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# REPEATED-DOSE TOXICOLOGICAL STUDIES OF HYDROALCOHOLIC EXTRACT OF *LANNEA KERSTINGII* ENGL AND K. KRAUSE (ANACARDIACEAE) AND IDENTIFICATION OF TOXICITY MECHANISMS

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**ABSTRACT:** *Lannea kerstingii* Engl. & K. Krause stem bark (Anacardiaceae) is used to treat anaemia, malaria, infections and Burili ulcer. The antioxidant, antihelmintic and trypanocidal properties have been demonstrated using appropriate *in vitro* and *in vivo* assays. But the effective doses have demonstrated toxic effects such as a significant decrease of body weight of the experimental animals. This work aimed to evaluate the 60 days repeated-dose toxicity of hydroalcoholic extract from *Lannea kerstingii* stem bark. After 60 days administration, *L. kerstingii* induced 50% of rats death; accumulation of gas in the digestive tract and a significant decrease of rats body weight. The determination of the toxic mechanism has shown that *L. kerstingii* decreased the daily food intake and the intestinal motility. The decrease of the intestinal motility by I.P. administration was higher than by P.O. administration. Naloxone, inhibited the effect of *L. kerstingii* on the intestinal motility. *L. kerstingii* stem bark hydroalcoholic extract possess a significant inhibition effect on ileum contraction. Therefore it can be toxic in prolonged use at higher doses and its use should be avoided in cases of constipation.

**INTRODUCTION:** The use of herbal plants as natural remedies, functional foods, and dietary supplements for health care has been increasing in the world. Market estimates suggest that the rate of growth in sales of traditional medicinal products in recent years has been between 5 and 18% per annum [1].

One of these traditional medicines is *Lannea kerstingii* Engl. & K. Krause (Anacardiaceae). *L. kerstingii* stem bark is used in Togo to treat anaemia and malaria [2-4].

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In West African countries such as Cote d'Ivoire, *L. kerstingii* stem bark and root are consumed by natives from Northern Côte d'Ivoire, as traditional remedies for the treatment of diarrhoea, gastritis, rheumatic, sterility, intestinal helminthiasis [5]. Also, in different West African areas, this plant species is prescribed against oedema, rickets, wounds, scurvy, scorbut, epilepsy [6]. The fruit of *L. kerstingii* is eaten raw in the Guinean pre-forest savannas of Ivory Coast [7]. In Benin, *L. kerstingii* leaves are used in the treatment of Buruli Ulcer [8].

The effectiveness of the traditional uses of *L. kerstingii* has been confirmed by various *in - vivo* and *in - vitro* assays. Pharmacological studies of *L. kerstingii* extracts have revealed several properties such as antihelmintic, antimicrobial and trypanocidal properties [9-11]. Our previous study has shown the antioxidant effect of *L. kerstingii* stem

bark hydroalcoholic extract using appropriate *in - vitro* and *in- vivo* assays [3], but the effective doses have demonstrated toxic effect during 28 days subchronic test. After 28 days administration, *L. kerstingii* at 1000 mg/kg increased significantly the relative weight of the spleen and decreased significantly the increment of body weight [12]. This increase in the relative weight of the spleen could be attributed to a toxic action or not. It's could be due to an inflammation or an increase of enzymes or peroxysomes synthesis [13-15]. Several authors agree that the change in body weight of the animals is a sign of toxicity [16-17]. According to our previous reports about the toxic activity of *L. Kerstingii* [12] on the body weight increment, we aim to study the 60 days toxicity of the hydroalcoholic extract of *L. kerstingii* stem bark and to determine it toxicity mechanisms.

## MATERIALS AND METHODS:

The study was conducted following an approved animal use protocol at from the institutional Ethical Committee for Teaching and Research (ref no. CNCB- CEER 2801/2010). Animal care and handling are conducted as conformed to accepted guidelines [18-20].

**Plant material:** *Lannea kerstingii* stem bark was collected at Bagbe (Togo) in July 2009. It was identified by Prof Kouami Kokou from the Botany department of University of Lome (Togo) and a voucher specimen was kept in the herbarium of the Laboratory of Botany and Plant Ecology (Faculty of Science/University of Lome) under the reference N° 10553 Akpagana.

**Preparation of hydroalcoholic extract:** The stem bark was washed in running water, then dried and ground to a powder. The powder was soaked in ethanol-water (50-50: v/v) for 72 h with manual discontinue agitation. The solution was filtered and evaporated using a rotary evaporator (yield: 12.34). The study was conducted in Animal Physiology Department, Faculty of Sciences, University of Lome, Togo.

**Preliminary phytochemical analysis:** Plant materials were screened for the presence of alkaloids, saponins, tannins, total phenols, anthraquinones, flavonoids, and sterols using the methods previously described by Tona et al [21].

**Animals:** Wistar rat either sex (150-200 g), provided by the department of Animal Physiology of University of Lome (Togo) were used. They were housed in a standard environmental condition and fed with rodent standard diets and water ad libitum.

**Subchronic toxicity test:** The repeat dose oral toxicity study was carried out according to OECD guideline 408 [18]. Because of rat death, instead of 90 days study we stopped at 60 days. The animals were divided into three groups of 8 animals each (4 males and 4 females). Group 1 received 10 ml/kg of distilled water and served as control. Group 2 and 3 received *L. kerstingii* hydroalcoholic extract at 500 mg/kg body wt. and 1000 mg/kg body wt. respectively. Doses of 500 and 1000 mg/kg are therapeutic doses used in our previous study[3]. Extract was administered daily for 60 days at similar time. Animals were observed at least twice daily for morbidity and mortality. Body weight of animals was evaluated daily.

On the 61$^{st}$ day, after an overnight fast, rats were anaesthetized with ether and blood sample for haematological and biochemical analysis were collected into tubes with or without EDTA respectively. haemoglobin (Hb), haematocrit (Ht), red blood cells count (RBC), white blood cells count (WBC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and platelet count were determined using automatic counter Sysmex (K21, Tokyo, Japan).

Biochemical analysis were performed in serum obtained after centrifugation of total blood without anticoagulant, at 2500 rpm for 15 min. Standardized diagnostic kits (Labkit®) and a Biotron® spectrophotometer were used for spectrophotometrical determination of the following biochemical parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, alkaline phosphatise (ALP), glucose (Glu), total proteins, γGT and urea.

Necroscopy of all animals was carried and the organ weights (heart, liver, kidney and spleen) were recorded. Each weighed organ was then standardized for percentage body weight of each rat (relative organ weight). Histological study of organs was done after sacrificing the animals on 61st day.

**Determination of the mechanism of action**

**Effect of *L. kerstingii* on daily food intake:** Rats were divided into three groups of 5 animals. Group 1 received 10 ml/kg of distilled water and served as control. Group 2 and 3 received *L. kerstingii* hydroalcoholic extract at 500 and 1000 mg/kg body wt once a day for 28 consecutive days. Food intake and body weight were recorded once a day at 24 h after the daily administration.

**Effect of *L. kerstingii* on the isolated ileum motility:** Rats were killed under ether anesthesia and a portion of ileum was removed and placed in oxygenated Krebs solution (NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $KH_2PO_4$, 2.5; gSO4, 1.2; $NaHCO_3$, 25; and glucose 11 nM) at room temperature. Isotonic contractions were recorded using Harvard isotonic transducer (Biopac system, MP 100) and displayed on a computer with Acq Knowledge (Acq Knowledge III software). After a maximal contraction obtained by adding Acetylcholine (Ach 320 nM), L. kesrtingii were added directly to the organ bath at the final concentrations ranging from 0.31 to 2.7 mg/mL. Concentration–response curve was obtained by the cumulative addition of *L. kerstingii* at 15-min intervals. All experiments were conducted in parallel with time-matched controls using the tissue from the same animal and adding an equivalent volume of vehicle. Contractions were measured as maximum inhibition in tension from one concentration to the next concentration and expressed as a percentage of inhibition of the contraction.

**Gastrointestinal transit test:** Three groups of 5 animals each fasted overnight were used. *L. kerstingii* extract was given orally to group 2 and 3 (500 and 1000 mg/kg, respectively), while group 1 was used as control. Five minutes later, 0.5 ml of a 3% charcoal suspension was administered orally to each rat. All the rat were killed by cervical translocation 30 min later and the distance travelled by the charcoal plug from pylorus to caecum was determined and expressed as a percentage of the total length of the small intestine. The assay was repeated after 7, 14 and 28 days of administration of *L. kerstingii* hydroalcoholic extract. To investigate the mechanism of action, another group of 5 rats received *L. kerstingii* (1000 mg/kg) by I.P. route and another group (5 rats) received naloxone (1 mg/kg) 30 min. after the administration of *L. kerstingii* (1000 mg/kg).

**Statistical analysis:** The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed by one way analyse of variance (ANOVA) with Tukey test to evaluated significant differences between groups. Values of $p< 0.05$ were considered significant. All statistical analysis were carried out using the Instat Statistical package (Graph Pad software, Inc. USA).

**RESULTS & DISCUSSION:**

**Phytochemical study:**

The phytochemical study did not detect any alkaloids, sterols, saponins and anthraquinones but has identified high concentrations of flavonoids, polyphenols and tannins. **(Table 1)**

**Subchronic toxicity test:**

In clinical evaluation, *L. kerstingii* caused 50% mortality of rats without severe behavioural changes at the end of the 60 days of treatment. The necropsy of rats has shown the presence of gas in the gastrointestinal tract. **(Fig 1)**

*L. kerstingii* at 500 mg/kg decreased significantly ($p<0.05$) rat body weight from the 6th to the 8th week but with 1000 mg/kg, the decrease was significant ($p<0.05$) from the 2nd to the 8th week (table 1). No significant differences were observed in organs relative weight between control and treated group except testis relative weight. *L. kerstingii* at 500 and 1000 mg/kg decreased significantly ($p<0.01$) testis relative weight **(Table 2)**. No clearly histological damage was observed in rat liver, kidney, spleen, colon, stomach and testis tissues treated with *L. kerstingii* hydroalcoholic extract when compared with control. No significant difference was observed in haematological and biochemical parameters **(Table 3** and **4)**. *L.*

*kerstingii* decreased, from the second week to the last week, the daily food intake (data are not shown).

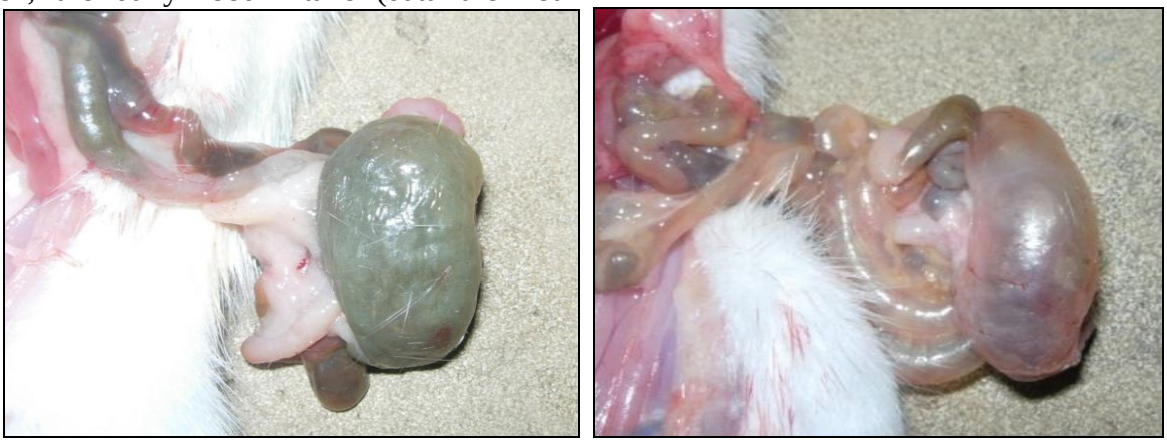

**FIG 1: EFFECT OF *L. KERSTINGII* ON THE GASTROINTESTINAL TRACT. REPRESENTATIVE RAT STOMACH FOR CONTROL (A) AND *L. KERSTINGII* TREATED RAT AT 1000 mg/kg (B) FOR 60 DAYS. NECROPSY OF RAT WAS CARRIED ON THE 61$^{ST}$ DAY, AFTER AN OVERNIGHT FAST.**

**TABLE 1: MEAN BODY WEIGHT (G) OF RATS AFTER 60 DAYS TREATMENT WITH 50% HYDROALCOHOLIC EXTRACT OF *L. KERSTINGII*.**

| Week | Extract (mg/kg) | | |
|---|---|---|---|
| | 0 | 500 | 1000 mg/kg |
| 0 | 134 ± 7.6 (8) | 132 ± 4.6 (8) | 129 ± 4.7 (8) |
| 1 | 148 ± 6.0 (8) | 133 ± 8.7 (8) | 130 ± 4.1 (8) |
| 2 | 161 ± 5.7 (8) | 134 ± 10 (8) | 130 ± 3.3* (8) |
| 3 | 165 ± 7.6 (8) | 136 ± 10 (8) | 137 ± 6.0* (7) |
| 4 | 171 ± 7.1 (8) | 146 ± 16 (7) | 143 ± 5.1* (7) |
| 5 | 179 ± 6.7 (8) | 149 ± 16 (6) | 143 ± 5.4* (6) |
| 6 | 175 ± 5.2 (8) | 140 ± 10** (6) | 138 ± 5.2** (6) |
| 7 | 180 ± 5.9 (8) | 146 ± 10* (5) | 143 ± 4.2** (4) |
| 8 | 174 ± 6.6 (8) | 137 ± 8.0** (4) | 138 ± 4.7** (4) |

The results are mean ± S.E.M.; with N=8; *P<0.05 (control group versus extract); **P<0.01 (control group versus extract).

**TABLE 2: MEAN RELATIVE ORGAN WEIGHT (%) OF RATS AFTER 60 DAYS TREATMENT WITH 50% HYDROALCOHOLIC EXTRACT OF *L. KERSTINGII***

| Parameters | Extract (mg/kg) | | |
|---|---|---|---|
| | 0 | 500 | 1000 |
| Heart | 0.43 ± 0.02 (4) | 0.48 ± 0.02 (4) | 0.46 ± 0.02 (4) |
| Liver | 2.42 ± 0.50 (4) | 2.41 ± 0.25 (4) | 2.38 ± 0.08 (4) |
| Spleen | 0.16 ± 0.01 (4) | 0.17 ± 0.01 (4) | 0.17 ± 0.01 (4) |
| Kidney | 0.58 ± 0.02 (4) | 0.59 ± 0.06 (4) | 0.59 ± 0.02 (4) |
| Testis | 0.61 ± 0.06 (3) | 0.40 ± 0.04** (3) | 0.31 ± 0.08*** (3) |
| Colon | 0.92 ± 0.06 (4) | 0.93 ± 0.11 (4) | 1.2 ± 0.13 (4) |
| Stomach | 0.67 ± 0.04 (4) | 1.1 ± 0.24 (4) | 0.97 ± 0.13 (4) |

The results are mean ± S.E.M.; (N) = number of animals; **P<0.01 (control group versus extract); ***P<0.0001 (control group versus extract)

**TABLE 3: HAEMATOLOGICAL PARAMETERS OF RATS AFTER 60 DAYS TREATMENT WITH 50% HYDROALCOHOLIC EXTRACT OF *L. KERSTINGII*.**

| Parameters | Extract (mg/kg) | | |
|---|---|---|---|
| | 0 | 500 | 1000 |
| WBC ($10^3$ /µl) | 5.2 ± 0.7 (4) | 4.7 ± 1.4 (4) | 4.2 ± 0.42 (4) |
| RBC ($10^6$ /µl) | 7.8 ± 0.32 (4) | 8.1 ± 0.82 (4) | 8.2 ± 0.45 (4) |
| Hb (g/dL) | 13.8 ± 0.20 (4) | 13.2 ± 0.20 (4) | 13.5 ± 0.24 (4) |
| Ht (%) | 40.1 ± 0.01 (4) | 40 ± 4.46 (4) | 40.4 ± 2.06 (4) |
| MCV (fl) | 51.5 ± 0.99 (4) | 49.1 ± 0.58 (4) | 49.6 ± 0.61 (4) |
| MCH (pg) | 17.63 ± 0.32 (4) | 16.03 ± 0.77 (4) | 16.52 ± 0.28 (4) |
| MCHC (%) | 34.38± 0.23 (4) | 32.67 ± 1.24 (4) | 33.40± 0.52 (4) |

| | | | |
|---|---|---|---|
| Platelet ($10^3$ /µL) | 794 ± 84 (4) | 640 ± 50 (4) | 638 ± 38 (4) |

The results are mean ± S.E.M.; (N) = number of animals.

**TABLE 4: BIOCHEMICAL PARAMETERS OF RATS AFTER 60 DAYS TREATMENT WITH 50% HYDROALCOHOLIC EXTRACT OF *L. KERSTINGII*.**

| Parameters | Extract (mg/kg) | | |
|---|---|---|---|
| | 0 | 500 | 1000 |
| AST (U/L) | 261 ± 92 (4) | 208 ± 18 (4) | 207 ± 15 (4) |
| ALT (U/L) | 62 ± 14 (4) | 40 ± 1.4 (4) | 55 ± 5.2 (4) |
| ALP (U/L) | 270 ± 55 (4) | 250 ± 47 (4) | 312 ± 29 (4) |
| Urea (mg/dL) | 27 ± 0.03 (4) | 26 ± 0.02 (4) | 28 ± 0.02 (4) |
| Glu (mmol/L) | 4.8 ± 0.08 (4) | 4.9 ± 0.17 (4) | 4.7 ± 0.23 (4) |
| Creatinine (mg/dL) | 8.0 ± 0.82 (4) | 10 ± 2.1 (4) | 9.2 ± 0.80 (4) |
| Total proteins (g/dL) | 5 ± 2.5 (4) | 5.1 ± 1.6 (4) | 6 ± 1.8 (4) |
| γGT | 3.4 ± 0.8 (4) | 3.9 ± 0.1 (4) | 3 ± 0.5 (4) |

The results are mean ± S.E.M.; with (N) = number of animal

*L. kerstingii* is known to possess a broad spectrum of traditional medicinal, pharmacological and therapeutic properties. Pharmacological studies of *L. kerstingii* extracts have revealed several properties such as antioxidant effect [3], antihelmintic [5, 9] and trypanocidal effect [10].

During the 28-day subchronic toxicity, we observed an increase in the relative weight of the spleen and a decrease in the body weight of rats. After the evaluation of the 28 days toxicity, OECD recommends the 90 days toxicity even if this plant is not traditionally used for a long time.

In this study, initially planned for 90 days, we have stopped at 60 days because of the death of rats (50%). The death of the rats is associated with an accumulation of gas in the digestive tract and decreased the rats body weight. Body weight changes are an indicator of adverse side effects, as the animals that survive cannot lose more than 10% of the initial body weight [16, 17].

Apart from the presence of gas in the gastrointestinal tract, *L. kerstingii* decreased significantly ($p < 0.05$) the daily food intake and the intestinal motility. The presence of gas in the intestine can be due to a bowel obstruction. There are three types of intestinal obstruction or occlusion. The first-type, mechanical occlusion is caused by the presence of an obstacle along the digestive tract. The second type is the functional occlusion. It is also known as paralytic ileus. Finally, there is mixed occlusion which is a combination of the two previous types [22].

**Spasmolytic activity:**

Rat ileum suspended in Krebs's solution under 1 g of tension after 30 min had a stable tension. Acetylcholine (ACh 320 nM) induced a phasic contraction in the tissue, reaching the peak within 30 s of contact. *L. kerstingii* hydroalcoholic extract, in a concentration-dependent manner (0.31-2.71 mg/ml) inhibited the ileum contraction induced by Ach 320 mM (**Fig. 2)**

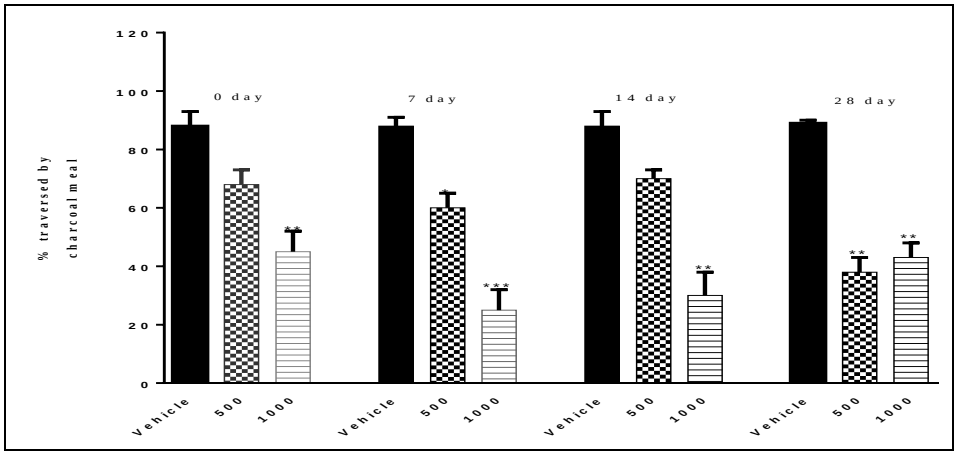


**FIG 2: EFFECT OF *LANNEA KERSTINGII* HYDROALCOHOLIC EFFECT ON THE INTESTINAL TRANSIT OF CHARCOAL MEAL IN RAT. VEHICLE (CONTROL), *L. KERSTINGII* HYDROALCOHOLIC EXTRACT (500 AND 1000 mg/kg) WERE ADMINISTERED BY P.O. 30 Min. BEFORE CHARCOAL ADMINISTRATION. THIRTY MIN. AFTER THE CHARCOAL**

**MOVEMENT IN INTESTINE WAS EXPRESSED AS A PERCENTAGE. THE ASSAY WAS REPEATED AFTER 7, 14, AND 28 DAYS ADMINISTRATION OF *L. KERSTINGII*. EACH COLUMN REPRESENTS MEAN ± SEM (N=5). *P<0.05, **P<0.01, ***P<0.0001 CONTROL GROUP VERSUS EXTRACT (ANOVA FOLLOWED BY TUKEY'S TEST).**

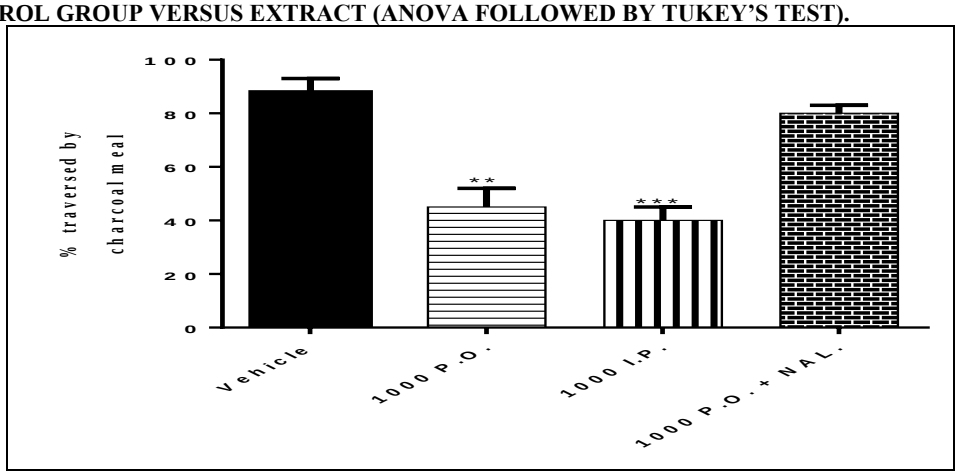


**FIG 3: EFFECT OF *LANNEA KERSTINGII* HYDROALCOHOLIC EXTRACT ON THE INTESTINAL TRANSIT OF CHARCOAL MEAL IN RAT. VEHICLE (CONTROL) AND *L. KERSTINGII* HYDROALCOHOLIC EXTRACT (1000 mg/kg) WERE ADMINISTERED BY P.O. (1000 P.O.); I.P. (1000 I.P.) AND P.O. BEFORE NALAXONE (1000 P.O. + NAL); 30 min. BEFORE CHARCOAL ADMINISTRATION. THIRTY MIN. AFTER THE CHARCOAL MOVEMENT IN INTESTINE WAS EXPRESSED AS A PERCENTAGE. EACH COLUMN REPRESENTS MEAN ± SEM (N=5). *P<0.05, **P<0.01, ***P<0.0001 CONTROL GROUP VERSUS EXTRACT (ANOVA FOLLOWED BY TUKEY'S TEST).**

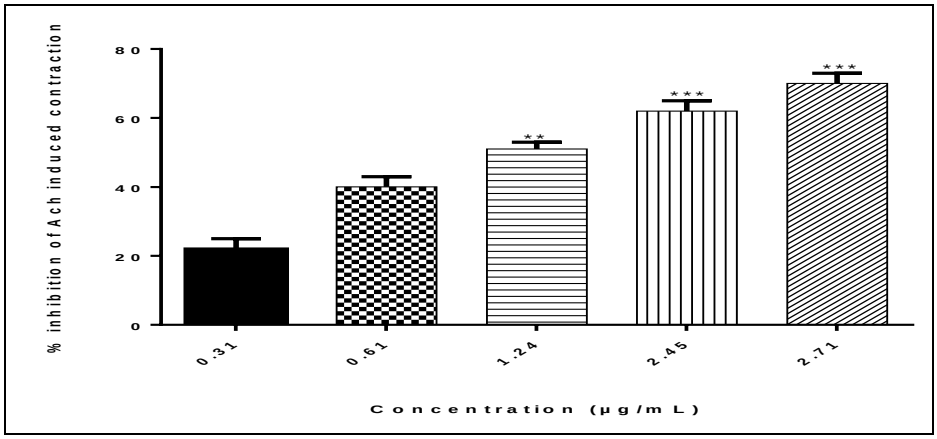


**FIG 4: EFFECT OF *LANNEA KERSTINGII* HYDROALCOHOLIC EXTRACT ON ISOLATED RAT ILEUM CONTRACTED BY ACH. AFTER A MAXIMAL CONTRACTION OBTAINED BY ADDING ACH (320 NM), *L. KESRTINGII* WERE ADDED DIRECTLY TO THE ORGAN BATH AT THE FINAL CONCENTRATIONS RANGING FROM 0.31 TO 2.7 MG/ML. EACH COLUMN REPRESENTS MEAN ± SEM (N=5). *P<0.05, **P<0.01, ***P<0.0001 VERSUS EXTRACT 0.3 µG/ML (ANOVA FOLLOWED BY TUKEY'S TEST).**

## Gastrointestinal transit test:

The results of the gastrointestinal transit test revealed that the hydroalcoholic extract (1000 mg/kg) inhibited the small intestinal motility of the charcoal marker in rat after single administration and after repeated administration during 7, 14 and 28 days **(Fig 2)**. After 28 days, *L. kerstingii* at 500 mg/kg induced also a significant ($p<0.01$) inhibition of the intestinal motility.

To identify the type of occlusion *L. kerstingii* induced; we have administered it (1000 mg/kg) by I.P. The administration of *L. kerstingii* by I.P. decreased also the intestinal motility **(Fig 3)**. Therefore it is not a mechanical obstruction but a functional obstruction. Our results are similar to those of Olatokunboh et al [23]. They have shown that the aqueous extract of *Lannea welwitschii* (400 mg/kg), a species very close to *L. kerstingii* induced a decrease of the intestinal motility. A lot of herbal medicines are traditionally used for their spasmolytic activity [24-26].

The use of naloxone 30 min after *Lannea kerstingii* administration inhibited it effect on the intestinal motility **(Fig 3)**. Naloxone, an antagonist of opiods receptors confirmed that the obstruction is not mechanic but functional and it may occur by

opioids receptors. This result is also in concordance with the phytochemical screening which shown the presence of tannins and polyohenols, and the traditional uses of *L. kerstingii* extract against diarrhea and gastristic [11, 27-29].

*L. kerstingii* it seems to be toxic after a long term administration at high doses. It's known that antidiarrheal plants can be toxic when they are used for a long period. For exemple, when rhubarb (*Rheum palmatum L*.; *Rheum tanguticum Maxim. Ex Balf.*, or *Rheum officinale Baill*.) is used as a purgative, long-term use usually leads to attenuation of its purgative activity, increase of the required dosages, and secondary constipation after drug withdrawal and even melanosis coli [30-32].

In conclusion, this study has shown that *L. kerstingii* hydroalcoholic extract, at therapeutic dose after long period administration is not toxic for rat liver, kidney and blood. But *L. kerstingii* extract possess a significant inhibition effect on ileum contractions. *L kerstingii* can also be a useful herbal medicine for the treatment of gastrointestinal spasms but it must be avoid in constipation. However, further studies are needed, to look for its possible action on other smooth muscle and to determine the mechanism of action.

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