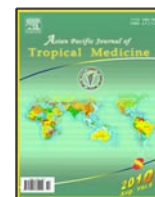




Contents lists available at ScienceDirect

## Asian Pacific Journal of Tropical Medicine

journal homepage: [www.elsevier.com/locate/apjtm](http://www.elsevier.com/locate/apjtm)

Document heading

Antidiabetic principles of *Loranthus micranthus* Linn. parasitic on *Persea americana*

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## ARTICLE INFO

## Article history:

Received 29 March 2010

Received in revised form 27 May 2010

Accepted 1 July 2010

Available online 20 August 2010

## Keywords:

Antidiabetic

isolation

*Loranthus micranthus*

Thin layer chromatography

Weakly acidic fraction

## ABSTRACT

**Objective:** To explore antidiabetic principles of the Eastern Nigeria mistletoe, *Loranthus micranthus* Linn. parasitic on *Persea Americana*. **Methods:** The weakly acidic fraction of the aqueous methanol extract of the leaves of *Loranthus micranthus* (Linn.) was isolated and tested for its antidiabetic activities. The isolation of the weakly acidic fraction was carried out following established physico-chemical based procedures. Furthermore, alloxan-induced diabetic rats were treated intraperitoneally (ip) with 250 mg/kg and 400 mg/kg of the weakly acidic fraction, glibenclamide 10 mg/kg (positive control) and 2 mg/kg of 3 % v/v tween 20 (negative control). The sugar levels of the treated and untreated animals were determined by withdrawing the blood at regular intervals and testing them with an automated glucometer. The phytochemical analysis of the acidic fraction was carried out using standard procedures. Chromatographic techniques were employed in the subsequent isolation and purification of the constituents of the weakly acidic fraction. **Results:** It was shown that the maximum effect of the weakly acidic fraction was obtained at 24 hours after administration and was found to be statistically comparable with that of the positive control. The phytochemical analysis revealed the presence of steroids, terpenoids, alkaloids, flavonoids, glycosides, carbohydrates, saponins, and acidic compounds in the crude extract and carbohydrates, flavonoids, terpenoids and oil in the weakly acidic fraction. Further purification of the weakly acidic fraction of the methanol extract using thin layer chromatography shows that toluene : methanol : diethyl amine (3:1:1) and chloroform: methanol: diethyl amine (9:1:1) are the best solvent system for the isolation of the various components of the weakly acidic fraction of the crude methanol extract of *Loranthus micranthus*. **Conclusions:** The present study has led to the conclusion that the weakly acidic fraction of the plant under study has the potent antidiabetic activity and that the various components can be isolated using basic chromatographic techniques.

## 1. Introduction

Diabetes mellitus (DM) is a chronic disorder of carbohydrate, lipid and protein metabolism characterized by persistent elevation of fasting blood glucose above 200 mg/dL, due to insufficient or complete cessation of insulin synthesis or secretion and/or peripheral resistance to insulin action[1]. DM is a global health issue[2] and in response to this global

health challenge, the WHO expert committee on diabetes mellitus recommended further evaluation of the folkloric methods of managing the disease because of high mortality and morbidity arising from its attendant complications and draw-backs associated with the use of conventional anti-diabetic drugs[1]. In pursuit of this goal, several medicinal plants are being investigated for possible hypoglycemic activities based on several approaches including ethnobotanical survey. Of the several indigenous plants used in the local treatment of DM in South-Eastern Nigeria is *Loranthus micranthus* (Linn.) an evergreen semi-parasitic plant that grows on branches of certain deciduous trees.

*Loranthus micranthus* Linn.(Loranthaceae) has been

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widely used in ethno medicine as antidiabetic, anticancer, antihypertensive, antimicrobial agent and others<sup>[3]</sup>. Different research teams have worked on various species of the plant and demonstrated some pharmacological activities which supported the acclaimed ethno medicinal uses<sup>[4–6]</sup>. Studies have shown that the composition and activities of *Loranthus micranthus* are host tree and harvesting period dependent<sup>[7]</sup>. In addition, it has been demonstrated that the extraction of the antidiabetic constituents of mistletoe requires the use of polar solvents such as water and alcohol in consonance with folkloric use of the aqueous and ethanol infusions<sup>[5]</sup>. In this present study, an attempt is made to isolate and identify the compounds with the greatest anti-diabetic property in the weakly acidic fraction of the methanol extract of *Loranthus micranthus* which is parasitic on the host tree, *Persea americana*. This is based on a previous work on the plant parasitic on *Azidrachta indica*<sup>[8]</sup> which identified the weakly acidic fraction of the methanol extract as having the greatest anti-diabetic activity.

## 2. Materials and methods

### 2.1. Plant material

Green leaves of *Loranthus micranthus* parasitic on *Persea americana* (Avacado) were collected in the month of June at Nsukka Town, South– Eastern Nigeria. Mr Ozioko, a taxonomist with the Bioresource Development and Conservation Programme (BDCCP) Center, Nsukka authenticated the materials. A voucher specimen with number BDCCP-07-532 was kept for reference purpose.

### 2.2. Extraction of the weakly acidic fraction

The extraction is based on the procedure described recently<sup>[9]</sup>. Briefly, it is a general procedure for the extraction of organic substances from a mixture. The leaves of *Loranthus micranthus* were dried in a shade to a constant weight and pulverized in a mechanical home grinder (Manesty, England). The powdered leaves (1 kg) were macerated with 3.5 L of 90% methanol in a screw fit container for 5 days. The resulting aqueous methanol extract (referred to as fraction C) was treated with dilute hydrochloric acid and partitioned with ether in a separating funnel. The ether extract was again partitioned with 0.5 N sodium bicarbonate. Next, the ether layer was partitioned with 0.1N sodium hydroxide and the sodium hydroxide extract gave the weakly acidic fraction (fraction B) while the sodium bicarbonate layer gave fraction A (strong acid fraction).

### 2.3. Chemicals and reagents

Diethylether, methanol, sodium hydroxide, sodium bicarbonate, hydrochloric acid, ammonium hydroxide, n-hexane, diethylamine, toluene, chloroform, ethylacetate, alloxan (Sigma Chemicals, USA). The chemicals are of analytical grade. Others are Tween 20, glibenclamide (Glanyl®,NGC), silica gel G60 coated on aluminium foil.

### 2.4. Animals

Wiser rats (90–120 g) of either sex were used. They were kept in standard laboratory conditions and fed with rodent commercial diet (Guinea Feed Nig. Ltd) and water ad libitum.

### 2.5. Phytochemical tests

Standard procedures<sup>[10]</sup> were followed for testing the constituents on the crude methanol extract and the weakly acidic fraction. The classes of constituents tested were glycosides, alkaloids, carbohydrates, saponins, tannins, flavonoids, resins, proteins, oils and steroids.

### 2.6. Anti-diabetic effect of the weakly acidic fraction of crude aqueous methanol extract

Diabetes was induced in the rats by intraperitoneal (ip) administration of alloxan monohydrate dissolved in normal saline at a single dose of 140 mg/kg body weight. Alloxan induces diabetes mellitus by selectively destroying the pancreatic  $\beta$  -cells, which are involved in the synthesis, storage and release of insulin<sup>[11–13]</sup>. Stable hyperglycemia was confirmed on the 5th day and rats with fasting blood glucose greater than 230 mg/dL were considered diabetic. Twenty of such rats were randomly grouped into four ( $n=5$ ) and used in this study. The animals were fasted for 12 hours prior to the experiment.

Groups I and II received (ip) 250 mg/kg and 400 mg/kg respectively of the weakly acidic fraction of the crude methanol extract (fraction C). Group III received 10 mg/kg of glibenclamide (positive control), while group IV received 2 mL/kg of 3% tween 20 solution (negative control). Blood samples were collected from the tail veins of the rats and blood sugar concentrations determined at 0, 1, 2, 3, 4, 5, 6, and 24 hours after drug treatments by using the One-Touch automated glucometer.

### 2.7. Analytical TLC of the weakly acidic fraction

Standard procedure<sup>[14]</sup> was followed in resolving the extract using thin-layer chromatography. Pre-coated silica gel plates (silica gel GF 254) were used. The plates were air dried, activated in oven at 150 °C for 30 min and cooled at room temperature. The weakly acidic fraction of the extract was dissolved in methanol and spotted on the plate, maintaining the same distance from one edge to another. The Foils were allowed to dry at room temperature and the spotted plates developed in different solvent systems as follows: toluene: methanol (1:1), toluene: methanol (3:1), chloroform: methanol (9:1), chloroform: methanol (7:3), toluene: ethyl acetate (3:1), chloroform: methanol: diethyl amine (9:1:1), toluene: methanol: diethyl amine(3:1:1), toluene: diethyl amine (19:1), hexane: ethyl acetate(7:3), hexane: ethyl acetate(8:2), hexane: methanol (19:1).

The solvent system was allowed to travel to a predetermined distance. The plates were removed from the chambers with a forceps, dried and spots viewed visually and under ultraviolet rays (wavelength of 254 and 366 nm).

The number of spots at each wavelength was counted.

### 2.8. Statistical analysis

The results were recorded as mean standard deviation and significance between treated and control groups were evaluated by the Student's *t*-test.  $P < 0.05$  and  $P < 0.001$  were considered significant<sup>[15]</sup>.

## 3. Results

### 3.1. Result of extraction

The yield of the crude aqueous methanol extract was

10.20% calculated with respect to the total weight of the starting material.

### 3.2. The antidiabetic activity

The effects of the two different doses of fraction B, glibenclamide and Tween 20 on the blood glucose level of the alloxan-induced diabetic rats were shown in Table 1. The result showed that the weakly acidic fraction induced a significant ( $P < 0.05$ ,  $P < 0.001$ ) dose related reduction in blood glucose concentration when compared to the positive control (glibenclamide). Table 2 showed the percentage reduction in blood sugar level of the animals by the various treatments and controls.

**Table 1**

Effects of single intraperitoneal administration of Tween 20 (3%), glibenclamide and 250–400 mg/kg of the weakly acidic fraction of the methanol extract of *Loranthus micranthus* on the blood glucose of the alloxan-induced diabetic rats.

Groups <sup>b</sup>	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	24 hr
I	477.0±90.0	493.0±51.3	425.0±56.6	472.5±56.6	425.0±56.6	365.5±75.3*	318.0±76.4*	189.5±17.3***
II	594.0±4.2	581.0±13.4	560.0±28.3	494.5±1.8	425.0±3.5	387.5±1.8	324.0±14.1*	198.5±1.1***
III	391.0±96.9	371.0±96.9	371.0±31.1	394.0±5.7	381.0±5.0	369.0±2.1*	341.0±7.1*	75.5±22.3***
IV	429.5±79.1	396.5±34.7	448.5±66.5	444.3±58.4	435.872.4	426.0±78.8	460.0±56.4***	462.0±58.1***

\*significant at  $P < 0.05$ , \*\*\*significant at  $P < 0.001$ , a: Values are means ± SEM with  $n = 5$  rats per group, b: all measurements of blood glucose level are in unit of mg/dL.

**Table 3**

Phytochemical analysis of the methanol extract and the weakly acidic fraction of the leaves of *Loranthus micranthus* parasitic on *Persea americana*.

Test	Crude methanol extract	weakly acidic fraction
Carbohydrate	++	+
Reducing Sugar	++	–
Glycosides	++	–
Flavonoids	+	+
Proteins	+	–
Resins	+	–
Oils	+	–
Steroids	++	++
Terpenoids	++	++
Acidic compounds	+	+
Alkaloids	+++	–
Tannins	+	–
Saponins	+	–

Key: + = present; ++/+++ = doubly/ more intensely present; – = absent

### 3.3. Phytochemical result

The result of the phytochemical analyses of both the methanol extract and the weakly acidic fraction (Fraction B) was shown in Table 3. It showed the presence of carbohydrates, glycosides, alkaloids, saponins, tannins, flavonoids, resins, proteins, oils, steroids, and terpenoids in the crude methanol extract with the alkaloid being highest in abundance. The weakly acidic fraction or Fraction B contained carbohydrate, flavonoids, steroids and terpenoids

**Table 2**

Percentage reduction in blood sugar level of diabetic rats following the different treatments.

Groups	Dose (mg/kg)	percentage reduction (%)
I (Fraction B)	250	60.30*
II (Fraction B)	400	66.60*
III (Glibenclamide)	0.1	80.70*
IV (3% Tween 20)	2 mL/kg	–7.10

\*Statistically not significant difference between extract treatment and positive control,  $P < 0.001$ .

(with carbohydrates and flavonoids being lower in the fraction compared to that in the methanol extract, while steroids and terpenoids remained unchanged in both the extract and fraction). The weakly acidic fraction also showed absence of reducing sugars, glycosides, proteins, resins, lipids, alkaloids, tannins, and saponins.

## 4. Discussion

The yield of the crude aqueous methanol extract was 10.20%. Studies have shown that yield depends on the method of extraction and the solvent used. For instance, similar yield (10%) was reported for *Viscum album* (the European version of mistletoe) with ethanol being used as the solvent and cold maceration method, as in the present work<sup>[16]</sup>. Recently, higher yields were reported but the method used was exhaustive soxhlet extraction with higher percentages of water added to the extracting solvent usually methanol<sup>[4, 17]</sup>. The result shows that the weakly acidic

fraction induced a significant ( $P < 0.05$ ,  $P < 0.001$ ) dose related reduction in blood glucose concentration when compared to the positive control (glibenclamide). Compared to the negative control (Tween 20), the test groups (I and II) and glibenclamide showed marked hypoglycemic effect. This is quite clearly shown by the percentage reduction in blood sugar level of the animals by the various treatments and controls. The weakly acidic fraction of the methanol extract exhibited statistically significant antidiabetic property and comparable to the standard drug, glibenclamide. This recent finding is in line with the folkloric use of the plant, *Loranthus micranthus* as an antidiabetic agent. It is an indication that the main active principle(s) should be in the weakly acidic fraction. The result is also in agreement with that reported for the methanol extract of the dried leaves of the Eastern Nigeria mistletoe, *Loranthus micranthus*[8]. As part of a clue to the possible mechanism of action, it has been recently reported that *Loranthus micranthus*, possesses potent immunostimulatory potentials[18] and that one of the main active immunostimulatory constituent is the flavonoids[17]. Immunostimulation is a major pathway of recovery from pathological damage secondary to diabetic complications. Flavonoids are known to be phenolic in nature and therefore, mostly weakly acidic. However, this plausible presumption can be proven only when the structures of the active constituents are unequivocally proven and further tested *in vivo*. Some pharmacokinetic parameters could be deduced from these results. It is noticeable that the hypoglycemic effects of the extracts and glibenclamide commenced an hour post administration and continued for up to 24 hours when the blood glucose level was lowest (reduction of 60.30 to 66 and 80.7% for the extracts and glibenclamide respectively). This suggests that the active antidiabetic principle(s) from the weakly acidic fraction has short onset and a long duration of action. It could therefore, be used for the management of postprandial hyperglycemia that occurs often in diabetes[19]. Furthermore, it is suitable for once-a-day administration to improve patient compliance and achieve an overall optimal therapeutic goal. Moreover, the safety of the plant extract had been established. The average acute toxicity ( $LD_{50}$ ) of the methanol extract of the plant harvested from five different host trees was reported as  $7954.68 \pm 1064.35$  with that of *Persea americana* derived mistletoe extract being highest as 11 000 mg/kg body weight[18]. This shows a high therapeutic index for mistletoe extract. The administered doses of 200 and 400 mg/kg body weight were quite within the safety range. However, it is possible that the purer the extract becomes, the smaller this margin of safety may become. In another related study, it was shown that there were no significant biochemical changes in the experimental rats when they received oral dose of 827 mg/kg body weight of the aqueous extract of the plant for 21 days[20]. With establishment of a potent antidiabetic activity and marginal safety of Eastern Nigeria mistletoe, *Loranthus micranthus*, various mechanisms of action could be proposed based on previous related studies. Documented reports have shown that extracts of *Fenugreek*, *Momordica charantia*, *Allium sativum*, and *Allium cepa* are antidiabetic agents which act by inducing the secretion of insulin by the pancreatic beta cells, by enhancing the regeneration of beta cells or by inducing the resistance of the

target cells to the actions of insulin[21–24]. Some plants were also shown to act through the inhibition of gluconeogenesis and glycogenolysis or the promotion of glucose through glycolysis[25]. Extracts from the European version of mistletoe, *Viscum album*, was reported to have the ability of stimulating insulin secretion from pancreatic beta cells[12]. As was stated above, extracts of Eastern Nigeria mistletoe is immunostimulatory in nature and could largely explain the mechanism behind its observed antidiabetic activity. Summarily, it could be suggested that *Loranthus micranthus* extract exert its hypoglycemic effect through any of these mechanisms. However, further detailed work at molecular level is needed to determine the actual mechanism(s) of action.

The result of the phytochemical analyses of both the methanol extract and the weakly acidic fraction (Fraction B) showed the presence of carbohydrates, glycosides, alkaloids, saponins, tannins, flavonoids, resins, proteins, oils, steroids, and terpenoids in the crude methanol extract with the alkaloid being highest in abundance. The weakly acidic fraction or Fraction B contained carbohydrate, flavonoids, steroids and terpenoids (with carbohydrates and flavonoids being lower in the fraction compared to that in the methanol extract, while steroids and terpenoids remained unchanged in both the extract and fraction). The weakly acidic fraction also showed absence of reducing sugars, glycosides, proteins, resins, lipids, alkaloids, tannins, and saponins. In line with the above observation, weakly acidic fraction contains weakly acidic flavonoids, slightly polar carbohydrates and moderately polar steroid and terpenoids.

Similar results were gotten from studies done using related plants. *Loranthus micranthus* harvested from Kola acuminata showed presence of alkaloids, cyanogenetic glycosides, saponins, flavonoids, tannins, proteins, and resins in the methanol extract of the dried leaves[26]. Comparatively, *Viscum album* which is also known to possess antidiabetic activity[27] was shown to contain the following constituents: lectins, viscotoxins, viscumin, alkaloids, flavonoids, glycosides, aglycones, triterpenes, saponins,  $\beta$ -amyryns, lupeol, oleanolic acid, ursolic acid,  $\beta$ -sitosterols, stigmasterol, sugars and palmitic acid[28–30]. Till date, there are no concrete evidences that lectins and viscotoxins are found in Eastern Nigeria mistletoe which obviously shows higher therapeutic margin of safety than the *Viscum album*. The main thrust of the present research is to navigate towards the eventual isolation and characterization of the active antidiabetic principle of this plant harvested from *Persea americana*. In a recent research work, a group of researchers had attributed the antidiabetic activity of the extracts of *Loranthus micranthus* harvested from neem plant, *Azadiracta indica* to the flavonoid content[8]. Similarly, several studies out on other plants had linked their blood sugar lowering activities to the flavonoid contents[13, 31]. Thus, the absence of reducing sugar, presence of flavonoid and the observed high antidiabetic activity of the weakly acidic fraction are clear indications that flavonoid was responsible for the recorded activity. This present fractionation of the methanol extract is a necessary step towards the final isolation of the bioactive principle(s) of the Eastern Nigeria mistletoe, *Loranthus micranthus*.

The result of the preliminary chromatographic studies on

the weakly acidic fraction showed that the solvent system of toluene:methanol:diethyl amine (3:1:1) and chloroform:methanol:diethyl amine (9:1:1) gave the highest number of spots, (3 spots) with Rf values of 0.4 to 0.8 each. Thus, these solvent systems gave the best resolution of the fraction and could be used to isolate the constituents weakly acidic fraction of the plant.

The weakly acidic fraction of the methanol extract of *Loranthus micranthus* (Linn.), parasitic on *Persea americana*, showed a dose dependent hypoglycemic activity which is comparable to glibenclamide. The antidiabetic activity is evidently due mainly to the flavonoid content of this plant. The activity could have been augmented by synergism with other phytoconstituents identified in the plant. The present observation is a prelude to the possible isolation of the pure compound(s) responsible for the antidiabetic activity of the weakly acidic fraction of the Eastern Nigeria mistletoe. Further studies involving physical methods, chemical methods, different techniques of chromatography and spectroscopy will lead to the eventual characterization of such isolated active compound(s).

### Conflict of interest statement

The authors declare no competing interest with the present work.

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