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In Vitro Cholinergic and Acute Toxicity Evaluations of *Salacia lehmbachii*

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ABSTRACT

Salacia lehmbachii has been implicated in the treatment of many disease conditions including gastrointestinal disorders by herbalists in South Eastern Nigeria. Our goals were first to assess the toxicity level of aqueous root extract of *S. lehmbachii* (ARESL) in mice and then to evaluate the cholinergic activity of the extract in isolated rabbit ileum**.** For acute toxicity study, mice of either sex were randomized into five groups of 10 mice and treated with 1000, 2000, 3000, 4000 and 5000 mg/kg body weight. Overt toxic signs were monitored for 48 hours. For cholinergic activity, ileal muscle strips were prepared and mounted in a tissue bath containing aerated De-Jalon's solution maintained at 36±1^{O}C. Dose-response relationships for acetylcholine (ACh) was studied isometrically in the presence of fixed concentrations of ARESL (0.25×10^{-2} and 0.5×10^{-2} mg/ml). Similarly, atropine control was investigated in the presence of ACh and the effect compared with those of the extract viz-a-viz amplitude, frequency as well as maximum achievable effects (E_{max}) and concentrations which inhibited 50% of maximum effects (IC_{50}). Data were computed by one-way analysis of variance (ANOVA) followed by Newman Keule's test as post hoc. Acute oral toxicity revealed no death but righting reflex revealed dose-dependent increase and LD_{50} was 631 mg/kg. Cholinergic activity revealed a significant (P <0.05) shift to the right of dose-response curve of ACh from pretreatment with extract 0.25 and 0.5×10^{-2} mg/ml and atropine 3.5×10^{-2} µg/ml. Since there was significant reduction in E_{max} and frequency of contraction, ARESL therefore possesses anticholinergic property and this explains why herbalists use it as antispasmodic agent.

INTRODUCTION

Cholinergic transmission involves the release of neurotransmitter called acetylcholine (ACh) when conducting electrical impulse reaches parasympathetic nerve terminus. ACh then diffuses across the synaptic cleft and excites receptors on the post-junctional membrane and triggers a series of chemical events resulting ultimately in a biological response such as muscle contraction [1]. Neuromuscular junctions, preganglionic neurons of the sympathetic nervous system, basal forebrain, brain stem complexes, and receptor for the merocrine sweat glands are all cholinergic [1]. In the brain, evidence suggests muscarinic receptors role in memory function and in the pathophysiology of affective illness [2-4] and schizophrenia [5, 6]. Due to their putative role in cognitive function, cholinergic transmission and especially muscarinic receptors have been a focus of research in Alzheimer's disease. Medicinal plants have provided the world with lead compounds which have had great significance in human as well as in animal health. This is mostly attributed to their large therapeutic widow and comparatively low side effects to allopathic medicine.

The plant *Salacia lehmbachii* belongs to celestraceae family and is a small woody herb of approximately 3 m of height. The leaves are simple, acuminate, ovate oblong, opposite and shining. The flowers are yellow on woody auxiliary tubercles while the fruits are orange, globose with one large seed at the center and two to four seeds immersed almost at the periphery of the pulp [7]. The herb is located in the tropical forest of South Eastern Nigeria and Cameroon [8] and aqueous root extract is reported to possess analgesic and anti-inflammatory property [9], anti-abortificient activities [10] and antidiuretic property [11] in Wistar rats. In South Eastern Nigeria, herbalists use root decoction in treating malaria symptoms and gastrointestinal disorder. Scientific evidence on isolated ileal activity of the root extract in animal model is unreported. Hence, the present study was aimed at assessing acute toxicity test in mice and *in vitro* cholinergic activity of the extract in rabbit ileum because of the great homology and similarity between the genomes of humans and laboratory animals [12].

MATERIALS AND METHODS

Drugs and chemicals

ACh and atropine were purchased from Sigma (UK), Salts from Zorka, Sabac (FR Yugoslavia). Following manufacturer's recommendations, salts were stored and always freshly prepared before the experimentations.

Plant material and preparation of extract

Fresh roots of *S. lehmbachii* were harvested without wholly uprooting the plant from South Western Region of Cameroon in January 2015. The plant was identified in Cameroon National Herbarium (CNH), Yaounde, with Voucher No. 40730/SRF/CAM. The roots were room dried at temperature of 25-28^{O}C for 3 weeks. Using a manual grinder, the dried root sample was ground to coarse powder and preserved in an air tight container. A I kg sample was water-macerated in 7.5 litres of distilled water for 3 consecutive days, filtered using Whatman filter paper and filtrate sun-dried at about 43±5^{O}C for another 3 days to obtain brick-red paste-like extract which was finally defatted to get dry polar aqueous extract [10].

Experimental animals

Healthy young adult rabbits weighing about 700 g were selected from Animal House Unit, Department of Pharmacology, University of Calabar and used for the experiment. The rabbits were housed in iron gauge cages and maintained under standard laboratory conditions of relative humidity (50±5%), temperature (28±2^{O}C) and a 12 h dark/12 h light cycle and received standard pellet diet (Agro Feeds, Calabar) and water *ad libitum*. Animal treatment was done in accordance with OECD's guidelines on animal care [13](OECD, 2014).

Phytochemical screening

ARESL has been screened following standard procedures in our previous study [13](OECD, 2014).

Acute toxicity test

Acute toxicity assay was carried out following Muhammed (2009) method with slight modifications. In brief, five groups of 10 mice were randomly selected and treated orally with varying doses of the extract and observed for overt toxicity signs for 24 h.

Preparation of rabbit ileul strip

Young fasted rabbits weighing about 2.5 kg were sacrificed with a swift blow on the head and 3-4 cm longitudinal segments of ileum were rapidly removed and immerged in De-Jalon's solution. Each segment was quickly threaded and mounted up in a 30 ml organ bath containing De-Jalon's solution with the following composition: NaCl = 9 g, KCl = 0.42 g, NaHCO3 = 0.5 g, CaCl2 = 0.06 g and glucose = 0.5 g [10]. One end of the thread was tied to a fixed tissue holder while the other end was tied to an isometric force transducer (UC 2 Gould-Statham, Oxnard USA) connected to an oscillograph (Universal Harvard Oscillogragh, UK). The solution was aerated with 95% O2 and 5% CO_2 maintained at a temperature of 36±1^{O}C. Isometric contractions were monitored under fixed passive force of 1 g. The preparation was allowed to stabilize for 40 min during which the bath solution was changed every 15 min.

Effect of ARESL on spontaneous ileal contractions

At equilibrium, baseline (100%) amplitude and frequency were established in the first 5 min from spontaneous rhythmic contractions. Then effects of ARESL (0.25×10^{-2} mg/ml) were investigated by exposing the tissue to the extract for 10 min followed by increasing cumulative double doses.

Effect of Induced ileal contractions on amplitude

At equilibrium, the amplitude of ACh was investigated separately and in combination with ARESL and atropine. Firstly, the tissue was challenged to increasing cumulative double doses of ACh ($0.25–10000\times10^{-5}$ mg/ml). Secondly, the tissue was incubated with ARESL (0.25×10^{-2} mg/ml; 0.5×10^{-2} mg/ml) for 5 min before being challenged again with ACh in increasing cumulative doses. Thirdly, the tissue was incubated with atropine (3.5×10^{-2} µg/ml) for 5 min before being challenged with ACh [10].

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple range test as post hoc test. Each value represented the Mean ± SEM. P <0.05 was fixed at the designed stage of the experiment [14].

RESULTS

Acute toxicity study

Acute toxicity study did not produce any death up to a dose of 5000 mg/kg body weight for 48 h. However, righting reflex revealed a dose-dependent increase as shown in Table 1. The LD_{50} of righting reflex was 631 mg/kg as shown in Figure 1.

Table 1: Results of acute toxicity test to determine LD_{50} of ARESL in mice after oral administration (n=10)

Group	Dose (mg/kg)	Log Dose	% Righting reflex	Corrected % righting reflex	Probit
1	1000	3	0	2.5	3.04
2	2000	3.3	30	30	4.48
3	3000	3.5	70	70	5.52
4	4000	3.6	90	90	6.28
5	5000	3.7	100	97.5	6.96

Cholinergic activity

Representative tracings showing the effects of a selected ARESL-and atropine-induced contractions on isolated rabbit ileum to ACh is shown in Fig. 2. Atropine seems to exhibit a complete blockage of cholinergic activity. Dose-response curves showing effect of ACh on ARESL- and atropine-induced ileal contractions are shown in Fig. 3. Pretreatment with ARESL and atropine shifted the dose-response curve of acetylcholine to the right. Concentrations of maximum response (E_{max}) and concentrations inhibiting 50% of maximum response (IC_{50}) are shown in Table 2. The extract and atropine significantly ($P \leq 0.05$) reduced both E_{max} and IC_{50}.

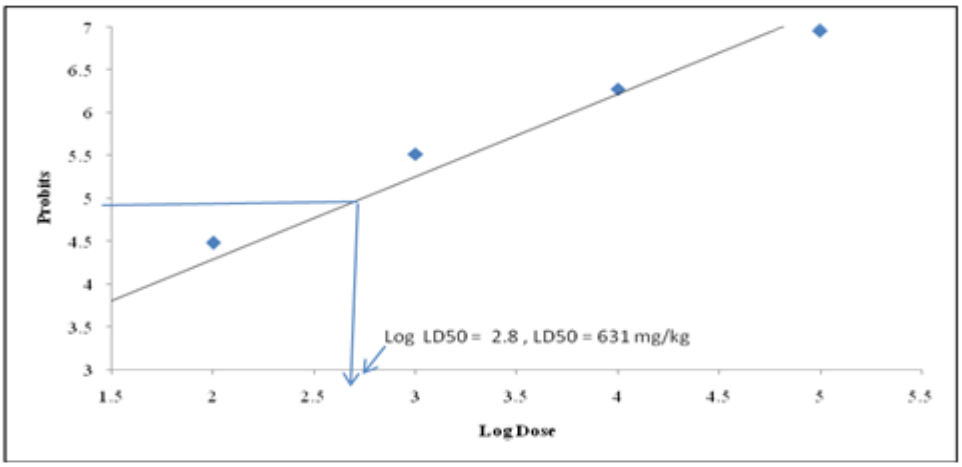
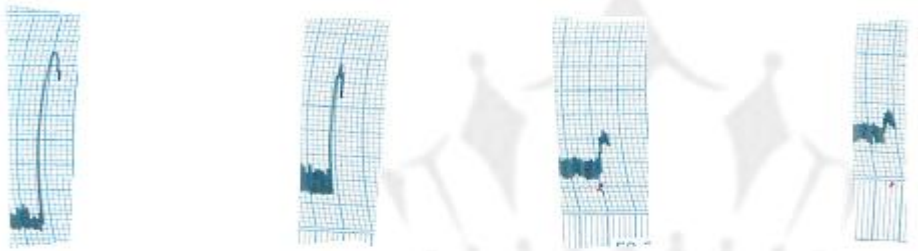


Fig. 1: Plot of log doses versus probits of righting reflex for calculation of LD50 of ARESL administered per oral



ACh Alone | ACh plus Extract (0.25×10^{-2} mg/ml) | ACh plus Extract (0.5×10^{-2} mg/ml) | ACh plus Atropine (3.5×10^{-2} µg/ml)

Fig. 2: Representative tracings showing the effects of a selected ARESL-and atropine-induced contractions on isolated rabbit ileum to ACh

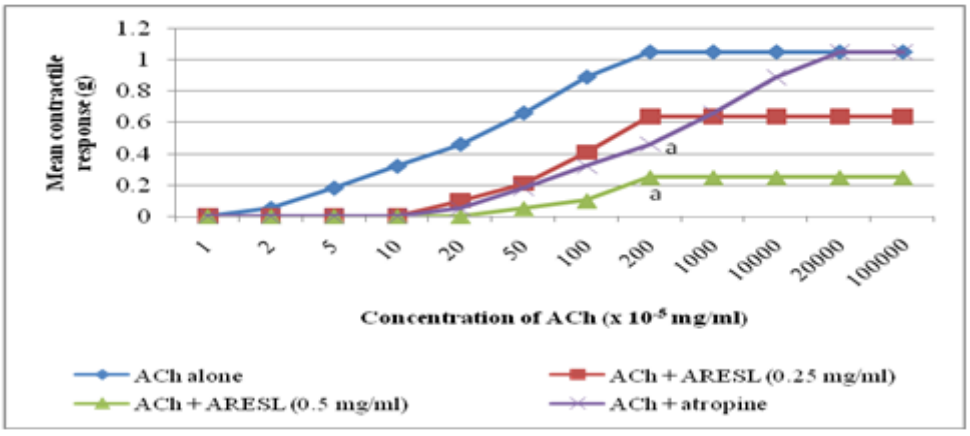


Fig. 3: Concentration-response curves showing effect of ARESL on ACh-induced ileal contractions.

Data represent Mean ± SEM (n = 5). $^{a}P \leq 0.05$

Table 2: Concentrations of maximum response (E_{max}) and concentrations inhibiting 50% of maximum response (IC_{50}) on isolated rabbit ileum.

Drug (mg/ml)	E_{max} (g)	IC_{50} (mg/ml)
ACh alone	2.10 ± 0.21	$108 \times 10^{-4} \pm 5.02$
ACh + ARESL (0.25×10^{-2})	0.79 ± 0.03[a]	$213\times 10^{-4} \pm 10.42$[a]
ACh + ARESL (0.5×10^{-2})	0.42 ± 0.02[a]	$276 \times 10^{-4} \pm 11.31$[a]
ACh + Atropine (3.5×10^{-2} µg/ml)	0.32 ± 0.01[a]	$321 \times 10^{-4} \pm 13.85$[a] (µg/ml)

ACh = Acetylcholine, ARESL = Aqueous root extract of *S. lehmbachii*. Data were presented as Mean ± SEM (n = 5). [a] $P \leq 0.05$

DISCUSSION

The value for acute toxicity study shows that *S. lehmbachii* is relatively safe. This is concluded from the Organisation for Economic Cooperation and Development Guidelines for testing of chemicals [13] which says that a substance that shows no overt toxicity up to 5000 mg/kg body weight is safe. Most medicinal plants fall into this group of chemical substances because there are no neurotoxic substances found in their secondary metabolites.

ACh is known to bind to ACh receptors at postganglionic cholinergic nerves and on smooth muscles to produce contraction. This was observed throughout the entire experiment with ACh. However, maximum achievable contractions of ACh were differentially reduced by extract and atropine. Atropine is recognized for being a tropane alkaloid and competitive antagonist of muscarinic ACh receptors in parasympathetic nervous system [15].

In the present experiment, atropine showed a competitive antagonism in the sense that at each blockage, increasing doses of ACh were able to surmount the blockage whereas that was not the case with the extract. In our earlier study to assess anti-abortificient activities of the extract in Wistar rats, it was realized that the extract had nonspecific uterine smooth muscle contractile antagonism with very high affinity for receptors and no efficacy [10].

In this study, the same antagonism is shown on ileal smooth muscle which begins to give clearer activity of the extract viz a viz smooth muscle of laboratory animal. The same noncompetitive

antagonism observed in uterine smooth muscle is observed in the present study with ileal smooth muscle. It can be suggested that the extract may have a potentiating effect on anticholinesterase thereby increasing ACh hydrolysis activity to acetate and choline and significantly blocking the activity of ACh. Another mechanism like presynaptic blockage of release of ACh is still possible because of the noncompetitive nature of this extract.

CONCLUSION

Aqueous root extract of *Salacia lehmbachii* possesses anticholinergic property and therefore supports its usage as antispasmodic agent by South Eastern Nigerian herbalists.

Competing Interests

Authors declare that there are no conflicting interests pertaining to the publication of this research work.

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