Manosrin A New Hypoglycaemic Compound From Anisopus Mannii N.E.Br.

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Abstract: The aqueous extracts of ten selected herbal traditional medicinal plants namely, Catunaregam tormentosa, Raulfia serpentine, Moringa oleifera and Anisopus mannii among others from the North Eastern Nigeria were screened ab initio in albino mice (n = 5) using standard procedures. The fraction with the highest hypoglycaemic activity was later extracted with 80% v/v MeOH using soxhlet extractor. The crude MeOH extract was partitioned with CHCl₃ and other solvents after TLC, and sub-fractions obtained were tested in diabetic mice (n = 5) using standard procedures to ascertain the most potent hypoglycaemic sub-fraction. This was further purified using HPLC and tested on diabetic mice (n = 5) with doses ranging from 1 to 10000 folds (µg/kg bw) and compared to standards drugs, Glibenclamide and Insulin, respectively. The sub-fraction was characterized using ¹H and ¹³C - NMR and two-dimensional correlation spectroscopy COSY, NOESY, HMQC and HMBC. A new compound "3, 23, 28-Trihydroxy-12-olean-3-0-(β-D-glucopyranosyl-(1,6)-β-D-glucopyranosyl-(1,6)-β-D-xylopyranosyl)-28-O-β-D-glucopyranosyl-(1,6)-β-D-glucopyranoside" (Manosrin) was obtained after characterization. Manosrin showed 45.15 and 67.97 % hypoglycaemic effect with 10 and 100 N folds dosages, respectively. The observed effects were almost at par with the standard drugs. The discovery of Manosrin from A. mannii N. E. Br. is as a new compound will add to the already existing list of known pharmaceutical hypoglycaemic compounds and a new development in the management of diabetes mellitus. Studies on toxicity, mechanism of Manosrin action will be published soon.

Key words: ab initio, Anisopus mannii, hypoglycaemic, Manosrin,

INTRODUCTION

The prevalence of Diabetes Mellitus (DM) in developing countries have been a source of concern to medical health practitioners over the years (1). Forecast by health statisticians points to a gorier outcome in the near future (2). The recent speculations about vaccine for DM (3) though a very good news to the Medicare world, may not be same for the African continent. This is because studies have shown that several Governments in the African continent have not been considering health care as priority when yearly budget are made (4), and if they do, such expenditures may not be wholly implemented as proposed. As such the use of alternative therapies in poor suburbs of Africa may remain a main stay for a long time. Alternative medicine from plants, such as roots, seeds, tree barks and leaves have been used for long time as traditional medicine. Some examples are Anogeissus acuminata (5), Catunaregam tormentosa and Raulfia serpentine [L] Benth. Ex. Kurz (6) and Anisopus mannii (7) among several others. Anisopus mannii (A. mannii) called kashe zaki in Hausa language (widely spoken in Northern Nigeria and in parts of West African) meaning “sweet destroyer”, is a perennial plant with leaves spread and petiole of about 1.3 to 2.0 cm long, bears a distinct gland at the apex, with blade about 5.7 - 7.6 cm long and stem twining to a height of 3.7 to 4.6 m (8). A. Mannii decoction is widely used in traditional medicinal preparation in northern Nigeria for treating diabetes, diarrhoea and haemorrhoid. Some reports opined that A. mannii is used also to treat fever (9), rheumatism and urinary tract diseases (10). Previous studies with the plants lead to the successful isolation of novel compounds, such as 17-naphthyridine alkaloid named anisopusin; 5-hydroxy-lup-20(29)-en-3-ylicosanoate, (6)-gingerdione, (6)-dehydroygerdione and fureric acid from acetone extract from its stem bank (11). The objective of this research was to screen ten traditional medicinal plants popularly used in north eastern Nigeria and to further identify potent hypoglycaemic compounds from that which exhibited the highest hypoglycaemic activity in a bid to carry out analysis leading to the purification and isolation of pure compounds that may be potent hypoglycaemic compounds.

MATERIALS AND METHODS

Plant Material

A. mannii specimen was obtained from Yola-North Local Government Area of Adamawa state Nigeria at an elevation of 176 m by Mr. Luka Danladi of the Department of Plant Science, Federal University of Technology, Yola. It was identified by Prof. Mohammed Saquib of the Department of Botany, Adamawa State University, Mubi Adamawa state, Nigeria. 1220 g of A. mannii powder was extracted with 80% v/v MeOH (CH₃OH) using a soxhlet extractor. After the reflux, filtration and evaporation of the methanol crude extract of A. mannii, the percentage yield was 19.90%. After partitioning, the chloroform fraction was 10.47% and the methanolic fraction was 9.45%. During the elution with the silica gel column chromatography of the chloroform fraction, five separate sub-fractions were collected as follows: 1.37% [22.77 g], [C₂ - 0.52% [6.39 g], C⁴ - 0.30% [3.24 g], CM - 2.91% [34.54 g] and CAM - 4.84% [59.10 g], respectively. For the methanol residue, four [4] sub-fractions were collected as follows: E - 0.08% [0.95 g], EA - 3.75% [45.77 g], EAM - 4.96% [60.54 g] and M - 0.53% [6.43 g].

Hypoglycaemic Activity

Male, albino mice weighing between 24 – 27 g were grouped randomly consisting of 5 in each. They were maintained on standard pellet and water ad-libitum.
Diabetes was induced by a single intraperitoneal injection of 180 mg/kg bw of alloxan monohydrate in normal saline water at a volume of about 0.5 ml. 100, 200, 400mg/kg bw of the obtained extract and fractions of A. mannii were administered using a feeding tube. The standard drugs were intraperitoneally administered, 0.5 iu/kg of Insulin and 1.0 mg/kg Glibenclamide as the positive control groups, respectively. The negative control group were administered distilled water (DW). Fasting blood glucose (FBG) was assessed hourly for a period of 4 hours using the method described by Zaruwa et al., (7).

**Purification and Isolation of Bioactive Compound**
The sub-fraction with the highest potency was subjected to purification by column gel chromatography technique. 8.0 g of the fraction was loaded into ODS gel in a 90 x 4.5cm column and semi-pure fractions were eluted with 300 ml diluted methanol and collected at intervals. The eluted fractions were run on analytical TLC to ascertain purity. Eluents with similar spots were combined and further separated using reverse phase recycling HPLC with a solvent system of MeOH:H2O (55:45). A pure compound was obtained after several recycling.

**Hypoglycaemic studies I**
All sub-fractions obtained from the partitioning were prepared into three doses of 100, 200 and 400 mg/kg bw based on the various concentrations obtained. The different doses were administered to groups (n =5) of normoglycaemic and alloxan induced diabetic ICR mice after an 18 h fast and monitored over a 4 h period. The effect of each sub-fraction was compared with standard hypoglycaemic drugs, insulin and Glibenclamide.

**Instrumentation**

\[ ^1H \text{-NMR and } ^1C \text{- spectra (400 and 100 MHZ) and two-dimensional correlation spectroscopy COSY; NOSEY; HMQC and HMB} \]

The dried powder of A. mannii MeOH extract was partitioned with chloroform and evaporated to dryness. The methanol sub-fractions were further separated using liquid phase partitioning (LPP) as described on Figure 1. ODS, Sephadex column chromatography, ELSD and HPLC were used to purify the methanol extract. Analytical TLC was used to compare the bands of all the fractions obtained and those with very similar characteristics were combined. The structures of the isolated fractions were elucidated by \[^1H\text{-}, ^1C\text{-NMR, DEPT 90, DEPT 135 and MS}.\]

**Structural Elucidation**
The structure of the isolated compound was elucidated using the nuclear magnetic resonance (NMR) and EI – MS.

**TLC/UV Spectrometric/HPLC analysis**

Preliminary analysis of the MeOH fraction of A. Mannii with 10% H2SO4 was spray was done alongside chemical constituent of phytochemical contents of A. mannii by HPLC using saponin purum standard in EtOH, Sigma-Aldrich Company, Germany. 1 mg of A. mannii (MeOH-fraction) was dissolved in distilled water (1 ml) and filtered with a 0.45 μm and 0.75 ml was analysed on a UV visible spectrophotometer (Shimadzu, Japan) to obtain a suitable spectral wave length for further analysis. 1 ml of the filtered solution was transferred into an amber bottle for HPLC analysis and compared with a standard. A Gemini – Nx 5 μ C18 110 A, 250 x 4.60 mm column (Thermo Finnigan- Auto sampler, Thailand). The mobile phase was 40% MeOH and using an isocratic program for 45 mins. The flow rate was 0.8 ml/min and the wavelength set at 218 nm.

**Extraction, isolation, purification and structure elucidation of selected (A. mannii) fractions by ODS gel chromatography**

The sub-fraction of interest (with the highest hypoglycaemic effect) was however administered in a fold (N), four folds (4N), ten folds (10N), a hundred folds (100N) and a thousand folds (1000N), respectively. The different doses were administered to groups (n =5) of normoglycaemic and alloxan induced diabetic ICR mice
after an 18 h fast and monitored over a 4 h period and compared to standard drugs Glibenclamide and Insulin.

Statistical Analysis
Statistical significance was established using one-way analysis of variance (ANOVA), and data were reported as mean ± standard error mean (s.e.m.). Significant difference was established at p < 0.05. Statistical analyses were carried out using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL).

Ethical clearance
All methods were ethically approved by the Chiang Mai University’s Animal Ethics Committee. Protocol Number 40/2552.

Results and Discussion

Hypoglycaemic activities of the methanolic fractions of A. manni
After the reflux, filtration and evaporation of the methanol crude extract of A. manni, the percentage yield was 19.90 %. After partitioning, the chloroform fraction was 10.47 % and the methanolic fraction was 9.35 %. For the elution with the silica gel column chromatography of the chloroform fraction, there were five separate sub-fractions as follows chloroform I, 1.87 % (22.77 g), chloroform II, 0.52 % (6.39 g), chloroform III, 0.30 % (3.24 g). Three separate bands on the column were observed by using chloroform as an eluent and were eluted and collected separately as chloroform I, chloroform II and chloroform III, while IV, Chloroform-methanol 2.91% (8.1) (35.54 g), V, chloroform-acetic acid-methanol (8:1:1) 4.84 % (59.10 g) were eluted with chloroform/methanol (8:1) and chloroform/acetic acid/methanol (8:1:1), respectively. For the methanol residue, it gave the following sub-fractions of i, ethyl acetate 0.08 % (0.95 g), ii, ethyl acetate-acetic acid (7:2) 3.75 % (45.77 g), iii, ethyl acetate-acetic acid-methanol 4.96 % (7:2:2) (60.54 g) and iv, methanol 0.53 % (6.43 g) by liquid phase partitioning (Figure 1).

The purified and elucidated compound from A. manni
The structural elucidation of the isolated compound from the methanolic sub-fraction of A. manni on the basis of the interpretation of 1H- and 13C-NMR and MS data (Gao et al., 2003), and comparative references estimation and verified by web NMR predictor (2011), gave a new compound called 3, 23, 28-Trihydroxy-12-oleanene-3-O-(β-D-glucopyranosyl-(1,6)-β-D-glucopyranosyl-(1,6)-β-D-glucopyranosyl-28-O-β-D-glucopyranosyl (1,6)-β-D-glucopyranoside (Manosrin) (Figure 2). The estimation of the pure compound was identified by comparison with the corresponding compounds in the following literatures with similar compounds: 1. Pentacyclic triterpene esters, 3β, 23, 28-trihydroxy-12-oleanene-23-caffic acid (12), 2. Longispinogenin 3-O-β-D-glucopyranoside, (13), 3. sitakoside XI-XX, 3-O-β-D-glucopyranosyl (1-6)-β-D-glucopyranosyl-(1-6)β-D-glucopyranosyl-28-O-β-D-glucopyranoside (14), 4. Platyicoside H, 3-O-β-D-glucopyranosyl-(1-6)β-D-glucopyranosyl-2β, 3β, 16α, 23-tetrahyxyolane-12-en-28-oic acid 28-O-β-D-glucopyranosyl-(1-4)-α-L-rhamnopyranosyl-(1-2)-α-L-arabinopyranoside (15). The molecular formula of the compound was determined by high resolution MS data given as C35H40O29. In the total 13C NMR, 59 signals were detected and the 13C NMR and DEPT experiment indicated 6 - C, 30 - CH, 17 - CH2 and 6 - CH3 (12). Hence the conclusion of the aglycone structure as ‘3β, 23, 28-trihydroxy-12-oleanene’, (12), which is C30H42O3. Comparing with the 13C NMR data of the compound 7-3-4 is very similar in the aglycone part with slight dissimilarities due to difference in the arrangement of the sugar moieties observed in compound 7-3-4. Five sugar moieties were detected in the compound with reference to 2, 3 and 4, which were estimated to be four hexose (C6H12O6) and one pentose (C5H10O5) giving a total of C29H36O28, accordingly (14). The fraction was obtained as a clear brownish film. Furthermore, a D absolute configuration of these sugar residues was assumed, consistent with the stereochemistry of naturally occurring monosaccharides. A comparison of the 13C- NMR spectral data with those of the sugar moieties of saponin in Gymnema sylvestre (13) indicated a glycosylation shift at the C-3 and C-28 positions while C-23 had no shift, leading to the conclusion that the glucosidic residue was attached to the C-3 and C-28 position of the compound. The 1H- and 13C-NMR spectra of the compound exhibited five sugars at the 1-6 linkages in the 6 105.7, 105.3, 105.5, 104.8 and 104.8, respectively. This estimation agrees in totality with the formula obtained from the MS data. Hence the name of Fr. 7-3-4 was deduced as ‘3, 23, 28-Trihydroxy-12-oleanene-3-O-(β-D-glucopyranosyl-(1,6)-β-D-glucopyranosyl-(1,6)-β-D-glucopyranosyl-28-O-β-D-glucopyranosyl-(1,6)-β-D-glucopyranoside’ (Manosrin), a triterpene saponin glycoside (Figure 2). The specific positions of the other individual Carbon spectra observed in the sugar moieties are still inconclusive namely: CH- 78.2, CH-78.0, CH-77.9, CH-77.8, CH-77.7, CH-77.6, CH-76.7, CH-75.6, CH-75.1, CH-75.9, CH-74.8, CH-71.6, CH-71.5, CH-71.3 and CH-71.1 (some carbon atoms overlapping). The triterpene saponin belongs to the family of saponin compounds. The chemical building blocks of triterpene saponins are four or five ring configurations of 30 carbons with oxygen atoms attached. Triterpenes are synthesized from five carbon isoprenoid units which create a steroidal structure such as cholesterol (C27H46O). They are mostly found in plants saponin glycosides which indicate the linkages of various sugar moieties to the triterpene unit (aglycone). The sugar moieties which could be either hexose or pentose can be easily detached from the triterpene in the mammalian gut by intestinal microflora. Thus, they are easily inserted into the cell membranes and modified in its molecular arrangement and affect the membrane fluidity. As pharmaceutical agents, several triterpene saponins have been observed to possess numerous biological activities, for example, triterpene saponin such as phytoestrol have been observed to decrease the absorption of cholesterol from the gastrointestinal tract and enhance its excretion (15), inhibit the action of enzymes responsible for the synthesis of cholesterol (16), (17). Vast majority of triterpene saponins have been reported to enhance the modulation of the mammalian immune system (18), (19). Other reported pharmaceutical effects are anti-inflammatory and improved blood circulation (19), (20), (21), (22), antimalarial and hypocholesterolemic (23), antihelminic (24), antisnake venom (25), antihypertensive (26), anti-carcinogenic effect (27), (28), hyperlipidaemic.
(29) and hypoglycaemic (30), (31). Several triterpene saponins were previously isolated and found to be hypoglycaemic, among which Senegins II – IV is desmethoxy senegen from the rhizome of Polygala senega var latifolia (32). Yoshikawa and Matsuda (33) attributed the inhibition of intestinal α-amylase to the hypoglycaemic activity of triterpene saponin. They averred that the hypoglycaemic effect of the triterpene saponin to the 3-O-glucoronide moiety and the 28-carbonyl group of the oleanolic acid glycoside are responsible for the exertion of the effect. Other characteristics observed with triterpene saponin was the suppression of gastric emptying by stimulation of the release of dopamine to act through the dopamine 2 receptor which stimulates the release of prostaglandins (33). The oleanolic acid constitutes the core of the discovered triterpene saponin. The oleanolic acid (saponins) have been reported to possess strong wound healing activity (34) which is one of the vital needs for diabetic patients. The elucidation of this new compound (Manosrin) (Figure 2) is only one of few compounds that have been isolated from A. mannii as a minor novel 1,7-naphthylidine alkaloid with an unprecedented skeleton, named anisopusine (1), was previously isolated while four other known compounds namely: 5α-hydroxy-lup-20(29)-en-3β-yl-eicosanoate (2), [6]-gingerdione (3), [6]-dehydrogingerdione (4), and ferulic acid (5) were also isolated from the plant (11). In an attempt to treat diabetes mellitus permanently, several plants were reported to yielded triterpene saponins as active principles which were observed to possess potent fasting blood glucose reduction (FBG) selected on the basis of their traditional usage as was A. mannii, among these medicinal plants are Mormodica charantia (35), Glycyrrhiza glabra (36), Panax ginseng (37), Platycodon Radix and Bellis perennis (29). Thus, the addition of A. mannii to the group of hypoglycaemic medicinal plants should trigger more research into other possible compounds that may be isolated from the plant with other biological characteristics. The compound Manosrin showed a 45.15 % FBG suppression in mice with the highest effect observed at the 4 h when administered with 3.2 µg/kg bw. Though the concentration that was used to achieve this was small, the main objective of this research had been archived. However, an increment in the dosage of the pure compound 32 µg/kg bw administered to other groups of diabetic mice lead to a further suppression of the FBG by 67.97%, while a further increase in dosage to 320 µg/kg bw lowered the hypoglycaemic effect by 29.03% only. Some behavioural hyperactivity was observed in the two groups of mice for reasons not well understood (Table 1), though the drastic drop in the blood sugar levels of the mice may have been responsible. Though, earlier results from the biological effect of the A. mannii sub-fraction lead to the speculation that the mechanism of action of the active principle may have enhanced insulin activity (or Thiazolidinedione action) as a way of FBG reduction in the diabetic mice, the effect of the pure compound could be said to suggest alternative mechanism in addition to that which was earlier speculated, because the lowering of the FBG suppression may have been exerted as a result of the effect of the excess dose of the compound leading to a negative feedback inhibition of the available amount of insulin present in the blood stream (38), (39). This implied that, the suppression of FBG in the diabetic mice may also be as a result of the action of the pure compound on the beta cells of the pancreas (sulfonylurea action), leading to hyper-secretion of insulin and a possible negative feedback inhibition leading to the observed reduction in hypoglycaemic effect. There are reports which implicated triterpene saponins for gastric emptying and the stimulation and release of dopamine to act on dopamine 2 receptors (33) which may have been responsible for the hyperactivity observed in the mice groups as the expectations of the dopamine levels are supposed to be reduced as observed by Ishida et al., (40).

**Fig 2:** Structure of the pure compound from the methanolic sub-fraction of A. mannii (Manosrin)

(3, 23, 28–Trihydroxy-12-oleanen-3-O-(β-D-glucopyranosyl-(1,6)-β-D-glucopyranosyl-(1,6)-β-D-xylpyranosyl)-28-O-β-D-Glucopyranosyl-(1,6)-β-D-glucuronide) (Manosrin)

**Table 1:** Hypoglycaemic effect of the methanolic pure compound isolated from A. mannii (Manosrin) compared to distilled water: DW and standard drugs, insulin: Ins and Glibenclamide: Glb and monitored 4 h post treatment. N: 0.128 µg/kg bw, 4 N: 1.28 µg/kg bw, 10 N: 3.20 µg/kg bw, 100 N: 32.00 µg/kg bw, 1,000 N: 320 µg/kg bw, Significant a: p<0.05, b: p<0.01, c: p<0.001

### Conclusion

Results from the model suggested that the peak values for the Manosrin could probably be between the 3.2 and 32 µg/kg bw. Though significant reduction of the FBG was obtained at a low concentration, this efficiency compares only with glibenclamide on a 1:1 basis but not with insulin. These findings are very interesting and they confirmed the use of these medicinal plants by the traditional health care givers and assure the patients in Nigeria, on the ancient wisdom in the use of these
hypoglycaemic plants in the management of what has been considered a global silent killer. This study like many others before it, has shown that medicinal plants could be relied upon if it is used with care like its allopathic counterpart. The use of medicinal plants around the world, especially in developing countries like Nigeria is inevitable considering the socioeconomic status of the majority of people in similar countries. The fact that, the health alarm from world bodies about the continual increase in the number of diabetic patients as a result of change in lifestyle and cuisine especially in the developing countries, makes this study worthwhile. It can be noted that, earlier on, diabetes was regarded in most communities in the developing countries, as a disease of the aged, affluent and the rich. This is no longer the case, though such ignorance contributed to the increasing numbers of diabetic patients in developed countries, especially Nigeria. The number of diabetic patients in the African sub-region will be reduced significantly if access to drugs and Medicare is made top priority. But considering the state of world economy, cheaper drugs can be made available to the teeming populace with the increase in the funding into research on medicinal plants and the subsequent reprocessing of the medicinal plants to mimic orthodox or allopathic medicines. The natural appearance and constituents of the processed medicinal plants are very important if they maintain potency because, it assures the presence of the natural adjuvant and the principles which exert synergistic effect within the framework of the medicine. The fact that, the main focus of the use of medicine of any kind is to heal, cure or reversal of disease conditions, research into the medicinal plants could stress more emphasis on the dosage and safety in such a way that the primary benefits of the medicinal plants would be received by those who need it, because orthodox medicines also have their side effects cum contra indications and are still allowed on the shelf and prescribed/administered to patients in hospitals or health centres. If the increasing number of people suffering from diabetes mellitus in developing countries would be assisted in managing their condition, considering the prevailing problems encountered by them, such as cost of the drugs, compliance to dosage regimen and numerous side effects, the time lapse between that drug development and marketing it or making it available to the consumer, the scarce funds needed for such research, energy required and other factors that slows the process would not make the drugs any cheaper nor readily available to those who need it. Therefore, as the world goes “green”, the realization that medicines both for prophylactic and therapeutic purposes abound in nature, would encourage the planting of more trees, reforestation programs and the reclaiming or a forestation of the desert parts of Africa and a significant reduction on the reliance of fossil sources of energy and an eventual increase in the budget for medicinal plant research for a better and healthier world. Studies on the mechanism of Manosrin action and toxicity are reported in another publication.

Reference


