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Full Length Research Paper

Phytochemical Screening, Chromatographic Evaluation and Antibacterial Activity of the Leaf Extracts of *Lawsonia Inermis* L

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The crude methanolic extract of *Lawsonia inermis* leaves was subjected to chromatographic separation by column and thin layer chromatography techniques. Hexane, ethyl acetate and methanol were used as solvent system. The eluted fractions of the preliminary column chromatography gave eleven (11) fractions. All the fractions had distinct colours ranging from green to reddish brown. The eluted fractions were spotted on TLC and six brands were yielded (LT-1, LT-2, LT-3, LT-4, LT-5, LT-6). The eluted fractions were screened for antibacterial activity. The eluted fractions, LT-2 – LT-6 exhibited some degree of antibacterial activity. Furthermore, the eluted compound fractions were assayed for minimum inhibitory concentration (MIC) using the 96-well microdilution technique. Phytochemical screening of the extract revealed the presence of alkaloids, flavonoids, saponins, phenols, tannins, steroids and anthraquinone.

Keywords: Column chromatography, *Lawsonia inermis*, minimum inhibitory concentration, phytochemical screening, thin-layer chromatography

INTRODUCTION

World Health organization (WHO, 2003) maintains that medicinal plants would be the greatest source to obtain a variety of drugs and play a key role in healthcare maintenance. It is estimated that about twenty-five percent (25%) of all modern medicines are directly or indirectly derived from plants (Gill, 1992). It has been further reported that about 80% of the populations of developing countries being unable to afford pharmaceutical drugs rely on medicinal plants to sustain their primary health care needs (UNESCO, 1996; Martinez *et al*. 1996; Mustapha 2006; WHO, 2011). This could be due to their availability, affordability and continuous efficacy when compared to the high cost of synthetic or modern medicines (Von-Maydel, 1996; Sofowora *et al*., 2013). Health is one of the most precious of all things (Adesina, 2000) and in recent times, there has been a shift from synthetic to the use of plant based products. This might not be unconnected to the fact that there are several reports of multiple drug resistance especially bacterial strains of important pathogens. The use of plants and plant based product to meet societal health needs, stems from the fact that commercial anti microbials commonly used in the treatment of infectious diseases are used indiscriminately (Gupta *et al*., 2008). There are also issues of adulteration, increasing toxicity, adverse effects on host and high cost (Shariff, 2001). Ethno pharmacologists, botanists, microbiologists, natural product chemists and other scientists are therefore searching for phytochemicals from plants that could give leads to the development of new effective drugs for treatment of infectious diseases and other diseases such as cancers (Cowan, 1999; Werner *et al*., 2007; Quattara *et al*., 2007). The studies of Perumalsamy and Ignacimuthu, (2000) showed that antimicrobials from

plant origin are effective in the treatment of infectious diseases with little or no side effects when compared to synthetic antimicrobials.

Though there are several synthetic antimicrobial drugs available at the present time against microbial infections, the fact remains that a large proportion of the world's population still does not have access to or cannot afford modern medicines, particularly in remote rural areas of developing countries.

Additionally, the continued usage of current antibiotic drugs is also posing a major problem of drug resistance in several microbial species. Thus, there is an urgent need for newer and inexpensive drugs of plants origin with no side effect and of good pharmacological activity that are able to act for longer periods of time before resistance sets in (Yamac and Bilgili, 2006; Okwu and Uchenna, 2009).

The plant, *Lawsonia inermis* (L) belongs to the 'Loose - Strife; family. It is popularly called 'Henna'. Lawsonia is named after Isaac Lawson, an 18th Century Scottish army doctor who was a friend of Linnaeus and inermis is a Greek word which means 'unarmed without spines' (Iyer *et al.,* 1998).

Several researchers have reported the different biological actions of *Lawsonia inermis* using various *in-vitro* and *in-vivo* test models. Henna leaves, flowers, seeds, stem bark and roots have been found to exhibit antioxidant, antidiabetic hyper-protective, hypoglycemic, antimicrobial anticancer and wound healing properties (Henna *et al*. 2010). These properties have been attributed to naphthoquinones including lawsone (Wren, 1998).

Henna leaves are used as a remedy in skin disease in the form of paste or decoction against burns, bruises and skin inflammation. Leaves in the form of paste have been used as external application for headache and in bringing down fever for the belief that it has cooling down effect. The purpose of this study is to evaluate the effect of fractionation of *Lawsonia inermis* (L) leaf extract on some selected pathogenic bacteria.

MATERIALS AND METHODS

Plant materials

The fresh aerial parts (roots, stem and leaf) of *Mitragyna inermis* were collected around Idu, near National Institute for Pharmaceutical Research and Development (NIPRD) Abuja. The plants were identified and authenticated in the herbarium of the department of Medicinal Plant Research and Traditional Medicine of National Institute for Pharmaceutical Research and Development (NIPRD), Abuja Nigeria. The samples of the plants were deposited in the herbarium for reference purposes. The voucher numbers is: voucher specimen No, NIPRD/H/6838 for *Lawsonia inermis*.

Preparation of plant materials

The leaf of the plant, *Lawsonia inermis* was separated, dried in a shade at room temperature. The completely dried parts were pulverized by grinding with electric blender.

Extracts preparation

The crude plant extracts were prepared by the methods of Olukoya *et al.* (1993), Aboh *et al.* (2014) with slight modifications. The samples were extracted using soxhlet apparatus on a rotary shaker with n-hexane, ethyl acetate, methanol and sterile distilled water respectively. Five hundred grammes of each pulverized parts was extracted using n-hexane for 48h.the residue was dried in oven, while the collected extract was concentrated through evaporation under reduced pressure, packed and kept in refrigerator for further biological investigations. The dried residue was further extracted with ethyl acetate for another 48h. The extract was also concentrated as above while the residue was dried and extracted with methanol, concentrated as above and finally extracted with sterile distilled water. All the crude extracts were filtered and concentrated through evaporation under reduced pressure then transferred into sterile sample bottles, labeled appropriately and kept in refrigerator for further use.

Sterilization of the plant extracts

The extracted parts above were filtered using the membrane filtration system as described by (Sultana, 2007), with slight modifications. The membranes are held in holders, supported on a frame. Fluids are made to transverse membranes by negative pressure. The filter membrane disc used is made of cellulose ester having a nominal average pore diameter of 30 micron (0.30 mm). The membrane was held firmly in a filtration unit which consists of a supporting base for the membrane, a receptacle for the fluids to be filtered, and a collecting reservoir for the filtered fluid. This was carried out under aseptic condition.

Preliminary phytochemical screening

The phytochemical screening for the presence or absence of phytochemicals of the crude extracts was carried out using standard protocols as described by Sofowora, (2013), Trease and Evans, (2002) and Aboh *et al.* (2014) with slight modifications. The following phytochemicals were screened for alkaloids, cardiac glycosides, tannins, flavonoids, phenols, saponins, anthraquinones, sterols and terpenes.

Standardization of plant extract

Five test tubes were labeled 1 to 5. A stock concentration of 50 mg/ml of the extract was prepared in the first test tube. 5 ml of distilled water was then introduced into the remaining four test tubes. The content of the first test tube was thoroughly mixed. 5 ml of this was withdrawn and added to the second test tube which was thoroughly mixed to obtain a concentration of 25 mg/ml. Another 5 ml was withdrawn from the second test tube and then transferred to the third test tube which was also thoroughly mixed to give a concentration of 12.5 mg/ml. In a like manner, a fourth concentration of 6.25mg/ml was prepared from the third test tube, and then 3.125 mg/ml was prepared from the last test tube. 5 ml was removed from the last bottle and discarded.

Fractionation of the crude methanolic extracts of *Lawsonia inermis*

Fractionation of the crude methanolic leaf extracts of *Lawsonia inermis* was carried out in two parts: Column and Thin Layer Chromatography. The protocols of Nwodo *et al.* (2010), Ode *et al.* (2011a) and Adefuye and Ndip, (2013) with slight modifications were employed.

Column chromatography

The crude leaf extracts of *L. inermis* were subjected to column chromatography to separate them into component fractions. The stationary phase (absorbent) used is column graded silica gel 60 G (MERCK) while combinations of hexane, ethyl acetate and methanol were used as mobile phase (solvent system). In the setting up of the column chromatography, glass column of internal diameter 80 mm and length 100 cm (Quickfit, England) was used. The lower part of the glass column was plugged with glass wool with the aid of glass rod. Sand bed was placed over the glass wool. The sand bed served to give a flat base to the column of the absorbent. The wet packing method was used in preparing the silica gel column. 25 g of silica gel (200-425 mesh particles, size Å pore) was wet packed with 250ml hexane solvent system. The slurry of the silica gel and hexane was poured down into the column carefully. The tap of the column was left open to allow free flow of solvent into a conical flask below. The set up was seen to be in order when the solvent drained freely without carrying the silica gel, sand, glass or wool into the tap. At the end of the packing process, the tap was locked. The column was allowed to stabilize for about 24 h. Slurry of the crude extract was prepared in a ceramic mortar by adsorbing 5g of the extract to 10g of silica gel in 10 ml of methanol. The slurry was gently loaded onto the packed column. The column was then eluted with solvent systems (mobile phase) gradually in order of increasing polarity using hexane, ethyl acetate and methanol, at a ratio of 2:1 v/v. The following ratios of the solvent combinations were sequentially used in the elution process: Hexane: ethyl acetate 100:0; 80:20; 60:40; 40:60; 20:80; ethyl acetate: methanol: 100:00; 80:20; 60:40; 40:60; 20:80; 0:100. The solvent systems (mobile phase) was continuously poured from the edge of the column with the aid of a dropper. The bottom outlet of the column was opened allowing eluent to flow through the column. As the eluent passed down the column, the compound fraction moved down the column. The separated fractions flowed out of the column where the different eluents were collected in properly labeled bottles. TLC analysis was carried out on fractions before they were evaporated over water bath. The weights of the dry fraction were recorded.

Thin layer chromatography

Analytical TLC plates were prepared by pouring homogenous silica gel (60, F_{254}, MERCK) slurry into aluminum plates by spreading technique. The silica gel layer was adjusted to 0.25 mm thickness. The coated plates were allowed to dry in air and activated by heating in hot air oven at 100-150ºC for 1 h.

Preparation of the development tank (mobile phase).

Solvent system used was hexane and ethyl acetate at the ratio of 4:1. v/v, then ethyl acetate (less polar) and methanol (more polar). The fractions obtained from the column chromatography were spotted with the help of capillary tube washed in acetone. Each fraction was applied as a single spot in a row along one side of the chromo plate, 2 cm from the edge and 1.5cm from edge (known as the origin). The spotted chromo plate was placed at an angle of 45º in the development tank containing the solvent system, covering the bottom of the plate by the solvent up to nearly 1cm.The solvent front was marked on the plate immediately after removing it from the chamber and allowed to dry. The mobile phase was not allowed to reach the end of the stationary phase. The plate was visualized and dried by hot air dryer. The plate was then viewed under daylight and UV light at 302 nm and 365 nm respectively. The plate was further exposed to iodine fumes in a chamber and finally sprayed with freshly prepared Vanillin reagent (0.16 g Vanillin powder + 14ml of methanol + 0.5 ml concentrated sulphuric acid. The plate was carefully heated at 105ºC for optimal colour development. Characterization of the different compounds identified was done by calculating Rf values. (Adefuye *et al*., 2013).

$$Rf = \frac{\text{Distance moved by the component from the origin to spot centre}}{\text{Distance moved from origin to solvent front}}$$

The fractions showing similar TLC mobility and band (that is, same RF) were pooled together. The fractions were kept at 4ºC in the refrigerator for further bioassay tests to confirm their biological activity.

Determination of minimum inhibitory concentration (MIC) of eluted fractions

The MIC was determined by using the micro-dilution method as described by Adefuye and Ndip, (2013) and Aboh *et al.,* (2014) with slight modifications. This assay was performed using round bottom 96-well microtitre plate. Two-fold serial dilutions of the eluted fractions were prepared in the test wells starting with 50mg/ml stock. The dilutions were 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, and 1.565 mg/ml. The bacterial strains were purified by standard bacteriological methods (Cheesebrough, 1984, CCLS, 2006). The bacterial strains were standardized to 0.5 MacFarland standard using Nephelometer (NCCLS, 1993) to give approximately 1.0 x 10^6 cfu/ml. The standardized cultures were maintained in sterile Muller Hinton agar. Eighteenth-hour broth culture of the *Candida albicans* was suspended into sterile Sabouraud dextrose liquid medium. It was standardized according to Clinical Laboratory Standards Institutes (CLSI, 2002) by gradually adding normal saline to compare its turbidity to McFarland standard of 0.5 which is approximately 1.0 x 10^6 cfu/ml using Nephlometer. Control wells were prepared using Ciprofloxacin 20mg/ml as positive for bacteria. Dimethyl sulfoxide (DMSO) was used as negative control. The plates were then sealed with parafilm and incubated at 37°C for 24 h for bacteria and yeast. After incubation, 40µL of 0.2 mg/ml of Tetrazonium dye (p-iodonitrotetrazoluim violet) was added to well and incubated for an hour, after which the uninhibited organisms would have converted the dye from blue to pink in the well. The MIC was defined as the minimum concentration at which growth was inhibited with no visible change. Sterility test was performed to verify whether the broth used in the assay was contaminated before test procedures. 50µL of broth was dispensed into a well, without both extract and inoculums.

Preliminary *In-vivo* efficacy study

Twenty five healthy male and female rats weighing between 250 g and 300 g were used. They were divided into five groups (I, II, III, IV, V), of the five rats each. Group I- Group IV were infected with a single dose of 1ml suspension of *Salmonella typhi* having being certified pure by morphological cultural and biochemical identification using protocols described by Cheesbrough, (2002) and CLSI, (2006). Daily monitoring of animals for clinical signs were taken, observed and recorded. Faecal droppings of each animal were collected for colony count. Infectivity was established eight days post- inoculation. Infected animals were treated with extracts and standard drugs for Group IV. Standard drug, Amoxycillin 20mg/mL was used for Group IV. The extracts, LI was administered in doses of 500mg/kg, 1000mg/kg and 1500mg/kg to Group I, II, III respectively. On the seventh day, the animals were sacrificed. Group V (control) was given only physiological saline.

RESULTS

Methanol extract of *Lawsonia inermis* was fractionated. Eleven (11) fractions (L1-L11) were eluted. The following phytochemicals were revealed in the eleven fractions: alkaloids, tannins, saponin, flavonoids, terpenes, cardiac glycosides, sterols, phenols, anthraquinones and carbohydrates. This is a confirmation of the phytochemicals that were present in the crude extracts. The details of the result are shown in (Table 1). All the fractions were further subjected to thin layer chromatography. Table 3 shows the details of the Thin Layer Chromatography profiles of the leaf extracts of *Lawsonia inermis.* The weight and percentage yield of the fractions of both plants and the retardation factor (Rf) values are also shown in the (Table 3). Fractions of *L. inermis* with same Rf values were pooled together to give the total of 6 fractions. They are:

LT1- fraction LT1
LT2- combination of fractions 2 and 3
LT3- fraction 4
LT4- fraction 5
LT5- combination of fractions 6 and 7
LT6- combination of fractions 8, 9, 10, 11

The fractions of the leaf extract from Table 4 showed the highest zone of inhibition against *Salmonella typhi* of 12 mm at 50 mg/ml with fractions LT5. This is closely followed by fractions LT4 and LT6 with mean zones of 10 mm each at 50 mg/ml. this is comparable to the positive control antibiotic which showed a mean zone of inhibition of 17 mm at 20 mg/ml. Fractions LT2 and LT3 showed a maximum of 6mm and 8mm at 50mg/ml respectively. No zone of inhibition was observed for LT1.

The minimum inhibitory concentration (MIC) of some of the methanol fractions of the *Lawsonia inermis* leaf extract against the test microorganisms showed similar MIC when compared to the control standard drug used (Table 5). In the experimental infection carried out, the plant extracts (LI) showed mild inhibitory effect against the infective organism *S. typhi* in wistar rats. Two extract concentrations, 500mg/kg and 1000 mg/kg of fractions LT5 were relatively safe experimentally for treatment. However, extracts at 1500mg/kg showed toxic effects on liver and kidney. Infectivity was established eight days

Table 1. Qualitative Phytochemical content of *Lawsonia inermis* fractions.

Constituents	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
Alkaloids	-	-	-	-	-	+	+	+	+	+	+
Tannins	-	-	-	-	+	+	+	+	-	-	-
Saponins	-	-	-	+	-	-	-	-	-	-	-
Flavonoids	-	+	+	+	-	-	-	-	-	-	-
Terpenes	-	-	-	+	-	-	-	-	-	-	-
Steroids	-	-	-	+	+	-	-	-	-	-	-
Glycosides	-	-	-	-	-	-	-	-	-	-	+
Resins	-	-	-	-	-	+	+	+	-	-	
Phenols	-	-	-	-	-	-	+	+	+	+	+
Anthraquinones	-	-	-	-	-	+	+	+	+	+	+

Key: L1- L11 = eluted fractions of *L, inermis*, + = present, - = absent

Table 2. Details of fractions from column chromatography of crude methanolic extract of *Lawsonia inermis* (L) leaf

Fractions	Eluent (Solvent System)	Ratio V/V	Colour of Fraction
LC1	Hex	100	Grey
LC2	Hex : EA	80 : 20	Greenish
LC3	Hex : EA	60 : 40	Dark green
LC4	Hex : EA	40 : 60	Dark green
LC5	EA	20 : 80	Light brown
LC6	Hex : EA	0 :100	Brownish
LC7	EA : Met	80 : 20	Brownish
LC8	EA : Met	60 : 40	Reddish brown
LC9	EA : Met	40 : 60	Reddish brown
LC10	EA : Met	20 :80	Reddish brown
LC11	Met :	100	Reddish brown

Key: Hex = Hexane, EA = Ethyl Acetate, Met = Methanol, LC = *Lawsonia inermis* fraction from Column chromatography

Table 3. Details of thin layer chromatography of methanolic extract of *Lawsonia inermis* leaf

Eluted Fractions	Weight of Fraction (g)	Percentage (%) Yield	Rf Value
LT- 1	0.065	1.63	0.19
LT- 2	0.213	5.33	0.34
LT- 3	0.212	5.30	0.45
LT- 4	0.302	7.56	0.61
LT-5	0.944	23.59	0.71
LT- 6	1.625	40.64	0.85

Key: Mobile System/ Solvent (Eluent) HEM = Hexane Ethyl Methanol Ratio of 4: 4: 1 (v/v)
LT = *Lawsonia inermis* fraction from TLC

post inoculation (Figure 1).

DISCUSSION

This study revealed the presence of different phytochemicals such as tannins, anthraquinone, flavonoids, and alkaloids in the fractions (Table 1). This shows that the leaf of *Lawsonia inermis* L is rich in bioactive phytocompounds. These phytocompounds have been reported to be associated with pharmacological activities (Edeoga and Eriata, 2001). For example, tannins possess anti-inflammatory and antibacterial properties, flavonoids are known to have diuretic and antibacterial properties (Tor-Anyiin and Orokpo, 2012). It was observed that all the fractions did not contain same phytochemicals.

The column chromatography profile of the crude methanol leaf extracts of *Lawsonia inermis* produced

Table 4. Zone of inhibition of the fractions of *L. inermis* against *Salmonella typhi* mean zone of inhibition (mm).

Fractions	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	Positive Control	Negative control
LT- 1	0.0 ± 0.0	0.0 ± 0.0	0.0. ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.00
LT- 2	5.0 ± 0.5	4.0 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	20.0 ± 0.0	0.00
LT- 3	8.0 ± 0.5	5.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	18.0 ± 0.5	0.00
LT- 4	10.0 ± 0.0	5.0 ± 0.0	4.0± 0.0	0.0 ± 0.0	20.0 ± 0.0	0.00
LT-5	12.0 ± 0.0	7.0 ± 0.0	5.0 ± 0.5	3.0 ± 0.5	17.0 ± 0.0	0.00
LT- 6	10.0 ± 0.0	8.0 ± 1.0	7.0 ± 0.0	3.0 ± 1.5	19.0 ± 0.5	0.00

Values are mean inhibition zone (mm) ± SD of the three replicates, p< 0.05.
Key: LT1 – LT6 = *Lawsonia inermis* fractions of TLC, Positive control = Chloramphenicol (20mg/ml), Negative control = Distilled water.

Table 5. Minimum inhibitory concentration (MIC) of methanol fractions of *Lawsonia inermis* against *Salmonella typhi*

Fractions	MIC (mg/ml)
LT-1	0.0 ± 0.0
LT-2	12.50 ± 0.00
LT-3	12.50 ± 0.50
LT-4	12.50 ± 0.00
LT-5	12.50 ± 0.00
LT-6	12.50 ± 0.00

*Values are mean inhibitory concentration (µg mL -1) ± of three replicates. LI- Fraction of *Lawsonia inermis*, Chloramphenicol 20mg/ml as positive control
DMSO- Negative control (0.00)

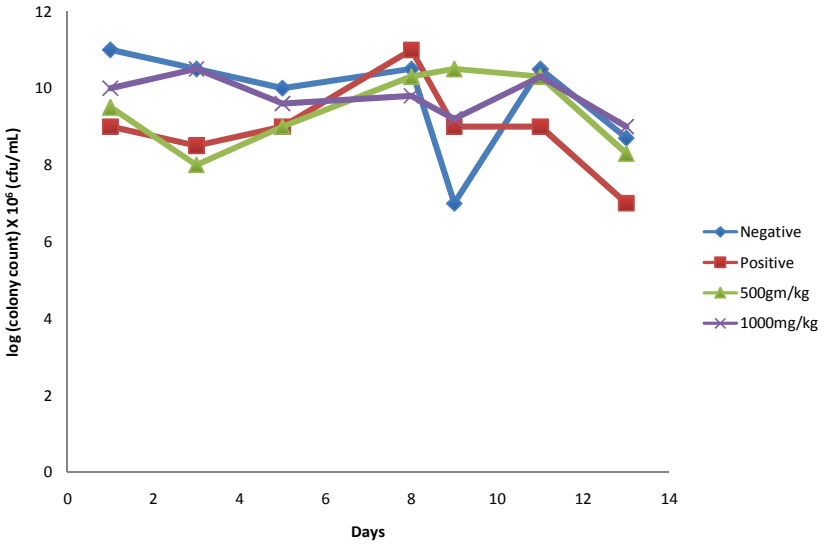

Figure 1. *In vivo* antibacterial activity of LI on *S. typhi*

eleven fractions, LC1 – LC11 (Table 2). The variation in the percentage recovery may be due to the differences in the solubility of the phytocompounds content of the plant extract in the solvent systems employed. This finding was reported by Kawo, and Kwa, (2011). Thin-Layer Chromatography is a technique that is selective specific and rapid in identification of chemical substances (Ode *et al*., 2011b). The TLC study of the crude methanol leaf extract of *L. inermis* produced six bands (LT-1 – LT-6) after pooling together of fractions with same retardation (RF) values (Table 3). The colour of the various fractions ranged from grrey in LC1 to dark reddish brown in LC11.

The fractions were assessed for antibacterial assay on *Salmonella typhi* (Table 4). All the fractions except LT-1 showed varying degree of activity on the test microorganism. LT-4 and LT-6 fractions showed 10mm mean zone of inhibition at 50 mg/ml while fraction LT-5 had 12mm mean zone of inhibition at 50 mg/ml. these compared favourably with the positive control drug, Chloramphenicol, which gave 19 mm mean zone of inhibition. LT-1 fraction did not inhibit the test organism, that is, there was no visible zone of inhibition. The least visible zone of inhibition observed at 50 mg/ml was 5 mm for fraction LT-2. Therefore, activity observed varied among the fractions. This could be due to the fact that the phytochemicals fractions in the *L. inermis* leaf vary in quantity. On the otherhand, it could be that the fractions with wider range of activity contain compound acting synergistically or additively (Nwodo *et al*., 2010). However, this needs to be ascertained. Furthermore, the fractions with greater zone of inhibition could be due to the presence of higher concentrations of the bioactive components. It is also known that TLC is a technique that is selective and rapid in identification of chemical substances (Ode *et al*., 2011a). Hefferon, (2012) reported a possible reduced biological activity of the fraction of some medicinal plants as compared to the crude extracts of such plants. The finding in this study is in agreement with the works of Nwodo *et al*. (2010) and Ode *et al*. (2011b) who reported that the decrease in activity after fractionation may be as a result of synergy between the active phytocompounds, which are separated into smaller entities with varying biological efficacy during the process of fractionation.

Conclusion

The richness of the phytochemicals observed in *L. inermis* could justify the multiple therapeutic indications for which the plant is used in ethnomedical practices.

Authors` Declaration

We declare that this study is an original research by our research team and we agree to publish it in the journal.

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