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**Characterization of potential hypoglycaemic agents from *Tapinanthus sessilifolius*
Parasitic on *Psidium guajava***

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ABSTRACT

Tapinanthus sessilifolius (Loranthaceae) (P. Beauv) Blume. commonly known as African mistletoe is a well-known medicinal plant in Africa and Europe. A bioassay-guided isolation of the plant constituents was carried out to identify potential anti-hyperglycemic agents. The hexane extract was discovered to be active which yielded two triterpenoids; betulinic acid and lupeol, responsible for the activity and their structures were elucidated by spectroscopic techniques. Other compounds from this plant identified by GC-MS technique were palmitic acid, hexadecanoic acid and stearic acid.

Key words: Traditional medicine, anti-hyperglycemia, Triterpenes, Mistletoe, Nigeria

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INTRODUCTION

The potential of medicinal plants as major sources of drugs and lead compounds for drug

discovery and synthesis are well known. The World Health Organization, recognizes and recommends the use of

medicinal plants as essential components of primary health care. Medicinal plants are also of great interest due to their use as nutraceuticals, pharmaceutical intermediates, and chemical entities for synthetic drugs. [1]

Diabetes mellitus is a heterogeneous metabolic disorder and the third major cause of death by chronic hyperglycemia due to lack or impaired insulin action and its complications such as nephropathy and retinopathy. There is an increased prevalence of diabetes globally especially in Nigeria. Presently, diabetes mellitus affects about 180 million people worldwide and it is forecasted to increase to 300 million by 2030 [2]. Drugs presently in use for the management of diabetes are associated with well-known side effects and toxicities such as weight gain, nitric acidosis and heart failure [5] therefore research has focused on exploiting plants and natural compounds with antidiabetic properties to serve as templates for the synthesis of new drugs with low toxicity and higher therapeutic index [4,5]. The anti-hyperglycemic effects that result from treatment with plants are often due to their ability to improve the performance of pancreatic tissue

thereby increasing insulin secretions or reducing the intestinal absorption of glucose.

Tapinanthus sessilifolius (Loranthaceae) commonly known as African mistletoe or all heal plant is a hemi-parasitic shrub that grows on dicotyledonous trees such as *Albizia lebbek*, *Terminalia mantaly*, *Terminalia catappa*, *Khaya senegalensis*, *Citrus grandis*, *Cola acuminata* and *Theobroma cacao* [4, 5]. Mistletoe attaches itself to the host by modified roots known as haustorium [6]. The leaves, stems, berries and flowers are majorly used in herbal medicine for treatment of headache, rheumatic pain, hypertension, ulcers and cancers. It is also claimed to possess low lipid density lowering effects. [7-9]. Scientific studies on the medicinal properties of this plant have confirmed its antioxidant, antimicrobial, laxative, hypoglycemic, anticonvulsant, anti-inflammatory, analgesic as well as the hypolipidemic effects [6, 8, 10-14].

Chemical constituents that have been isolated from this plant include 6-[2-hydroxy-4-(4-hydroxyphenyl) butyl]-5,6-dihydropyran-2-one and 2-[(4-hydroxyphenyl)ethyl]-2,6-

dioxabicyclo-1]-nonan-3-one, quercetin, catechin, quercetin, rutin and avicularin [17[11]]. Others are flavonoid glycosides such as 2-hydroxy-4,6-dimethoxychalcone-4-O-glycoside, 2-hydroxy-3,4,6-trimethoxychalcone-4-O-glycoside, 2-hydroxy-4,6-dimethoxychalcone-4-O-apiosyl-(1@2) glucoside, (2R)-5,7-dimethoxyflavanone-4-O-glycoside, (2S)-3,5,7-trimethoxyflavanone-4-O-glycoside, (2S)-homoeriodictyol-7-O-glycoside, and rhamnazin-3-4-di-O-glucoside. Also phenylpropanoids, cinnamic acid derivatives, caffeic acids, ferulic acids and sinapic acids have been isolated from mistletoe. The present study seeks to identify some bioactive constituents from the hexane extract of *Tapinanthus sessilifolius* growing on *Psidium guajava* hosts which are responsible for the plant's hypoglycaemic activity using chromatographic and spectrometric techniques in order to establish a scientific basis for the use as a medicinal plant in the management of diabetes and its complications.

Materials and Methods

Plant Material:

The leaves of *Tapinanthus sessilifolius* were harvested from the host *Psidium guajava* between October and November 2019 at Jos, Plateau State of Nigeria. The plant material was identified and an authenticated voucher specimen (No. FHI 105336) was deposited at Forestry Research Institute, Ibadan.

Phytochemical investigations

Preparation of extract

The fresh leaves of *T. sessilifolius* were air-dried for seven days and ground into coarse powder. About 250 g of the powdered leaves were successively extracted with hexane, ethyl acetate and methanol using Soxhlet apparatus. The extracts were then concentrated to small volumes *in vacuo*.

Chemicals, equipment and instrumentation

Column chromatography were carried out in glass columns (80 × 4 cm) with silica gel (60-230 mesh, Merck & Co., Darmstadt, Germany). Thin-layer chromatography was carried out on aluminum sheets pre-coated with silica gel (silica gel 60 F₂₅₄, 0.25 mm, Merck & Co., Inc., U.S.A.) and Nuclear magnetic resonance (NMR) spectra (400 MHz) were obtained on a Bruker AMX 400

NMR spectrometer (Bruker Inc., Germany). Alloxan monohydrate was purchased from Sigma Chemical Co St. Louis M.O., (USA). Accu-Check Active Glucometer (model GB with Accustrips Roche, Mannheim Germany).

Isolation and identification of the compounds

The hexane extract, three grams (3.0 g) was subjected to a normal column chromatography separation, and eluted using hexane: ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 30:70, 0:100). The fractions 8 - 15 eluted with 10% ethyl acetate in hexane yielded two white amorphous powders TSLH8 (254 mg) and TSLH15 (374 mg). These were identified by spectroscopic techniques using carbon and proton NMR

Animals

Animals used for the study were male and female healthy mice that weighed between 18-30 g and Wistar albino rats, with an average weight of range 150 -250 g and bred at $27 \pm 3^{\circ}\text{C}$, 65 % relative humidity, 12 h day–night light exposure, and housed in different cages in the animal house, department of Pharmacology and Toxicology,

Animal facility Centre NIPRD, Nigeria. They were fed on a standard pellet diet with water given *ad libitum*.

Swiss albino mice of both sexes (18 – 30 g) and Wistar rats (150 - 250 g) were obtained from the Anima Facility Center of NIPRD where they were maintained under ambient conditions. They were house in acrylic cages with wood shavings as bedding. Animals were feed *ad-libitum* with standard rodent pellet and clean drinking water from the municipal source. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Academies Press [15]

Acute toxicity test

The safety of the extract was assessed by determining the acute toxicity which was carried out in five groups, each consisting of five (5) mice. Group 1 – 4 were administered with varying doses of extract at 10, 100, 1000 and 2000 mg/kg p.o., while group 5 served as control and was administered normal saline at 10 ml/kg.

Signs and symptoms of toxicity and mortality were observed over a period of 24 h. Animals were monitored for 14 days.

Induction of diabetes

Diabetes was induced by 80 mg/kg i.v injection of freshly prepared 10% alloxan monohydrate to overnight-fasted rats. After a stabilization period of 10 days, animals with blood glucose higher than 300 mg/kg were selected and randomly placed into five groups of five rats each. The effect of the hexane extract (TSH) and the fraction obtained from the hexane extract of *Tapinathus sessilifolius* (TSHF) were tested in diabetic rats.

Studies on the effect of *Tapinathus sessilifolius* on Alloxan-induced hyperglycaemia

The animals were treated with hexane extract of *Tapinathus sessilifolius* (TSH) as follows:

- Group 1: Alloxan + normal saline (10 ml/kg) – Negative control
- Group 2: Alloxan + Chlopropamide (400 mg/kg) – Positive control
- Group 3: Alloxan + Hexane extract of TS (200 mg/kg)
- Group 4: Alloxan + Hexane extract of TS (400 mg/kg)

Group 5: Alloxan + Hexane extract of TS (800 mg/kg)

Group 6: Normal saline (10 ml/kg) – Non diabetic normal

Treatment was administered orally for a duration of 28 days. Blood glucose was determined at an interval of seven days from blood obtained from the tail vein by glucose oxidase method using Acuchek.

In another experiment, different set of animals were treated with the fraction obtained from the hexane extract of *Tapinathus sessilifolius* (TSHF) in the following manner

- Group 1: Alloxan + normal saline (10 ml/kg) – Negative control
- Group 2: Alloxan + Chlopropamide (500 mg/kg) – Positive control
- Group 3: Alloxan + TSHF 200 mg/kg
- Group 4: Alloxan + TSHF 400 mg/kg
- Group 5: Alloxan + TSHF 800 mg/kg

Blood glucose was determined using Acuchek after 2 h interval after single oral administration of the fraction isolated from the hexane extract of *Tapinathus sessilifolius* (TSHF).

Statistical analysis

The data obtained from the study were expressed as mean \pm SEM for the number (n = 5) of animals in the groups. They were analyzed with Two-

Way Analysis of Variance (ANOVA), followed by Bonferroni *t*-test or Student-Newman-Keuls post hoc tests, using GraphPad® InStat version 6.0 (GraphPad Software Inc., San Diego, USA). $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Characterization of Lupeol

The compound did not show a visible spot on TLC plate under daylight or UV light (256-365 nm) after development in hexane: ethyl acetate (75:25), however it gave a purple colour characteristic of terpenoids on spraying with Vanillin-Sulphuric acid reagent ($R_f = 0.78$). The proton spectrum (Table 1) showed the presence of vinyl methylene protons at δ_H 4.69 ppm (d, $J = 1.68$ Hz) and 4.57 ppm, a vinyl methyl at 1.69 and six tertiary methyl singlets between 0.77 - 1.04 ppm. The H-3ax and H-19 protons were observed at 3.19 (dd, $J = 5.0, 11.2$ Hz) and 2.38 (ddd, $J = 5.8, 11.0, 11.0$ Hz) respectively. The J modulated ^{13}C spectrum showed a total of 30 carbon atoms including seven methyls, and one quaternary carbon attached to a vinyl methylene group. There was no carbonyl carbon and this was

confirmed by its IR spectrum. Exact measurement of molecular ion M^+ observed at $m/z = 426.38$ gave the molecular formula $\text{C}_{30}\text{H}_{50}\text{O}$. The spectral data was typical of a pentacyclic triterpene of the lupane type [17, 18] and was thus identified as lupeol.

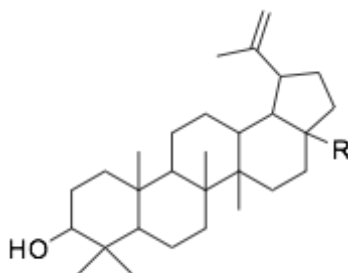
Characterization of Betulinic acid

Betulinic acid was isolated as white crystal, it was also non-visible under UV light after TLC development, and turned purple after spray with vanillin-sulphuric acid reagents followed by heating which was characteristic of a terpenoid. ^1H and ^{13}C NMR data for this compound (Table 1) revealed close similarity to that of the isolated lupeol except that the ^{13}C spectra for this compound revealed five tertiary methyl groups instead of six as seen in lupeol, and the IR spectrum revealed the presence of a carbonyl signal. Comparison with literature data [17, 18] revealed this compound to be betulinic acid. The main difference was at C-28 where a carboxylic acid signal is present for betulinic acid unlike the methyl signal for lupeol.

Table 1: ^1H and ^{13}C NMR data for Betulinic acid and Lupeol 400MHz CDCl_3

	Betulinic acid	Lupeol	Betulinic acid	Lupeol
Position	^1H	^1H	^{13}C	^{13}C
1	1.63, 0.89	1.63, 0.89	38.94(t)	38.94(t)
2	1.63	1.63	27.65(t)	27.65(t)
3	3.19	3.19	79.23(d)	79.23(d)
4	-	-	39.09(s)	39.09(s)
5	0.68	0.68	55.53(d)	55.53(d)
6	1.52, 1.37	1.52, 1.37	18.55(t)	18.55(t)
7	1.40	1.40	34.52(t)	34.52(t)
8	-	-	41.07(s)	41.07(s)
9	1.20	1.20	50.68(d)	50.68(d)
10	1.18	1.18	37.40(s)	37.40(s)
11	1.44, 1.18	1.44, 1.18	21.16(t)	21.16(t)
12	1.63, 1.04	1.63, 1.04	25.38(t)	25.38(t)
13	1.65	1.65	38.29(d)	38.29(d)
14	-	-	43.06(s)	43.06(s)
15	1.69, 1.08	1.69, 1.08	27.68(t)	27.68(t)
16	1.95, 1.21	1.95, 1.21	35.81(t)	35.81(t)
17	-	-	43.23(s)	43.23(s)
18	1.55	1.55	48.54(d)	48.54(d)
19	2.38	2.38	48.21(d)	48.21(d)
20	-	-	151.20(s)	151.20(s)
21	1.90, 1.46	1.90, 1.46	30.08(t)	30.08(t)
22	1.90, 1.06	1.90, 1.06	40.23(t)	40.23(t)
23	0.95	0.95	28.21(q)	28.21(q)

24	0.77	0.77	15.59(q)	15.59(q)
25	0.38	0.38	16.34(q)	16.34(q)
26	1.04	1.04	16.20(q)	16.20(q)
27	0.98	0.98	14.78(q)	14.78(q)
28		0.80	178.9(s)	18.23(q)
29	4.69, 4.57	4.69, 4.57	109.54(t)	109.54(t)
30	1.69	1.69	19.53(q)	19.53(q)



Lupeol: R = CH₃

Betullinic acid: R = COOH

Chemical Structure of Lupeol and Betullinic acid

Acute toxicity

The preliminary acute toxicity tests showed no signs of toxicity was observed in treated animals at all the doses tested. The behaviour of treated animals was similar to control group. No mortality was recorded throughout the 14 days observation period at a dose up to 2000 mg/kg.

Effect of hexane extract *Tapinathus sessilifolius* (TSH) on Alloxan-induced hyperglycaemia

Administration of alloxan to normal rats caused an increase in the level of blood glucose. Treatment of hyperglycaemic rats with the hexane extract of *T. sessilifolius* caused reduction of the blood glucose level. The reduction in blood glucose level was significant at doses 200 – 800 mg/kg from day 7 of treatment. This effect was

however not dose dependent as 200 mg/kg reduced the levels to lower values compared to 400 mg/kg and 800 mg/kg (Table 2). Treatment of diabetic rats with chlopropamide caused decrease in blood glucose level which was significantly different from the negative control from day 14 of administration of the standard drug.

Table 2: Effect of hexane extract of *T. sessilifolius* (TSH) on blood glucose level in alloxan-induced diabetic rats over 28 days

	Blood glucose level (mg/dl)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Diabetic untreated	395.0 ± 7.6	380.4 ± 7.4	384.0 ± 7.5	369.2 ± 0.2	380.7 ± 5.0
Diabetic + CPR 400 mg/kg	368.2 ± 12.4	271.6 ± 11.2	185.2 ± 10.1 ^{ac}	169.4 ± 9.5 ^{ac}	158.4 ± 10.6 ^{ac}
Diabetic + TSH 200 mg/kg	373.6 ± 19.2	159.0 ± 9.8 ^{ac}	172.0 ± 12.0 ^a	167 ± 13.3 ^a	144.08 ± 12.96 ^{ac}
Diabetic + TSH 400 mg/kg	350.0 ± 23.7	214.6 ± 20.0 ^a	290.4 ± 16.4	206.2 ± 9.8 ^b	246.1 ± 25.0 ^b
Diabetic + TSH 800 mg/kg	399.2.8 ± 6.4	264.0 ± 4.5 ^b	213.2 ± 6.2 ^b	202.6 ± 10.4 ^b	270.2 ± 3.7 ^b
Non-diabetic normal rats	107.3 ± 1.2	99.0 ± 0.93	105.0 ± 1.2	98.0 ± 0.9	102.0 ± 1.2

CPR – chlopropamide; TSH - hexane extract of *T. sessilifolius*

Data expressed as value ± SEM (n=5), Two-way ANOVA followed by Bonferroni post hoc.

Significance p<0.05 Treatment vs control ^ap<0.001, ^bp<0.01

^a Significantly different from Group 1 at p<0.001, ^b significantly different from Group 1 at <0.01, ^c significantly different from Group 2 at p<0.001, ^j significantly different from Group 5 at p<0.001.

Effect of isolated fraction from hexane extract of *Tapinathus sessilifolius* (TSHF) on Alloxan-induced hyperglycaemia

Administration of the fraction isolated from the hexane extract (TSHF) produced a reduction in blood glucose levels. This was observed between 4 - 8 hours after treatment in all fraction treated groups. Administration at 200 mg/kg was the most effective dose as significant activity was

recorded from 2 h post administration which lasted up to 24 after treatment. The lowest blood glucose level was also recorded at 6h for 200 mg/kg. This is similar to blood glucose obtained in normal non-diabetic animals. Increasing dose of the fraction did not result in increasing activity. The blood glucose lowering. The effect of the chlopropamide was significantly different from the negative control groups (Table 3).

Table 3: Effect of isolated fraction from hexane extract of *T. Sessilifolus* (TSHF) on blood glucose level of alloxan-induced diabetic rats over a 24h period

Treatment (mg/kg body wt)	Blood glucose level (mg/dl)					
	0 h	2 h	4 h	6 h	8 h	24 h
Control	373.0 ± 6.0	387.0 ± 7.3	382.0 ± 5.4	371.0 ± 7.0	383.8 ± 4.0	368.2 ± 6.0
TSHF 200	345.0 ± 10.7	179.9 ± 13.2 ^{ac}	137.8 ± 6.7 ^{ac}	99.6 ± 11.0 ^{ac}	101.8 ± 7.3 ^{ac}	179.6 ± 21.0 ^{ac}
TSHF 400	373.4 ± 5.1	274.0 ± 13.6	240.0 ± 6.6 ^b	154.0 ± 18.0 ^a	141.4 ± 14 ^a	228.0 ± 5.5 ^b
TSHF 800	377.0 ± 7.0	278.2 ± 8.7	219.0 ± 10.0 ^b	140.4 ± 13.0 ^a	156.0 ± 14 ^a	302.0 ± 14.0
CPR 400	343.0 ± 23.0	273.5 ± 5.4	201.0 ± 7.6 ^b	144.6 ± 23.0 ^a	139.4 ± 14.0 ^a	196 ± 25.0 ^a

CPR – chlopropamide; TSHF – fraction isolated from hexane extract of *T. sessilifolius*

Data expressed as value ± SEM (n=5), Two-way ANOVA followed by Bonferroni post hoc. Significance p<0.05 Treatment vs control ^ap<0.001, ^bp<0.01

DISCUSSION

The effect of hexane extract and a fraction isolated from the hexane extract was evaluated in

laboratory models of diabetes. Diabetes was induced by the administration of alloxan.

The hexane extract caused a decrease in blood glucose level in alloxan-induced rats. The hexane

extract's effect at 200 mg/kg was similar to that of chlorpropamide 400 mg/kg which suggests that the hexane extract might be acting either by stimulating the pancreatic cells to release insulin or enhance glucose utilization. On daily administration, the extract caused a significant decrease in blood glucose level compared to negative control and this was maintained for a period of 28 days, suggesting that the extract might be protecting the pancreas from further damage by alloxan and possibly stimulating the residual cells to release insulin and also decrease oxidative stress through the radical scavenging effect of the extracts [19]. Alloxan induces selective cytotoxicity in pancreatic β -cells resulting in decrease of insulin [19]. The extract exhibited anti-hyperglycaemic properties against alloxan-induced diabetic rats. This effect suggests the presence of bioactive antidiabetic principles in *T. sessilifolius* that might act through renewal of β -cells in the pancreas and stimulates pancreatic insulin secretion, or aid in recovery of partially destroyed β -cells. Betulinic acid, a constituent of the hexane extract, can inhibit different enzymes related to carbohydrate/lipid absorption and metabolism, such as α -amylase, protein tyrosine

phosphatase1B, glycogen phosphorylase, and diacylglycerol acetyl [20-22,29]. The data indicate that *T. sessilifolius* (African Mistletoe) possesses significant anti-diabetic activity on induced diabetic rats, its anti-diabetic activity appears to be highly dependent on the host plant. [7,12].The secondary metabolite found in *T. sessilifolius* includes saponins, flavonoids and terpenoids. Several studies have implicated flavonoids and terpenoids in some hypoglycemic activities and more especially the terpenoids [23]. There is a wide range of bioactivities and bioassays of lupeol which suggest its useful medicinal properties with a diversity of action against various ailments as an hypoglycaemic and anti-lipideamic agents [23].Many experiments have shown that triterpene have adequate properties with several anti-diabetic mechanisms that can inhibit enzymes involved in glucose metabolism, prevent the development of insulin resistance and normalize plasma glucose and insulin levels.[23]Triterpenes are also promising agents in the prevention of diabetic complications because they have strong antioxidant activity that inhibit the formation of advanced glycation end products (AGE) implicated in the pathogenesis of

diabetic nephropathy, embryopathy, neuropathy and impaired wound healing.[N24,25].Treatment can also be achieved through the inhibition of α -glucosidases and α -amylases which delay the absorbance of carbohydrates in the intestine, leading to a decrease in the postprandial insulin level useful in treating diabetes [25,26].Lupeol also inhibits PTP 1B .PTP 1B negatively regulates insulin signaling by catalyzing the dephosphorylation of both the IR and insulin-receptor substrate-1 (IRS1) in a non-competitive manner, indicating that they may bind to the enzyme-substrate complex or interact with a specific binding site distinct from the active site of the enzyme [27].These evidences strongly support that lupane-type triterpenoids can be a lead moiety for the development of new PTP1B inhibitor and activated protein kinase 3 (AMPK 3) activator and improve glucose uptake and lowering blood glucose [28,29].

Conclusion

The data obtained from this study showed that the hexane extract of the plant *T. sessilifolius* and the isolated fraction possesses blood glucose

lowering actions which may be attributed to its phytoconstituents that include betulinic acid and lupeol which are bioactive triterpenes with hypoglycemic properties

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