.18 *m*	126.12, 128.65	128.26
4	128.65	7.18 *m*	126.12, 128.65	128.26
5	128.26	7.27 *m*	138.99, 128.65	128.26
6	138.99	-	-	-
7	35.06	2.79 *m*	40.49, 138.99	128.26
8	40.49	3.43 *m*	35.06,	138.99, 171.64
9	-	-	-	-
10	171.64	-	-	-
11	51.94	4.2 *m*	28.07, 171.64	31.60, 177.95
12	-	-	-	-
13	177.95	-	-	-
14	42.33	2.19 *m*	16.87, 26.80, 177.95	11.32
15	26.80	1.40 *m*	11.32, 42.33	16.87, 177.95
		1.59 *m*	11.32, 42.33	16.87, 177.95
16	11.32	0.86 *dd*	26.80	42.33
17	16.87	1.10 *d*	42.33	26.80, 177.95
18	28.07	1.85 *m*	31.60, 51.94	171.64, 176.44
		2.00 *m*	31.60, 51.94	171.64, 176.44
19	31.60	2.19 *m*	28.07, 176.44	51.94
20	176.44	-	-	-
NH_2	-	3.7 *br s*	-	-

ESI-MS gave a molecular ion peak at m/z 195.2 for (M + Na)$^+$ in the positive mode and at 171.2 for (M-H)$^-$ in the negative mode. These indicated that CM-3 has a molar mass of 172.2. ^{13}C-NMR indicated that CM-3 is a 10-carbon compound with a relative molar mass of 172. Using HSQC and HMBC connectivities the structure of the carbon skeleton of the compound was constructed below:

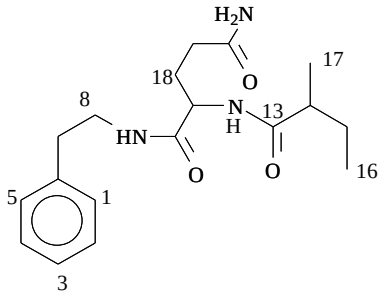

Figure 3

Discussion

C. membranaceus has clearly demonstrated potentially both acute and chronic effects on blood glucose levels. This outcome was evident in the similarities observed between the blood glucose lowering effect of both extracts (AECM, EECM) and the conventional drug insulin (2u/kg). It has been suggested that hypoglycaemic actions of the

diabetic agents in diabetic rats could be mediated by insulin action or by other mechanisms such as stimulation of glucose uptake by peripheral tissues, inhibition of endogenous glucose production or activation of gluconeogenesis in the liver and muscular tissues (Burcelain *et al*., 1995). The hypoglycaemic effect of the plant extracts in diabetic rats may therefore be possible through any of these mechanisms.

Preliminary phytochemical screening of both extracts of *C. membranaceus* showed the presence of reducing sugars, alkaloids and terpenoids. Our findings suggest that these chemical constituents in the plant extracts may be responsible for the biological activity on blood sugar. In contrast to a previous study on the ethanol extract of *Butea monosperma*, phytochemical analysis revealed that poly-phenols, sterols and flavonoids were responsible for the hypoglycaemic effect on the streptozotocin-induced diabetic rats (Bavara and Narasimhacharya, 2008).

In a study where galegine was isolated from *Galega officinalis* (Fabaceae), the outcome was significant as it formed the basis for the synthesis of metformin, an oral hypoglycaemic agent (Sneader, 1985). Similarly, our study isolated N[N-(2-methylbutanoyl)]-2-phenylethylamine from *C. membranaceus*, which can subsequently form the basis for the synthesis of N[N-(2-methylbutanoyl)]-2-phenylethylamine analogues leading to the formation of more effective and less toxic isolates that could be developed into a standard hypoglycaemic agent if it has hypoglycaemic activity. The prospects of this study are very high with significant impact in the management of diabetes mellitus.

4. Conclusion

This study has revealed that both 96% ethanol and aqueous extracts of *C. membranaceus* exhibited promising hypoglycaemic activity in streptozotocin-induced diabetic rats. Again, the study has shown that N[N-(2-methylbutanoyl)glutaminoyl]-2-phenylethylamine is an isolate from *C. membranaceus* and may be one of the active constituents responsible for the observed activity. This research therefore provides support to the use of *C. membranaceus* by indigenous people in the management of diabetes in Ghana.

5. Acknowledgement

The authors are grateful to Department of Pharmacognosy and Herbal Medicine, School of Pharmacy, University of Ghana and Centre for Plant Medicine Research at Mampong-Akwapim, Ghana for providing support for this study.

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