ORIGINAL ARTICLE

BIOLOGICAL ACTIVITY OF LEAVES EXTRACTS AND ACTIVE *ENT*-KAURANE FROM *Erythroxylum ferrugineum cav.* (ERYTHROXYLACEAE)

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ABSTRACT

Background: *Erythroxylum ferrugineum Cav*. (Erythroxylaceae) is a small tree endemic of Madagascar used in the form of decoction in traditional medicine for the treatment of dysentery. **Objectives**: The main aim of this study was to investigate the potential inhibitory activity of crude extracts and isolated compounds against causative agents of diarrheal Disease. **Methods:** Plant extracts and compounds were obtained from the air-dried powder of leaves of *E. ferrugineum* by cold maceration and liquid chromatographic technics. Pure compounds were identified by direct comparison of their physical and spectroscopic characteristics with those published in the literature. Effect of crude extract on intestinal motility was evaluated in vivo by intestinal transit test. Antibacterial activities were tested using micro broth dilution method. The cytotoxicity of the most active fraction and isolated compounds was assessed on vero cells lines using the MTT assay. **Results:** *E. ferrugineum* leaves extract yielded 40.05 %, slightly affected the intestinal motility and showed a moderate antibacterial activity against *S. typhi*, *S. enterica* and *S. aureus*; with minimum inhibitory concentration (MIC) ranging from 125 to 250 µg/ml. Its polar part (86.63 %), was non-cytotoxic (50% cytotoxic concentrations: CC50 > 200 µg/mL) and displayed the strongest antibacterial activity (MIC=100µg/mL). A phytochemical investigation of the DCM extract led to the isolation of a non-toxic new diterpene named *ent*-kauran-15α, 16β, 17-triol (CC50 > 200 µg/mL) and a known derivative identified *ent*-kauran-16β, 17-diol (CC50=67.040 ± 1.257 µg / mL), which had a MIC of 100 µg / ml against *S. aureus* and 2 strains of *Salmonella*. **Conclusions:** The results of this study contribute to validate the traditional use of *E. ferrugineum*. The chemical structures of the compounds obtained may aid the design of antibacterial agents.
Keywords: *Erythroxylum ferrugineum, antibacterial, Cytotoxicity, diterpenes.*

1. INTRODUCTION

Diarrhoea diseases are responsible for millions of death in the world each year. Among the causal agents, bacteria are one of the most common pathogens [1, 2]. This disease is characterized by an increase in the volume and weight of daily stool secondary to malabsorption, intensification of intestinal motility, altered mucosal transport, as well as secretory or osmotic dysfunctions [3, 4]. In developing countries, particularly in rural and marginalized areas, the impact of diarrhoeal infection is exacerbated by relatively reduced number of conventional drugs for its treatment as well as lack of adequate and affordable healthcare, among others [1]. The uses of traditional medicines are still important in these countries but, only few medicinal plants have been controlled clinically, or studied chemically and biologically [4]. Herbal medicines have played an important role in the past in the discovery and isolation of new drugs and the World Health Organization has recommended the studies of traditional medical practices in the Diarrhoea Disease Control Program [5].

Erythroxylum ferrugineum Cav. is a small tree endemic of Madagascar where it is used to make tool handles. The decoction of its leaves is taken orally as to treat dysentery. The leaves of this specie contain 1.4% lipids, aromatic compounds and traces of alkaloids [6]. However, no biological studies on this specie have been reported in the literature to the best of our knowledge. Previous phytochemical investigation on *Erythroxylum* genus (Erythroxylaceae) revealed the occurrence of tropane alkaloids, diterpenes, flavonoids and triterpenes [7]. Some biological activities have been reported for the genus, such as anti-inflammatory, cytotoxic and antioxidant [7].

This research was designed to justify the widespread traditional use of *E. ferrugineum* in the treatment of dysentery through the search for antibacterial fractions and the isolation and characterization of secondary metabolites responsible for this activity.

2. MATERIALS AND METHODS

2.1. General experimental procedures

TLC was performed on aluminium silica gel 60 F254 (Merck) plates (0.2 mm layer thickness). Spots were visualized on TLC either by UV lamp (254 and 366 nm) or by heating after spraying with 20% H_2SO_4 (v/v) or $FeCl_3$ solution. Different mixtures of *n*-hexane, DCM, AcOEt and MeOH were used as eluting solvents. Melting points were measured on a Buchii melting point apparatus.

High resolution mass spectra were obtained with a QTOF Spectrometer (Bruker, Germany) equipped with a HESI source. 1D- and 2D-NMR spectra were recorded in deuterated solvants on Bruker ARX 500 NMR spectrometer (proton at 500 MHz and carbon 13C at 125 MHz) and AVANCE DMX 600 NMR spectrometer (proton at 600 MHz and carbon 13C at 150 MHz). All chemical shifts (δ) are quoted in parts per million (ppm) using a residual solvent signal as secondary reference relatively to tetramethylsilane (TMS) as internal standard, while coupling constants (*J*) are given in Hz.

2.1.1. Plant material

E. ferrugineum Cav. known as "Menahy, Hazomby, Hazomainty, Hazombiby, Taimboalavo" were collected from Alaotra-Mangoro region, in the East of Madagascar on August 2017. The plant name has been checked with http://www.theplantlist.org (kew-2801401) and specimen was identified by the botanist at the Botanical and Zoological Park of Tsimbazaza (PBZT). A voucher was deposited in the "Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique" at the Sciences Faculty of Antananarivo University for future references.

2.1.2. Extracts preparation and fractionation

The air-dried and powdered leaves (300 g) of *E. ferrugineum* were extracted with ethanol 80% (2 L) under room temperature for one week and filtered. The filtrate (1.43 L) was evaporated under reduced pressure till dryness to give ethanolic extract (120.15g). A part of this extract (35.67g) was partitioned using successively 360 ml of *n*-hexane (hex), 198 ml of dichloromethane (DCM) and 60 ml of ethyl acetate (EtOAc) to yield three fractions, namely hex (1.45 g), DCM (1.37 g) and AcOEt (0.14 g) extracts, respectively. A brown powder (30.90 g) was obtained by washing the last residue with ethyl acetate, namely IFA. It showed fluorescence under UV 365 nm and gave a green coloration with ferric chloride reagent on TLC (R_f 0.76; with a mixture of AcOEt-EtOH 8:2).

A part of the DCM extract (1.33 g) was subjected to liquid chromatography over silica gel (Merck MN silica gel 60 M : 0.04–0.063 nm), eluted with a gradient of *n*-hexane/EtOAc (9:0 → 0:1) and then EtOAc/MeOH (9:1 → 0:1) to give a total of 309 fractions of 100 ml each, which were combined based on TLC analysis to aﬀ ord 6 main lots labelled [fr.1 (1–88), fr.2 (89-109), fr.3 (110-133), fr.4 (134-148), fr.5 (149-245) and fr.6 (246–309)]. Compound 1 (0.20 g) was separated from lot fr.2 after repeated recrystallizations in ethyl acetate and methanol. In the same way, compound 2 (0.40 g) was purified from lot fr.4. Its structures were determined by means of spectroscopic analysis and by comparison of their NMR data with those reported in the literature [8].

2.2. Biological tests

2.2.1. Intestinal transit test

A method described by Wong and Way (1981), Sairam et *al.,* (2003), Marona and Lucchesi (2004) with slight modification was used to test effect of the ethanolic extract on intestinal motility [9, 10, 11]. All procedures were conducted in accordance with the principles of laboratory animal use and care according to the European Community guidelines (EEC Directive of 1986 ; 86/609/EEC) and were approved by the Animal Ethical Committee of the Faculty of Sciences, University of Antananarivo —Madagascar. SWISS mice of both sexes, weighing between 25 – 30 g were obtained from the animal facility at the IMVAVET (Institut Malgache des Vaccins Vétérinaires) and fasted for 18 hours prior to the experiment. The animals were randomly separated in 5 groups of 3 mice each and received the treatment by oral gavage administration. The negative and positive control groups were treated with distilled water and loperamide (5 mg/kg/animal), respectively. Animals in groups 3, 4 and 5 received various dosages of ethanolic extract. After 60 min, the animals received a suspension of active charcoal 10% in a solution of 5% agar and 0.2 ml/animal, by gavage administration. The mice are sacrificed by cervical dislocation after 60 min. The entire intestine were immediately removed; then the distance the meal has travelled through the intestine as indicated by the charcoal is measured and expressed as per cent of the total distance from the pylorus to the caecum. Student's t-test (Microsoft Excel 2013) is used to compare the control and the drug-treated group. Significance difference was accepted at a probability level of p < 0.05.

2.2.2. Antibacteria activity

The microdilution method on Muller Hinton broth was used to test the susceptibility of bacteria (*Staphylococcus aureus* ATCC25923 and *Salmonella enterica* NR4311, three clinical isolates of *Salmonella* from the Centre Pasteur of Cameroun: *Salmonella typhimurium CPC*, *Salmonella enteritidis CPC* and from the University Teaching Hospital of Yaoundé: *Salmonella typhi CHU*). The tests were carried out on 96-well microplates according to the M07 A9 protocol described by

CLSI (2012) [12]. For this, two-folds serial dilutions of extracts were carried out in the Muller Hinton broth to obtain volumes of 100µL per well. One hundred microliters of a bacterial suspension (1.5×10^6 CFU/mL) were added into each well containing the test substances to obtain final concentration range of 500-6.25 µg/mL. The percentage of DMSO in the first wells was 0.4% and showed no effect on bacterial growth. Ciprofloxacin was used as a positive control and the plates were covered and incubated at 37°C for 24 h. MICs were determined by the addition of 20 µl of rezasurin (a lamar blue TM Cell Viability Reagent) solution to the wells and re-incubated for 30 min. Membrane dehydrogenases from viable cells reduce the blue-colored dye to formazan pink. The MIC was determined as the lowest concentration of test substance which hindered bacterial growth, marked by no change in color of the medium. All the tests were performed in duplicates at two different occasions.

2.2.3. Cell viability test-MTT assay

The cytotoxic effect of antibacterial fraction and compounds was assessed using the MTT assay [13], targeting Vero cells (African Vero Monkey) cultured in complete medium containing 13.50 g/L DMEM (Gibco, Waltham, MA USA), 10% foetal bovine serum (Gibco, Waltham, MA USA), 0.21% Sodium bicarbonate (Sigma-Aldrich, New Delhi, India) and 50 µg/mL Gentamicin (Gibco, Waltham, MA USA). Essentially, Vero cells at 104 cells/100 µL/well were seeded into 96-well flat-bottomed tissue culture plates (Corning, USA) in complete medium. After 24 h of incubation, media was refreshed and 4 µL of serially diluted extracts solutions (at 1.6, 3.13, 6.25, 12.5, 25, 50, 100 and 200 µg/mL) were added and incubated again for 24 h in a humidified atmosphere at 37°C and 5% CO_2. 10% and 0.4% DMSO (v/v) were taken as positive (0% growth) and negative controls (100% growth) respectively. Twenty microliters of a stock solution of MTT (5 mg/mL in 1X phosphate buffered saline) were added to each well, gently mixed and incubated for additional 4 h. After spinning the plate (1500 rpm, 5 min), the supernatant was carefully removed and 200 µL of 100 % DMSO (v/v) was added to dissolve the formazan. Formazan formation was read on a Magelan Infinite M200 fluorescence multi-well plate reader (Tecan) at 570 nm. The 50% cytotoxic concentrations (CC50) of extracts were determined by analysis of dose – response curves using Graphpad prism 7.0 software.

3. RESULTS

3.1. Spectroscopic data of compound 1

ent-kauran-16β, 17-diol: HR ESI-MS; m/z 329.2841 (calc. for $C_{20}H_{34}O_2Na^+$ 329.245), 1H NMR (CDCl$_3$, 500 MHz) δ(ppm); 0.72 (3H, s, H-19), 0.78 (3H, s, H-18), 0.94 (3H, s, H-20), 3.6 (1 H, d, *J* = 12 Hz, H-17), 3.7 (1 H, d, *J* = 12 Hz, H-17), 13C NMR (CDCl3, 125 MHz) δ(ppm); 17.8 (C-20), 18.3 (C-11), 18.6 (C-2), 20.4 (C-6), 21.6 (C-19), 26.3 (C-12), 33.2 (C-4), 33.5 (C-18), 37.3 (C-14), 39.4 (C-10), 40.3 (C-1), 42.0 (C-3), 42.2 (C-7), 44.8 (C-8), 45.4 (C-13), 53.4 (C-15), 56.1 (C-9), 56.7(C-5), 66.3 (C-17), 81.3 (C-16).

3.2. Structure elucidation of compound 2

Compound 2 was obtained as colourless needles, melting point: 192-194 °C. Its HR ESI-MS spectrum in positive mode suggested the molecular formula $C_{20}H_{34}O_3$ from the sodiated molecular ion peak [M+Na]$^+$ at m/z 345.2823 (calc. for $C_{20}H_{34}O_3Na^+$ 345.240). The NMR data of compound 2 were similar to those of compound 1 (Table 1). Indeed as 1, 1H, HMQC, 13C and DEPT spectra of compound 2 exhibited among the 20 carbons observed the three methyl singlet at δH 0.72 / δC 21.5, δH 0.78 / δC 33.5, δH 0.93 / δC 17.7, oxymethylene signal at δH 3.6 / δC 65.8 (3.65 and 3.66 each 1 H, d, J = 12 Hz), the oxygenated quaternary carbon at δC 80.6.

The differences between spectra of compounds 2 and 1 was the resonance of an oxymethine (δH 3.40 (s) / δC 83.1) in NMR spectra of 2 instead of the methylene H15 (δHa 1.34 / δC 53.4; δHb 1.51 / δC 53.4) in compound 1 indicating a hydroxyl group at C15 position in compound 2. This assignment was confirmed by HMBC correlations of proton H17 (-CH2-OH) and carbon C15 (δC 83.1); proton H15 (-CH-OH) and carbons C14 (δC 35.6) and C9 (δC 55.6); protons H14 (-CH2) and carbons C15 (δC 83.1), C14 (δC 35.6) and C12 (δC 25.5); proton H9 (-CH-) and carbons C15 (δC 83.1), C12 (δC 25.5) and C20 (δC 17.7). Thus compound 2 was identified as *ent*-kauran-15, 16, 17-triol, a new derivative. The relative configuration of compound 2 was established by NOESY correlations and by comparison with the previously reported data [14,15] which established the relative configuration of compound 2 as *ent*-kauran-15a, 16β, 17-triol (Figure 1).
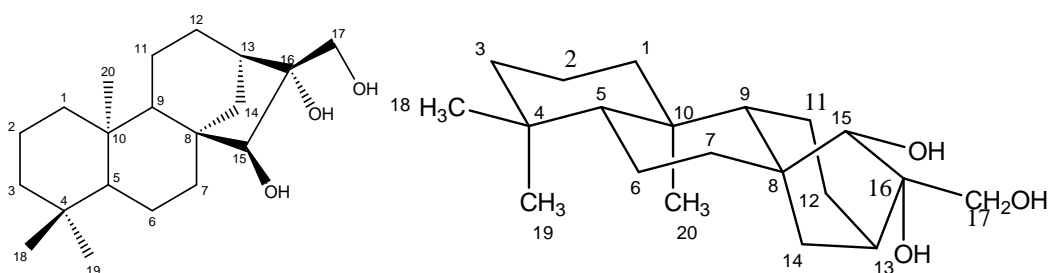

Figure 1: The figure presents the relative configuration of compound 2

Table 1: Comparison of the NMR data of the two diterpenes isolated from *E. ferrugineum*:

Carbon Number	*ent*-kauran-16β, 17-diol (Compound 1)		*ent*-kauran-15α, 16β, 17-triol (Compound 2)	
	δc (in ppm)	δH (in ppm)	δC (in ppm)	δH (in ppm)
1	40.3	0.64, 1.70	40.3	0.64,1.71
2	18.6	1.26, 1.52	18.5	1.30,1.58
3	42.0	1.43, 1.53	42.1	1.59
4	33.2	-	33.2	-
5	56.7	0.69	56.1	0.71
6	20.4	1.22, 1.50	19.4	1.18, 1.55
7	42.2	1.00, 1.27	35.7	1.29
8	44.8	-	47.5	-
9	56.1	0.91	55.6	1.02
10	39.4	-	39.5	-
11	18.3	1.57	18.4	1.59
12	26.3	1.51	25.5	1.43
13	45.4	1.95 (m)	43.2	2.01 (m)
14	37.3	1.47, 1.91	35.6	1.47, 1.77
15	53.4	1.34, 1.51	83.1	3.40 (s)
16	81.3	-	80.6	-
17	66.3	3.6, 3.7 (each 1 H, d, J = 12 Hz)	65.8	3.65, 3.66 (each 1 H, d, J = 12 Hz)
18	33.5	0.78 (s)	33.5	0.78 (s)
19	21.6	0.72 (s)	21.5	0.72 (s)
20	17.8	0.94 (s)	17.7	0.93 (s)

m: multiplet, d: doublet, s: singlet

3.3. Effect on intestinal motility, and antibacterial activities

The ethanolic extract slightly affected the distance travelled by charcoal meal through the intestine and these effects depend on the dose administered (figure 2). Ethanolic extract at a dosage of 500 mg/kg and loperamide (5 mg/kg) caused the charcoal meal to travel 37.22 ± 3.94 % and 38.48 ± 2.73 % of the total intestinal length, respectively (figure 2) which were significative when compared to those of the negative control group (54.78 ± 2.08 %), ($P<0.05$).
The susceptibility to the ethanolic extract was investigated on a wide range of bacteria strains including *S. aureus ATCC25923*, *S. enterica NR4311*, *S. typhiridium CPC*, *S. enteritidis CPC* and *S. typhi CHU*. The results show that this extract was active against three pathogens out of the five strains used, particularly *S. typhi*, *S. aureus* and *S. enterica* (Table 2). The polar part of its extract namely IFA, displayed the strongest activity against all tested bacteria while the Hex and DCM extracts exhibited antibacterial activity against *S. aureus* and *S. typhi*, respectively. Either *ent*-kauran-16β, 17-diol (Compound 1) or *ent*-kauran-15α, 16β, 17-triol (compound 2) has a moderate antibacterial activity against *S. typhi*, *S. enterica* and *S. aureus* (table 2).
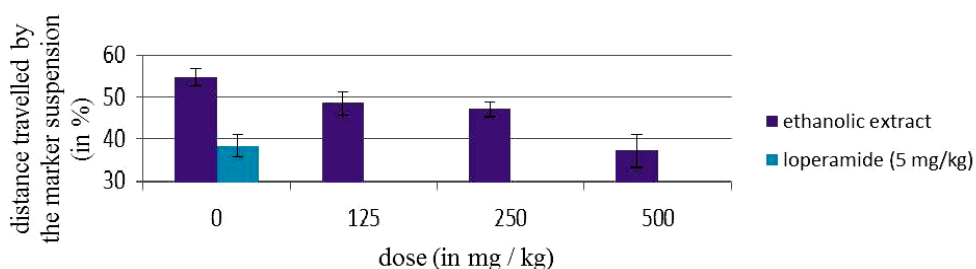

Figure 2: The figure showed the effect of ethanolic extract from *E. ferrugineum* and loperamide on gastrointestinal motility in SWISS mice, expressed as percentage of the small intestine travelled by the faeces containing a charcoal meal (means ± s.e.m; n=3).

3.4. Cytotoxic activity of fraction IFA, compounds 1 and 2

Cytotoxicity values (CC50) of *ent*-kauran-16β, 17-diol (compound 1), *ent*-kauran-15α, 16β, 17-triol (compound 2) and the polar fraction IFA on vero cell lines were determined by MTT assay but only compound 1 showed cytotoxic activity with a CC50 value of 67.04±1,257 µg/mL (table 2). IFA and compound 2 did not exhibit significant cytotoxicity (CC50 above 200 µg/mL).

Table 2: The title showed the antibacterial and cytotoxic activities of extracts, fractions and compounds from *E. ferrugineum.*

		MIC (µg/mL)					CC50 (µg/mL)
		S. typhi	*S. enterica*	*S. aureus*	*S. enteritidis*	*S. typhimurium*	Vero cells
Crude extract	**Ethanolic extract**	**125**	**125**	**250**	**>500**	**>500**	**-**
Fractions	Hex extract	>250	>250	250	-	-	-
	DCM extract	250	>250	>250	-	-	-
	AcOEt extract	>250	>250	>250	-	-	-
	IFA	100	100	100	-	-	> 200
Compounds	*ent*-kauran-16β, 17-diol	100	100	100	-	-	67.04±1,257
	ent-kauran-15α, 16β, 17-triol	100	100	100	-	-	> 200
Control	Ciprofloxacin	<1	<1	<1	<1	<1	-

MIC: Minimal Inhibitory Concentration; CC50: 50% Cytotoxic Concentration.

4. DISCUSSION

Diarrheas still a global problem of public health and many people have customarily used plant(s) or plant(s) derived preparations to combat these disorders [1-4]. Only few of these preparations have been controlled scientifically. Thus the decoction of leaves of *E. ferrugineum* is used in Malagasy traditional medicine to treat dysentery but no biological studies on this specie have been reported in the literature. In the present study, we have performed in vivo and in vitro assays in order to evaluate the inhibition of intestinal motility, antibacterial activities and cytotoxic action of extracts from the leaves of this specie. The main aim was to justify the widespread traditional use of *E. ferrugineum* and to investigate the potential activity of crude extracts and isolated compounds against some causative agents of diarrheal Disease.

It is known that the majority of herbal medicines used as antidiarrheal delay gastrointestinal processes and suppress gut motility [2]. In addition, antimicrobial activity plays a pivotal role in the treatment of diarrhoea caused by pathogens such as *Salmonella and Staphylococcus* [4]. Since the ethanolic extract of *E. ferrugineum* caused a dose-dependent decrease on intestinal motility and was active against some enteric bacteria, which would validate its use as traditional remedy. Indeed the percentage of the small intestine travelled by the charcoal meal can be used as parameter for the intestinal motility measurement [16].

As the determination of the active components provides baseline information on potential usage of plant extracts [4], we focused our work on fractions and compounds from the ethanolic extract. Tannins have been linked with antimicrobial activities [16, 17], not surprising therefore to observe activities in the fraction IFA since it probably contained tannins as major constituents. Indeed the presence of tannins was confirmed by positive reaction with ferric chloride. Moreover, Kaurane diterpenes are mostly found in different plant species belonging to Erythroxylaceae [18, 19]. Diverse biological activities have been described for the kaurane diterpenes and many of them, including antimicrobial, antiparasitic, cytotoxic, inductor of apoptosis and antispasmodic activities are referred to in the review published by Pablo *et al.,* (2007) [20]. Two *ent*-kauranes namely the known *ent*-kauran-16β, 17-diol and the new ent-kauran-15α, 16β, 17-triol were isolated from the DCM extract of *E. ferrugineum*. There were no differences between the sensibilities of tested bacteria to these isolated kauranes but only one (*ent*-kauran-16β, 17-diol) presented a cytotoxic activity. These results showed that the presence of an additional hydroxyl at C15 position in *ent*-kauran-15α, 16β, 17-triol has decreased its cytotoxic effect. The cytotoxicity of many kauranes is largely documented in literature [20, 21, 22]. However, in vivo data are necessary in determining the potential usefulness of these products.

5. CONCLUSION

The findings of this study represent a significant contribution to the chemistry, pharmacology and toxicity of *E. ferrugineum* specie, and suggest that this plant constitutes a promising treatment to fight enteric bacteria and motility-related diarrhea. This study is the first to report the inhibitory effect on intestinal motility, antibacterial activity and cytotoxicity of leaves of *E. ferrugineum* and these biological activities may explain the benefits of using this plant in the treatment and management of dysentery. Via this study, a known *ent*-kauran-16β, 17-diol and a new *ent*-kauran-15α, 16β, 17-triol, were isolated from *E. ferrugineum* for the first time. This study also introduces tannins and *ent*-kaurane diterpenes as potential antibacterial principles of this plant.

6. REFERENCES

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