International Journal of Pharmaceutica Analytica Acta

Research Article

# The Anti-Diabetic Activity-Guided Isolation-Targeted Fractionation of Crude Extracts and Fractions of *Loranthus micranthus* Parasitic on *Kola acuminata*

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**Submitted:** 08 April 2021; **Approved:** 12 April 2021; **Published:** 23 April 2021

**Citation this article:** Osadebe PO, Johnson Ajinwo OR. The Anti-Diabetic Activity-Guided Isolation-Targeted Fractionation of Crude Extracts and Fractions of *Loranthus micranthus* Parasitic on *Kola acuminata*. Int J Pharma Anal Acta. 2021 April 23; 4(1): 001-007.



ISSN: 2694-0159

ISSN: 2694-0159


## ABSTRACT

Diabetes Mellitus (DM) is a global threat that demands urgent pharmacological interventions. The disease is progressing and estimated to attain astronomical proportions if unchecked. Currently, 422 million people are diabetic. The mortality from DM was 1.6 million deaths in 2012 with an economic burden of 1.31 trillion US dollars in 2015; an indication that the search for cheaper sources of anti-diabetic medications cannot be over-emphasized.

*Loranthus micranthus* parasitic on *Kola acuminata* was sourced in the month of April and successfully fractionated by sequential partitioning in solvents based on increasing polarity to obtain the following four fractions; n-hexane, chloroform, ethyl acetate and methanol fractions respectively. The phytochemical screening of the crude methanol extract disclosed the following classes of compounds; flavonoids, tannins, alkaloids, terpenoids, steroids, saponins, glycosides, reducing sugar, fats and oils. Seven bioactive compounds were identified from the GCMS analysis of the crude methanol extract. The compounds were Benzene, 1,2,3-trimethyl, 2,4-Di-tert-butyl phenol, Tetradecane, Hexadecanoic acid methyl ester, Octadecanoic acid methyl ester, Hexanedioic acid bis(2-ethylhexyl) and Diisooctyl phthalate. The anti-diabetic activities of the extracts and fractions were determined on alloxan-induced diabetic rats at dose levels of 200 mg/kg and 400 mg/kg using the glucometer. The activities of the extracts and fractions were in a dose dependent manner, with better hypoglycemic effects achieved from the 400 mg/kg doses. The activity of the 400 mg/kg chloroform fraction was comparable to that of glibenclamide at the 12 hr time interval. A comparison of the anti-diabetic activities of the fractions showed that the activity was in this order: chloroform > ethyl acetate > n-hexane > methanol. There is justifiable evidence from this work that *L. micranthus* sourced from *K. acuminata* possess significant anti-diabetic activities and thus deserves further preclinical studies for the therapeutic development of this herbal drug. This is the first time report of the compounds responsible for the anti-diabetic activities of *L. micranthus* parasitic on *K. acuminata*.


**Keywords:** *Loranthus micranthus*; *Kola acuminate*; Anti-diabetic

## INTRODUCTION

DM is the 7th killer disease today with a fatality of 1.6 million people in 2012 [1]. The International Diabetes Federation (IDF), recent forecast projected that by 2045, 629 million adults would suffer from DM [2]. There is huge number of undiagnosed diabetics which further heightens the burdens from the disease. The majority of which live in low income countries where access to decent healthcare is beyond their reach. There is an urgent need to search for cheaper remedies to the health challenges posed by DM.

The use of medicinal plants is as old as the history of man, given that every human society has formal or informal records of the use of plant parts for therapy. While there have been heavy dependence on woody and herbaceous plants, few societies reckon with parasitic plants as potent sources of pharmacological activities. Mistletoe is a hemiparasitic plant popularly referred to as bird lime and all heal. In England the plant is called children's matches or golden bough. The plant belongs to the order of Santalales of which the two largest families are Loranthaceae and Viscaceae. In Nigeria there are two main species; the Northern specie; *Loranthus begweensis* Linn and the Eastern Nigeria specie; *Loranthus micranthus* Linn. Mistletoes have several traditional names in Nigeria. These are: Kauchin in Hausa, Afomo onisana in Yoruba and owube, or awurisi in Ibo [3,4]. The plant is widespread in other African countries with species such as *Tapinanthus vittatus* from southern African and *Evianthemum uluguvense* from Kenyan documented [5]. The European specie; *Viscum album* has been extensively researched into and has gained prominence as an anti-cancer agent with commercially available brands such as Iscador, Abnobaviscum, Helixor and fermented V. album [6-8].

In Nigeria the knowledge of the health properties of mistletoe is very scanty, as many communities are unaware of the importance of this unique plant. Most farmers tag mistletoes as invasive weeds which must be eradicated to save their trees from poor yield or outright death [9]. The invasive nature of mistletoes have resulted in its presence on numerous trees such as *Citrus sinensis, Kola acuminata, Pentaclethra macrophylla, Manikara zapota, Azadirachta indica, Persia americana, Baphia nitida, Psidium guajava, Theobroma cacao* etc. Indigenous uses of the plant for the treatment of diseases such as diabetes, hypertension, epilepsy, infertility, rheumatism, headaches, menopausal problems and cancer have been documented. Mistletoes have been used as antispasmodic and anti-diuretic [3,10-12].

The awareness of the plant's usefulness remained largely undisclosed by herbalists until the 1990s', when an article on Mistletoe by Kafaru E. appeared in the Guardian newspaper in 1993, where the plant was described as an "all-purpose herb" 13. This was followed by PAX publication on Mistletoe: Nature's wonder herb [14] in 2007, which resulted in an avalanche of researches into mistletoes from various trees for potential bioactivities. The anti-diabetic, antimicrobial, anti-motility, anti-hypertensive, hepato-protective and immuno-modulatory activities have been reported for *Loranthus micranthus* parasitic on several host trees [15-18].

Preliminary studies have shown that *Loranthus micranthus* parasitic on *Kola acuminata* indicated anti-diabetic activities *in vivo*. This work on anti-diabetic activity-guided isolation-targeted fractionation of crude extracts and fractions of *Loranthus micranthus* parasitic on *Kola acuminata* is aimed at identifying the active principles responsible for the anti-diabetic activity of the plant.

## MATERIALS AND METHODS

Plant Material: Fresh leaves of *Loranthus micranthus* sourced in the month of April were obtained in Oba in Nsukka LGA of Enugu State. The plant was identified by a botanist and a voucher specimen deposited in the Department of Botany, University of Nigeria, Nsukka.

**Extraction procedure**: The *L. micranthus* leaves were dried at a temperature of 25°C under a shade for one week. The leaves were pulverized and weighed quantities of the *L. micranthus* were extracted with aqueous methanol using soxhlet extractor.

Fractionation: The obtained crude methanol extract was successfully partitioned into n-hexane, chloroform, ethyl acetate and methanol fractions by the method of sequential solvent partitioning.

**Preliminary screening:** The phytochemical constituents of the

ISSN: 2694-0159

crude methanol extracts were determined according to the method prescribed by Trease and Evans [19].

### GC-MS analysis of the crude extracts

The Gas Chromatography Mass Spectrometry (GC-MS) analysis of the crude methanol extract of the leaves of *L. micranthus* parasitic on *K. acuminata* was quantitatively determined using an Agilent 7890B GC system coupled with an Agilent 5977A MSD with a Zebron-5MS column (ZB-5MS 30 m × 0.25 mm × 0.025µm) (5%-phenylmethylpolysiloxane). The GC-grade helium served as the carrier gas at a constant flow rate of 2 mL/min. The crude extract was dissolved with ethanol and filtered before use. The column temperature was maintained at 60°C and gradually increased at 10°C per minute until a final temperature of 300°C was reached. The time taken for the GC-MS analysis was 30 min. The compounds were identified based on computer matching of the mass spectra with the NIST 11 MS library (National Institute of Standards and Technology library).

### Anti-diabetic screening

This study was designed in accordance with the ethically approved experimental procedures adopted by the department of Experimental Pharmacology and Toxicology, of the Faculty of Pharmaceutical Sciences, University of Port Harcourt. Adult mixed-gender wistar rats weighing between 120-170g, bred by the animal house unit of the department of Experimental Pharmacology and Toxicology were used for the study. The rats were housed in spacious cages, to allow for free movement at room temperature, sufficient humidity and 12/12 light/dark cycle. The animals, had access to standard laboratory animal feed and water prior to the commencement of the experiment.

The anti-diabetic screening of the extracts and fractions of *L. micranthus* obtained from *K. acuminata* was carried out. Their baseline blood sugar level was determined before the experiment. The rats were fasted for 12 hours and hyperglycaemia induced by the intraperitoneal administration of 100mg/kg alloxan monolydrate. Alloxan; a hydrophilic unstable analogue of glucose which selectively destroys beta cells of islets of pancreas, resulting in the elevation of blood glucose level, decrease in protein content, with a concomitant increase in levels of cholesterol and triglycerides [20]. Alloxan and its product dialuric acid are known to develop a redox potential forming superoxide radicals. These reactive oxygen species simultaneously increase the cytosolic calcium concentration, induce rapid destruction of β-cells [21] with a decrease in the islets cell numbers; resulting in cell damage, and cell death [22]; inducing Type I diabetes mellitus. After three days, blood samples were withdrawn from the tail vein of the rats and the blood sugar levels determined using the accucheck glucometer. The diabetic rats (blood sugar > 120mg/dl) were randomly divided into 10 groups ($n$ =4). 200mg/kg dose of the crude methanol extracts and fractions was administered intraperitoneally to the first four groups of the diabetic rats separately. The next four groups received 400mg/kg dose of the extracts. The ninth group received 2ml/kg of 3% tween 80 solution (negative control), while the tenth group of rats received 10mg/kg of glibenclamide (positive control). Blood samples (< 0.1ml) were collected from the tail vein of the rats for the determination of blood sugar concentration at these intervals: 0, 1, 2, 4, 12, 24, 72 hours after treatment.

### Statistical analysis

The analytical tool used was Graph pad Prism version 8 for calculation of the mean values ± Standard Error of Mean (SEM). The one-way ANOVA was used to determine statistical difference between means. A $p < 0.05$ was considered statistically significant.

## RESULTS

Table 1 shows the result of phytochemical analysis of the crude methanol extract of *L. micranthus*. The pharmacologically important classes of secondary metabolites are contained in the mistletoe. There is a heavy presence of alkaloids and flavonoids, with moderate amounts of terpenes and tannins in the plant compared to the other phytoconstituents.

The GCMS chemical characterization of the crude methanol extract was carried out and the results presented in table 2. The interpretation of GC-MS mass-spectra was based on the NIST library of the equipment. The individual spectrum were matched with that of the library and the following parameter; molecular weight, structure, retention time and fragmentation patterns compared. Seven bioactive compounds were ascertained from the spectral match.

The results of the anti-diabetic evaluation of the extracts and fractions of *L. micranthus* are presented in figure 1 below. The results showed a rapid onset of activity by the crude methanol extracts and the 400 mg/kg doses of the n-hexane, chloroform and ethyl acetate fractions. The chloroform fraction was comparable to the standard drug; glibenclamide. The hypoglycemic effects of the extracts and fractions were in a dose dependent manner and continued until the 24hr. After 72hr, there was a slight rise in hyperglycemia. The extracts and fractions were significantly more active than the negative control ($p > 0.05$). Optimum anti-diabetic activities were achieved at the 12hr by the extracts and fractions.

Further evaluation of the anti-diabetic activities of the extracts/fractions were carried out by calculating the percentage reduction of Fasting Blood Glucose Levels (FBGL) as seen in figure 2 below.

A break-down of the percentage reduction of FBGL in the rats displayed in figure 2, revealed that the chloroform fractions were the most potent of the fractions. Both the 200 mg/kg and 400 mg/kg doses achieved the highest % reduction in all the time intervals investigated with the exception of the 2hr only. The activity of the 400

**Table 1:** Result of phytochemical test on the methanol extracts of *L. micranthus* parasitic on *K. acuminata* harvested in the month of April.

| Phytochemical constituents | Abundance |
|---|---|
| Flavonoids | +++ |
| Saponins | + |
| Tannins | ++ |
| Alkaloids | +++ |
| Proteins | + |
| Fats and oil | + |
| Steroids | + |
| Acidic compounds | - |
| Terpenoids | ++ |
| Carbohydrates | + |
| Glycosides | + |
| Reducing sugar | + |
| Resins | Trace |

Key: + = present in low quantity; ++ = present in moderate quantity; +++ = present in heavy quantity; - = absent.

ISSN: 2694-0159

**Table 2:** GCMS Analysis Results.

| No. | Compounds | Retention time (RT) | Molecular Formula | Documented Pharmacological Activities |
|---|---|---|---|---|
| 1 | Benzene, 1,2,3-trimethyl | 6.221 | $C_9H_{12}$ | Antifungal [23,24] |
| 2 | 2,4-Di-tert-butyl phenol | 10.563 | $C_{14}H_{22}O$ | Anti-oxidant [25,26], Anti-microbial, anti-fungal, antiviral, anti-inflammatory, Insecticidal, nematicidal, allelopathy and autotoxicity [26]. |
| 3 | Tetradecane | 12.635 | $C_{14}H_{30}$ | Antifungal and Antibacterial [27]. |
| 4 | Hexadecanoic acid, methyl ester | 20.298 | $C_{17}H_{34}O_2$ | Antioxidant, antimicrobial, antifungal, antimalarial, hypocholesterolemic, nematicidal, anti-androgenic flavour, haemolytic and 5-Alpha reductase inhibitor [28-30]. Release of insulin Stimulation, Anti-diabetic activities [31] |
| 5 | Octadecanoic acid, methyl ester | 22.167 | $C_{19}H_{38}O_2$ | Antifungal, antitumor activity, antibacterial [32,33]. alpha-glucosidase inhibitor [34] |
| 6 | Hexanedioic acid, bis(2-ethylhexyl) | 23.560 | $C_{22}H_{42}O_4$ | Antimicrobial [35] |
| 7 | Diisooctyl phthalate | 24.885 | $C_{24}H_{38}O_4$ | Antifungal, Antibacterial, Antiviral and Antioxidant activities [36]. |

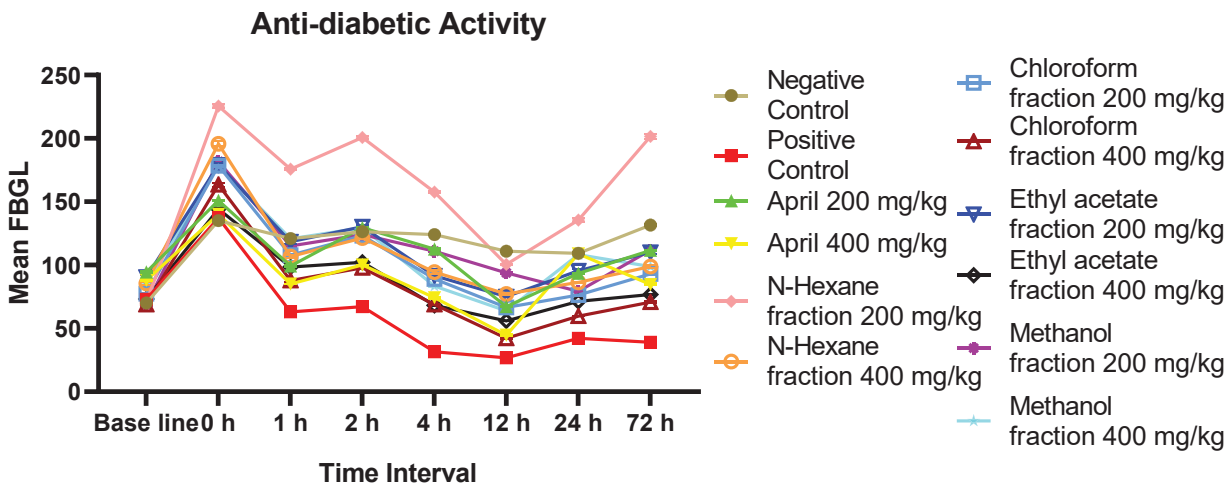



**Figure 1:** A bar chart showing the effect of extracts and fractions of *L. micranthus* on FBGL (Mean value ± SEM) in hyperglycemic rats.

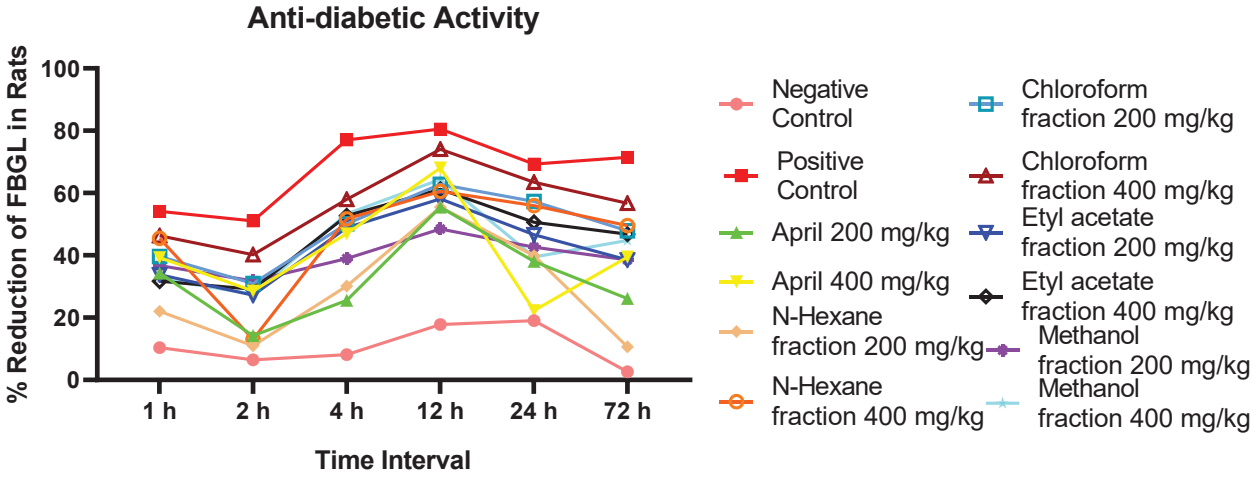



**Figure 2:** A bar chart showing the % reduction of Fasting Blood Glucose Levels (FBGL) in rats after administration of the extracts and fractions of *L. micranthus.*

ISSN: 2694-0159

mg/kg dose was comparable with that of the standard drug at 12 and 24hr respectively.

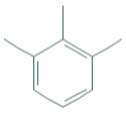
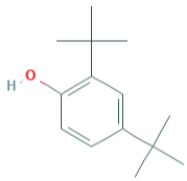
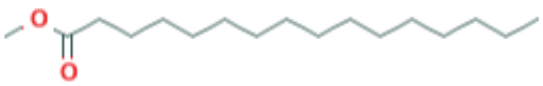
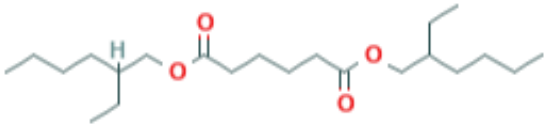
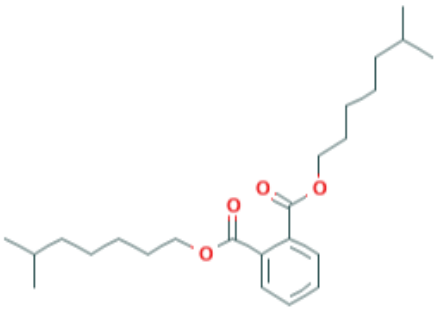
## DISCUSSION

The results of the phytochemical tests of the crude methanol extract of the *L. micranthus* parasitic on *K. acuminata* shown in table 1 indicated that therapeutic classes of secondary metabolites were among the compounds present in the plant. An important finding is the abundance of alkaloids and flavonoids in the mistletoe. These classes of compounds have been linked to several biological activities. Again, the results showed a moderate presence of tannins and terpenoids, which are of pharmacological importance. The slight presence of saponins, steroids, glycosides and proteins further underscores the potentials of this plant as a therapeutic agent.

The GCMS analysis further demonstrated the presence of seven highly bioactive compounds (structures shown in table 3): Benzene, 1,2,3-trimethyl, 2,4-Di-tert-butyl phenol, Tetradecane, Hexadecanoic acid methyl ester , Octadecanoic acid methyl ester , Hexanedioic acid, bis(2-ethylhexyl) and Diisooctyl phthalate in *L. micranthus*. These compounds are reported for the first time in *L. micranthus* parasitic on *K. acuminata*. Previous work on *L. micranthus* parasitic on *Azadirachta indica* (sourced from India), collected in the month of April, revealed the presence of 2,4-Di-tert-butyl phenol, Tetradecene, Hexadecanoic acid, methyl ester, octadecanoic acid methyl ester amongst other compounds, with no mention of Benzene, 1,2,3-trimethyl and Diisooctyl phthalate [31]. The researchers reported that, Hexadecanoic acid methyl ester and octadecanoic acid methyl ester, possessed anti-diabetic activities.

The anti-diabetic activities of Tetradecane has not been documented in literature. Previously, the compound was found to be the second major bioactive compound with an abundance of 34.61% in the investigations of the antibacterial activity of volatile component and various extracts of *Spirulina platensis* [27].

**Table 3:** The structures of the bioactive compounds from the GCMS analysis.

| SERIAL NUMBER | COMPOUND | MOLECULAR FORMULA | STRUCTURE |
|---|---|---|---|
| 1 | Benzene, 1,2,3-trimethyl | $C_9H_{12}$ |  |
| 2 | 2,4-Di-tert-butyl phenol | $C_{14}H_{22}O$ |  |
| 3 | Tetradecane | $C_{14}H_{30}$ |  |
| 4 | Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ |  |
| 5 | Octadecanoic acid, methyl ester | $C_{19}H_{38}O_2$ |  |
| 6 | Hexanedioic acid, bis(2-ethylhexyl) | $C_{22}H_{42}O_4$ |  |
| 7 | Diisooctyl phthalate | $C_{24}H_{38}O_4$ |  |

Structures culled from: National center for biotechnology information (2021). PubChem compound summary. Retrieved April 7, 2021 from https://pubchem.ncbi.nlm.nih.gov/compound/

Similarly, Diisooctyl phthalate is reported to possess antifungal, antibacterial, antiviral and antioxidant activities. Recently, the anti-diabetic activity and chemical composition of Sanbai Melon Seed Oil was carried out and Diisooctyl phthalate was indicated as one of the major bioactive compounds identified [36]. Benzene, 1,2,3-trimethyl has been reported as the major component from the GC–MS analysis of the dried leaves of *T. danielli*; a plant with significant antifungal activities [23]. Again, Ojinnaka and co-workers have documented Benzene, 1,2,3-trimethyl as one of the chemical constituents from the GC-MS analysis of the seed (Fruit) extracts of *Buchholzia Coriacea* Engler (Capparaceae), popularly known as wonderful kola in Nigeria; while investigating the antifungal activities of the plant [24]. The anti-diabetic activities of the seed extract of *Buchholzia Coriacea* have been reported in literature [37].

Hexanedioic acid-bis-(2-ethylhexyl) ester is a plasticizer derivative, which was previously described from natural sources by Elleuch L, et al. 2010, from fractions of a terrestrial *Streptomyces sp*. TN262 that demonstrated mild antimicrobial activities [35].

The compound; 2,4-Di-tert-butyl phenol, has been identified in the leaves of three species of Loranthaceae. Namely: *L. micranthus*, (Linn.), *L. pentapetalus* (Roxb.) and *Viscum ovalifolium* (Wallich ex Candolle) [26]. Literature survey revealed that the compound is highly bioactive with ten documented activities; anti-oxidant, anti-microbial, anti-fungal, antiviral, anti-inflammatory, cytotoxicity, Insecticidal, nematicidal, allelopathic and autotoxicity [25,26]. Recently, the anti-virulence properties of the agent were reported from the compound's restriction of the adhesion and invasion of the pathogen; *P. aeruginosa* into the A549 lung alveolar carcinoma cells [38]. The compound has been identified in the GCMS analysis of the bioactive fractions of *L. micranthus* parasitic on *A. indica* in the investigations of the anti-diabetic activities of the plant [31].

The results of the anti-diabetic screening (shown in figure 1), of the crude extract and fractions of *L. micranthus* demonstrated that the 200 mg/kg and 400 mg/kg doses exhibited anti-diabetic activities. There is clear evidence of a dose-effect relationship in the activities of the extract/fractions. The 400 mg/kg doses performed better than the 200 mg/kg dose levels.

An analysis of the results of figure 2, showed that the percentage reduction of the Fasting Blood Glucose Level (FGBL) at different intervals after administration of the fractions, revealed that the chloroform fraction reduced FGBL significantly after 12hr of administration, followed by the ethyl acetate fraction. This is suggestive of moderately polar constituents being the most bioactive compounds in the plant. The activity of the chloroform fraction was comparable to that of glibenclamide.

After the 72hr, the result clearly showed that the fractions of *L. micranthus* possessed significant hypoglycaemic effect. The antihyperglycemic results obtained are in consonance with the work of Govindappa, et al. 2015 [31] on the effects of *L. micranthus* parasitic on *A. indica* in alloxan induced rats.

A comparison of the anti-diabetic activities of the fractions showed that the significant activity was in this order: Chloroform > ethyl acetate > n-hexane> methanol. A correlation of the results of significant activity and the results of the phytochemical tests demonstrated that the chloroform fraction which gave the optimum activity had high abundance of tannins, flavonoids and alkaloids. This finding is resonated by Eliakim-Ikechukwu and co-workers, who reported that flavonoids, alkaloids and saponins present in *anacardium occidentale* L. stem-bark had blood glucose reduction activity [39].

## CONCLUSION

In conclusion, the methanol extract of *K. acuminata* sourced in the month of April and the four fractions obtained have been investigated for anti-diabetic activities. The study showed that the plant could be employed in the management of DM and thus validates the indigenous uses of the plant in the treatment of DM. Further evaluation of the findings of the study, indicated that the chloroform fraction was the most bioactive of the fractions with comparable activities to that of glibenclamide. The activities of this plant are due to the presence of the seven identified bioactive compounds from the GCMS analysis. 2,4-Di-tert-butyl phenol is considered the most bioactive compound responsible for the observed anti-diabetic activity. Thus, the compound merits further research as a potential therapeutic in the treatment and management of DM.

### Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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