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BIOASSAY-GUIDED FRACTIONATION, MECHANISM OF ACTION AND TOXICOLOGICAL EVALUATION OF ANTI-NOCICEPTIVE PRINCIPLES OBTAINED FROM *ERYTHROPHLEUM IVORENSE*

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ABSTRACT

The present study describes the bioactivity guided identification of anti-nociceptive compounds found in stem bark extract of *Erythrophleum ivorense*. The stem bark of the plant was air-dried, grounded and macerated using absolute methanol which was then filtered. The filtrate was evaporated using rotary evaporator and Crude Methanol Extract (CME) was obtained. Bioactivity-guided fractionations of CME was performed using partitioning, thin layer, and open column chromatography. Partitioned fractions (20 mg/kg), E. ivorense-fractions (EiF-1 to EiF-6) (20 mg/kg), EiF-1 (5, 10, 20 mg/kg), fractions (F1 to F8) (20 mg/kg), and semi-purified fractions (50 mg/kg) were screened for anti-nociceptive and anti-inflammatory activities. Animals in each group were administered with extract or fractions intraperitoneally. The mechanism of action and toxicological evaluation of semi-purified fraction were also studied. Data were analyzed using One-Way Analysis of Variance (ANOVA) and the level of significance of the data were taken at $p<0.05$. Partitioned CME yielded butanol (34.7 g), ethyl-acetate (27.0 g), dichloromethane (17.2 g), and n-hexane (6.1 g) extracts. Chromatographic fractionation of ethyl acetate yielded six fractions (EiF-1 to

EiF-6). Purified fraction (EiF-1), the only fraction with anti-nociceptive activity yielded sub-fractions (F1 to F8). Screened fractions (F1 to F8) produced F3 as the fraction with highest activities. Further purification of F3 yielded semi-purified fractions coded as F3C1, F3C2, F3C3, F3C4, F3C5-mother liquor (F3C5-MQ) and F3C5-crystal. F3C5-MQ produced a central anti-nociceptive effect while F3C2 indicated peripheral effect. Atropine significantly reversed the anti-nociceptive effect of F3C5-crystal in both phases of formalin test. At the doses studied, the fraction was fairly non-toxic. In conclusion, the semi-purified fractions exhibited peripheral and central anti-nociceptive activities. The central anti-nociceptive effect of F3C5-MQ may be mediated via muscarinic receptor.

KEYWORDS: *Erythrophleum ivorense*, Anti-nociceptive, chromatography, partition.

INTRODUCTION

Natural products have been recognized as a source of medicines since ancient times across the world for treating and preventing human diseases.[1] They have been derived from various sources including plants, microorganisms, marine organisms, vertebrates and invertebrates.[2] Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drugs discoveries because of the unmatched availability of chemical diversity.[3] Despite major scientific and technological progress in combinatorial chemistry, drugs derived from natural products still make an enormous contribution to drug discovery today. Natural sources possess chemical diversity and therefore serve as an important reservoir of bioactive leads in the development of new drugs by providing novel templates for new drugs, as well as patterns for structural modifications to produce more potent and safer drugs.[4] Natural products and their derivatives represent over 60% of all drugs clinically used worldwide where natural products from medicinal plants alone contribute to 25% of the total drugs.[5] Natural products and related drugs are reportedly used for example as antibacterial, anticancer, anticoagulants, anti-nociceptive, anti-parasitic and immunosuppressant agents to treat 87% of all category of diseases.[2] More than 28% of new chemical entities introduced into the markets are derived from natural products.[6] A good proportion of the world population particularly those living in developing countries like Nigeria depend mostly on natural products for their health needs.

There are several traditional claims regarding the usefulness of Erythrophleum ivorense in pain, inflammation and convulsion.[7] Previous study on this plant revealed sedative and anticonvulsant effect,[8] anti-nociceptive and ant-inflammatory effect of methanol extract of

E. ivorense was also reported in our previous study.[9] This study thereby designed to investigate the bioactive compounds responsible for the reported antinociceptive effect and also to determine the probable mechanism of action.

MATERIALS AND METHODS

Plants Materials

Collection and identification of plant materials

Fresh stem bark of *Erythrophleum ivorense* was collected from a village in Iwo Local Government area of Osun State, South West, Nigeria. The plant was identified by Mr Bernard Omomoh of the herbarium unit, Department of Botany, Obafemi Awolowo University Ile-Ife, where voucher specimen was deposited (voucher number 16878).

Preparation of plants material

1kg of the stem bark of *Erythrophleum ivorense* was dried and reduced to coarse powdery form using electric blending machine. Air-dried powder of *Erythrophleum ivorense* was macerated in 5litres of 75% methanol and placed on a mechanical shaker for 24 hours to extract. It was then filtered and concentrated to dryness using Buchi Rota Vapour R110. The crude extract obtained were preserved in the freezer until ready for use.

Animal Materials

The animals used in the study were mice (Swiss strain, male, 20-30g). They were obtained and housed in Animal House, College of Health Science Ososgbo, Ladoke Akintola University of Technology Ogbomoso. They were kept in standard cages with a maximum of six animals in a cage. The animals were housed under standard environmental conditions in the Department of Pharmacology and Therapeutics, College of Health Science Ososgbo. Animals were fed with standard diet (Ladokun feeds Ltd, Ibadan) for two weeks prior to experimentation and allowed free access to clean drinking water.

Acute Toxicity of CME

The intraperitoneal median lethal dose (LD50) of the plant extract was conducted and calculated in mice according to the method of modified.[10] Lorke (1983) using thirteen (13) mice. In the initial phase, three (3) groups of three mice each was treated with the extract at doses of 10mg/kg, 100mg/kg and 1000mg/kg body weight intraperitoneally and observed for signs of toxicity and death for 24 hours. In the second phase, animals were divided into four (4) groups each containing a mouse and were injected intraperitoneally with four more

specific doses of the extract at 10mg/kg, 20 mg/kg, 40 mg/kg and 80 mg/kg based on the result of the first phase. The LD50 was calculated as the square root of the product of the maximum dose for all survival and minimum dose for all death.

Bioactivity-guided fractionation of CME

A total of 85g of Crude methanol extract of *Erythrophleum ivorense* was suspended in distilled water and then partitioned between n-hexane and water using separating funnel. At this stage of partitioning, the organic (n-Hexane) phase was pooled and was concentrated using rotary evaporator to yield a dark brown extract. This procedure was carried out for dichloromethane, ethyl-acetate and butanol, to obtain the n-hexane fraction (n-HF; 6.1 g; 7.1% w/w), dichloromethane fraction, (DCMF; 17.2g; 20.2w/w), ethyl acetate fraction (EAF; 27.0 g; 31.8% w/w) and butanol fraction (BF; 34.7 g; 40.8% w/w). The antinociceptive profile of the extract and fractions were determined by means of the selected assays. Bioactivity-guided studies on the fractions showed that EAF produced the highest activities in all the experimental models.

Column chromatography of EAF

EAF (10.0 g) was separated in a silica gel column (60 cm in length and 7.5 cm in diameter) eluted with gradient mixtures of ethyl-acetate and methanol and the fractions were collected in aliquots of 10 ml in test tubes. The collected fractions were subsequently pooled into six broad fractions, EiF-1 – EiF-6, based on the similarity of constituents visualized on silica gel pre-coated thin layer chromatography (TLC) plates developed with mixtures of methanol and dichloromethane. Further activity-guided studies on the fractions showed that EiF-1 (1.25 g; 12.5%w/w) was the only fraction with pharmacological activities and since there wasn't any study carried out on *Erythrophleum ivorense* in order to establish the chemical principles responsible for the reported activities. Hence, EiF-1 sub-fraction was therefore selected for further fractionation to isolate the active ingredient.

One one-tenth gram (1.1 g) of EiF-1 was dissolved in dichloromethane (5 ml), adsorbed on silica gel (20 g) and triturated completely. It was then allowed to stand or air-dried for about one hour before loading onto the column (15 mm x 300 mm). The component was eluted with gradient mixtures of n-hexane and ethyl acetate and the fractions were collected in aliquots of 15 ml in test tubes. The collected fractions were subsequently pooled into eight components (F1 to F8) based on the similarities of the constituents as visualized on silica gel precoated thin layer chromatographic plates. Fractions were concentrated using a rotary

evaporator. Activity guided studies on the fractions demonstrated significant activity with the F 3 fraction necessitating further fractionation.

Fraction (F 3, 0.5 g) was dissolved in 2 ml of dichloromethane and the solution later adsorbed on silica gel (10 g). It was then triturated completely and air-dried for about one hour before parking onto the column. Components eluted with gradient mixtures of n-hexane and ethyl acetate, the collected fractions were pooled into five semi-purified fractions (F3C1, F3C2, F3C3, F3C4 and F3C5). While concentrating F3C5 two substances emerged a) a whitish substance which crystallized (F3C5-crystal) out of solution; soluble in methanol and insoluble in dichloromethane and; b) the main compound (mother liquor) (F3C5-MQ) a yellowish green, oily mixture. The concentrated fractions obtained were suspended in 5 % DMSO prior pharmacologic testing. Semi-purified fractions (F3C1, F3C3, F3C4, and F3C5-crystal) obtained from the purification of F3 were too low in yield for in-vivo tests, so semi-purified fractions F3C2 and F3C5-MQ were subjected to pharmacologic testing since they were enough for the studies.

Analgesic Studies

Effect of fractions, subfractions and semi-purified fractions on acetic acid-induced writhing test

Writhing in mice was induced according to the method described by Koster *et al*., 1959[11] The mice were randomly divided into nine groups, group 1 received normal saline (10ml/kg, i.p) while one of the partitioned fractions, subfractions (EiF-1 to EiF-6) or semi-purified fractions (F3C2 and F3C5-MQ) (20 mg/kg, i.p) was given to groups (2-9). Each mouse was given 0.6% aqueous solution of acetic acid and then placed in an observer box. The animals were pretreated for 30minutes before acetic acid administration. Nociception was evaluated by counting the number of abdominal constriction for 20 minutes after administration of acetic acid. Percentage protection against abdominal constriction was taken as an index of analgesia Acetylsalicylate (ASA, 150mg/kg, i.p), served as reference drug.

Effect of fractions, sub-fractions and semi-purified fractions on Hot plate

In this experiment the pain episode was induced by thermal stimulus as described by Hunskaar *et al.*, 1986.[12] The mice were randomly divided into nine groups, group 1 received normal saline (10ml/kg, i.p) while one of the fractions, sub-fractions or semi-purified fractions (20mg/kg, i.p) was given to groups (2-9). The animals were pretreated for 30minutes, each mouse was placed in a hot plate maintained at 55±0.5º Nociception was

evaluated when the animal began to lick its hind paw or attempt to jump out of the hot plate. The time taken to lick the hind paw was taken as reaction time Anti-nociception was expressed as the increased in reaction time. Morphine sulphate (5mg/kg, i.p) served as reference drug.

Effect of fractions, sub-fractions and semi-purified fractions on Formalin-induced paw lick

In this experiment paw lick in mouse was induced by formalin according to the method described by Hunskaar and Hole 1987.[13] The mice were randomly divided into nine groups, group 1 received normal saline (10ml/kg, i.p) while one of the fractions or compounds (20mg/kg, i.p) was given to groups (2-9). Each mouse was injected at right hind paw with formalin (1%, 2ul). The animals were pretreated for 30minutes before injection of formalin. Nociception was evaluated when the animal began to lick its paw at 0-5 minutes (early phase) and 20-30 minutes (late phase). Anti-nociception was expressed as the reduction in duration of paw lick. Morphine sulphate (5mg/kg, i.p) served as reference drug.

Anti-inflammatory Activity

Effect of fractions, sub-fractions and semi-purified fractions on carrageenan-induced paw lick

Pedal inflammation in mice was induced according to the method described by Winter *et al*., 1962.[14]

The mice were randomly divided into nine groups, group 1 received normal saline (10ml/kg, i.p) while one of the fractions, sub-fractions or semi-purified fractions (20mg/kg, i.p) was given to groups (2-9). Edema was induced by sub-plantar injection of 0.1ml of freshly prepared 1% carrageenan into the right hind paw of each mouse. The animals were pretreated for thirty minutes before sub-plantar injection (0 hour). Inflammation was evaluated by increased in paw volume, paw volume was measure at 0 and 3 hours after carrageenan injection using cotton thread. Anti-inflammation was expressed as reduction or increased in percentage inhibition of paw volume. Indomethacin (5mg/kg, i.p) served as reference drug. The percentage of inhibition for each mouse was obtained as follows:

$$\text{Percentage inhibition} = \frac{(Vt - Vo)\ \text{control} - (Vt - Vo)\ \text{treated}}{(Vt - Vo)\ \text{control}} \times 100$$

Where Vo, is the paw volume before injection of carrageenan.
and Vt, is the paw volume after injection of carrageenan.

Assessment of possible mechanism of action of F3C5-MQ

To investigate some possible mechanism of action by which the F3C5-MQ exerts its anti-nociceptive activity, mice were pretreated with antagonists of notable mediators of the nociceptive pathway in the formalin test.

a) Opioid system

Naloxone (2 mg/kg) was administered intraperitoneally as described by Moniruzzaman et al. (2015);[15] thirty minutes after administration of the F3C5-MQ fraction (20 mg/kg, i.p) or vehicle (10ml/kg, i.p) or morphine (5 mg/kg, i.p). The nociceptive response to formalin injection was then scored 30 minutes after administration of crystal or vehicle or morphine.

b) Muscarinic cholinergic system

Atropine (5 mg/kg/body weight) a nonselective muscarinic receptors antagonist was administered[16] (Eric and Wonder, 2011) intraperitoneally prior to the administration of the F3C5-MQ (20 mg/kg, i.p) or vehicle (10 ml/kg, i.p) or morphine (5 mg/kg, i.p); nociceptive response to formalin injection was scored 30 minutes after administration.

c) Adenosinergic system

Theophylline (10 mg/kg, i.p) a nonselective adenosine receptors antagonist was administered to a group of mice 30 minutes, after administration of F3C5-MQ (20 mg/kg, i.p) or vehicle (10 ml/kg, i.p) or morphine (5 mg/kg, i.p). The nociceptive response to formalin injection was scored 30 minutes after administration of the test substance.

Sub-chronic administration

Fifteen adult rats randomly assigned to three groups of 5 rats each were used to study the effects of subchronic administration of E.ivorense crude extract and fractions on biochemical and histological parameters of liver and kidney injury. Animals in group 1 received vehicle (5 % DMSO), groups 2 and 3 were administered F3C5-crystal at 25 and 50 mg/kg while animals in group 4 were administered CME at 50 mg/kg. Treatments were administered orally at 10 ml/kg via an oral cannula for 28 days (Amole et al., 2006). Animals in all groups weighed weekly using a weighing balance (Mettler Toledo Type BD6000, Greifensee, Switzerland), and observed closely for changes in their physical characteristics, food, and water intake. At

the end of the experimental period, animals were anesthetized with diethyl-ether, blood taken via a cardiac puncture for estimation of serum alkaline phosphatase, aspartate transaminase, alanine transaminase, urea and total protein. Animals were then sacrificed by cervical dislocation following which the liver and kidneys were dissected out and processed for general histological study.

Biochemical tests

Blood samples were collected into lithium heparin bottles and centrifuged at 3500 rpm for 10 minutes using a general purpose centrifuge (Uniscope SM112, Surgifriend Medicals, England) for separation of serum. The serum was assayed either immediately or stored at 20^{o}C. Alanine, aspartate transaminase (ALT, AST) levels using colorimetric methods, colour change measured at 546 nm was used to assess concentration of oxaloacetate hydrazone and the pyruvate hydrazones formed with 2,4-dinitrophenylhydrazine. Alkaline phosphate (ALP) was measured spectrophotometrically using appropriate assay kit following the instruction of the manufacturer. Serum urea was measured using colorimetric reaction with urea assay kits. Total protein level was measured according to the direct Biuret method. All kits were purchased from RANDOX (UK).

Histological procedure

At the end of the experimental period rats were observed for changes in their physical characteristics and then sacrificed by cervical dislocation. The liver and right kidney were dissected via an abdominal midline incision Organs were observed grossly, fixed, sectioned and processed and stained with haematoxylin and eosin for general histology. The liver and kidneys were fixed in 10% formol saline by total immersion for a period of 24 hours following which they (liver and kidneys) were sectioned (3-5mm thickness) The tissues were dehydrated at room temperature by passing them through increasing concentrations of ethanol. Dehydrated tissue was then cleared in two changes of xylene (each lasting one hour) at room temperature following which they were infiltrated n two changes of molten paraffin wax at 60 °C (one hour each) and then finally embedded in paraffin wax using a multiblock plastic embedding mould. The paraffinized tissue blocks were mounted for sectioning (5ụm thickness) on a rotary microtome (Bright B5143, Huntington, England). Sections were then transferred into a water bath (40°C) to enable spreading of the folded tissue sections. These sections were then mounted on clean glass slides, dried to 40°C using a slide drier to ensure adherence of tissue section to the slides.

Staining procedures

To remove paraffin (which is poorly permeable to stains), sections were deparafinized in two changes of xylene (2 minutes each); following which the sections are passed through 2 changes of alcohol (absolute) at 4 minutes each. Sections were hydrated in descending concentrations of alcohol allowing for staining with an aqueous dye. Haematoxylin and eosin staining was carried out as described by Drury and Wallington (1980).[17] Following hydration of tissue sections in descending concentrations of alcohol up to water, tissue sections are then rinsed in distilled (3 minutes) and stained in haematoxylin for 15- 20 minutes. Sections are rinsed in water (2-3 minutes) to remove excess hematoxylin, differentiated in acid alcohol (2-3 seconds); rinsed again to remove excess acid and then counter stained in alcoholic eosin (3- 4 minutes). Excess eosin stain is washed off by rinsing under running water. Sections are dehydrated through ascending grades of ethanol, cleared in xylene and mounted synthetic resin medium (DPX) using glass cover slips.

Photomicrography

Slides were examined under a Carl Zeiss microscope (Axioscope 40, Germany) with a digital camera attached. Photomicrographs of representative sections of the liver and kidney were observed and captured at different magnifications.

Statistical analyses

Results of parametric tests were expressed in terms of mean±SEM. In the assays involving comparison of more than two means, one-way ANOVA was used, followed by the Student Newman-Keuls test when statistical difference was detected among the groups. P-values less than 0.05 were considered statistically significant

RESULTS

Acute Toxicity Test

Acute toxicity study showed that the crude methanol extract is fairly toxic. Some of the signs of toxicity observed were reduced motor activity, ataxia, scratching and increased respiratory rate and death. The crude methanol extract showed that 89.4mg/kg body weight killed 50% of population.

Effect of fractions (BF, EAF, DCMF, n-HF), sub-fraction and semi-purified fractions of *Erythrophleum ivorense* on Acetic Acid-Induced Writhing

Table 1 showed that BF, EAF, DCMF, EiF-1, F3C5-MQ and F3C2 produced significant ($p<0.05$) inhibition of acetic acid-induced writhing at the rate of 87.1, 89.4, 65.0, 68.2, 41.2 and 22.4% respectively compared with control. And pretreatment with acetylsalicylic acid (ASA, 150mg/kg) significantly inhibited acetic acid induced writhing by 85.8% lower in potency to BF and EAF.

N-HF (20mg/kg) did not significantly ($p>0.05$) inhibit acetic acid-induced contraction of the stomach of mice.

Table 1: Effect of fractions (BF, EAF, DCMF, n-HF, EiF-1), sub-fraction and semi-purified fractions of *E. ivorense* on acetic acid induced writhing.

Pretreatments	Doses (mg/kg)	Number of writhes**	% Inhibition
Control	0	31.7±2.30	0
BF	20	4.1±0.40	87.1*
EAF	20	3.6±0.22	89.4*
DCMF	20	11.9±0.45	65.0*
n-HF	20	30.8±0.40	9.1
EiF-1	20	8.6±0.25	68.2*
F3C2	20	19.5±0.75	41.2*
F3C5-MQ	20	25.7±0.24	22.4*
ASA	150	4.8±0.30	85.8*

**Values are recorded as means±SEM (n=5).

*Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests.

Effects of fractions (BF, EAF, DCMF, n-HF, EiF-1), sub-fraction and semi-purified fractions of *E. ivorense* on thermal stimulus-induced by hot plate in mice

Table 2 showed that BF, EAF, EiF-1and F3C5-MQ significantly ($p<0.05$) prolonged the reaction time (seconds) in hot plate test, EiF-1and F3C5-MQ shows greater effect even more than the standard drug at the same doses to noxious heat, while DCMF (2.8±0.40), HF (2.8±0.5) and F3C2 (2.5±0.27) did not increase reaction time to heat compared with control (2.2 ±0.20).

Table 2: Effect of fractions (BF, EAF, DCMF, n-HF), sub-fraction and semi-purified fractions of *E. ivorense* on thermal stimulus-induced by hot plate induced writhing.

Pretreatments	Doses (mg/kg)	Reaction time(s)**
Control	0	2.2±0.20
BF	20	6.3±0.50*
EAF	20	6.6±0.45*
DCMF	20	2.8±0.40
n-HF	20	2.8±0.50
EiF-1	20	14.5±0.75*
F3C5-MQ	20	12.1±0.21*
F3C2	20	2.5±0.27
Morphine	5	10.2±0.25*

**Values are recorded as means±SEM (n=5). *Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests

Effects of fractions (BF, EAF, DCMF, and n-HF), sub-fraction and semi-purified fractions *Erythrophleum ivorense* on Formalin induced paw licks

BF, EAF, EiF-1 and F3C5-MQ produced significant ($p<0.05$) inhibition of acute (36.1, 42.7, 40.8, 24.5%) and chronic (49.2, 55.3, 18.0, 61.2%) phases of formalin-induced paw licks respectively. While, DCMF and F3C2 produced significant ($p<0.05$) inhibition of inflammatory phase in the rate of 63.7, and 65.6% but not the neurogenic (0.1, 0.1%) pain induced by formalin respectively when compared with the control. (Table 3).

Table 3: Effects of fractions, sub-fraction and semi-purified fractions on Formalin-induced paw licks.

Pretreatments	Doses (mg/kg)	Latency of paw licks**			
		First phase	%inhibition	Second phase	%inhibition
Control	0	55.1±0.45	0	146.1±0.95	0
BF	20	35.2±0.30	36.5*	74.2±0.75	49.2*
EAF	20	31.6±0.42	42.7*	65.3±0.25	55.3*
DCMF	20	54.5±0.33	0.1	53.0±0.32	63.7*
n-HF	20	54.7±0.43	0.1	146.3±0.92	0
EiF-1	20	32.6±0.82	40.8*	119.8±0.45	18.0*
F3C5-MQ	20	41.6±0.97	24.5*	56.7±1.65	61.2*
F3C2	20	54.7±0.34	0.1	50.3±0.56	65.6*
Morphine	5	30.3±0.27	45.0*	46.0±0.42	68.4*

**Values are recorded as means±SEM (n=5). *Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests

Effect of fractions (BF, EAF, DCMF, and N-HF), sub-fraction and semi-purified fractions on carrageenan induced rat paw edema at 3 hours (peak edema period)

Injection of carrageenan into the hind paw induced a progressive edema reaching its maximum at 3 hours. BF, EAF, DCMF, EiF-1, F3C5-MQ and F3C2 significantly ($p<0.05$) inhibited the paw edema sizes at the rate of 58.3, 60.0, 54.5, 50.0, 66.7, 41.7% respectively, while, N-HF did not significantly ($p>0.05$) inhibit paw edema sizes compare with control. (Table 4).

Table 4: Table Effects of fractions, sub-fraction and semi-purified fractions on carrageenan-induced mice paw edema.

Pretreatments	Doses (mg/kg)	Paw sizes (cm)**		%Inhibition
		Before carrageenan	After carrageenan	
Control	0	2.0±0.20	3.2±0.22	0
BF	20	1.9±0.22	2.4±0.23	58.3*
EAF	20	2.0±0.20	2.6±0.29	60.0*
DCMF	20	2.0±0.20	2.5±0.50	54.5*
n-HF	20	2.0±0.20	3.1±0.45	8.3
EiF-1	20	2.2±0.17	2.8±0.20	50.0*
F3C5-MQ	20	2.1±0.18	2.5±0.41	66.7*
F3C2	20	2.0±0.20	2.7±0.21	41.7*
Indomethacin	5	1.9±0.21	2.3±0.25	68.7*

**Values are recorded as means±SEM (n=5). *Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests.

Possible mechanism of action of F3C5-MQ

Atropine reversed the anti-nociceptive effect of F3C5-MQ in both phases of formalin test significantly, also abolished the anti-nociception caused by morphine in the second phase but not in the first phase significantly (Table 5).

Anti-nociceptive effect of morphine was completely antagonized by naloxone but failed to modify the anti-nociception due to semi-purified fraction. In contrast, treatment with theophylline did not significantly modify the anti-nociceptive effect caused by F3C5-MQ or morphine against both phases of formalin-induced nociception (Table 6 and 7, respectively).

Table 5: Effect of atropine on anti-nociceptive effect of F3C5-MQ in formalin test.

Pretreatment	Doses (mg/kg)	Duration of paw licking (s)**			
		First phase	%inhibition	second phase	%inhibition
Control	0	67.5±2.31	-	110.1±0.95	-
F3C5-MQ	50	65.8±1.42*	2.5	108.6±1.20*	1.4
Morphine	5	26.5±0.67	60.7	109.2±2.70*	0.8

**Values are recorded as means±SEM (n=5). *Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests.

Table 6: Effect of naloxone on anti-nociceptive effect of F3C-5-MQ in formalin test.

Pretreatment	Doses (mg/kg)	Duration of paw licking (s)**			
		First phase	%inhibition	second phase	%inhibition
Control	0	66.9±0.78	-	106.1±1.45	-
F3C5-MQ	50	45.7±0.85	31.7	59.3±1.37	44.1
Morphine	5	66.1±0.67*	1.2	104.6±1.47*	1.4

**Values are recorded as means±SEM (n=5). *Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests.

Table 7: Effect of theophylline pretreatment on anti-nociceptive effect of F3C5-MQ on formalin test.

Pretreatment	Doses (mg/kg)	Duration of paw licking (s)**			
		First phase	%inhibition	second phase	%inhibition
Control	0	67.2±1.40	-	107.1±1.45	-
F3C5-MQ	50	48.7±1.24	27.5	56.1±2.05	47.6
Morphine	5	26.9±0.58	60.0	47.6±0.97	55.6

**Values are recorded as means±SEM (n=5). *Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests.

Effects of subchronic administration of *E. ivorense* crude extract and fractions

Effect of CME and F3C5-MQ on the body weight

The changes in the mean values of the initial and final body weights of the rats treated with 25 mg/kg and 50 mg/ kg of semi-purified fraction are shown in Table 8. The result revealed no statistical significant changes in the body weights of animals treated at 25 mg/kg and 50mg/kg body weight compared with control. However, there was a significant reduction in the body weight of animals treated with CME at a dose of 50 mg/kg.

Table 8: Effects of F3C5-MQ and CME on the body weight of mice treated at dose 50mg/kg.

Treatments	Dose (mg/kg)	Weights**		Change in weights
		Initial	Final	
Control (2% DMSO)	0	180.6±2.15	189.8±1.87	9.2
F3C5-MQ	25	185.1±1.90	194.6±2.10	9.5
F3C5-MQ	50	182.3±2.05	191.5±0.93	9.2
CME	50	185.7±2.26	184.6±1.64*	-1.1

**Values are recorded as means±SEM (n=5). *Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests.

Effect of CME and F3C5-MQ on biochemical parameters

Table 9 shows the results of the effect of sub-chronic administration of extract and fractions on biochemical parameters. Serum levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total protein, urea and creatinine did not record significant alterations in F3C5-MQ treated groups as compared to the control group. However, there was a significant increase in the levels of AST, ALT, ALP, total protein, urea and creatine following administration of 50 mg/kg dose of CME compared to control.

Table 9: Effect F3C5-MQ and CME on Biochemical parameters.

Treatment	Dose (mg/kg)	Biochemical parameters					
		AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total protein(mg/dl)	Creatine (mg/dl)	Urea (mg/dl)
Control	0	145.1±2.06	99.33±2.20	3.04±0.50	61.98±2.10	92.93±2.60	8.07±0.22
F3C5MQ	25	144.8±2.06	98.90±2.06	2.90±2.06	61.20±2.06	92.50±2.06	7.80±2.06
F3C5MQ	50	141.6±1.72	98.1±1.53	3.12±0.95	59.85±1.53	91.87±3.25	8.05±0.65
CME	50	185.1±3.4*	123.5±1.76*	6.09±0.97*	64.20±3.14*	95.07±1.35*	9.12±0.98*

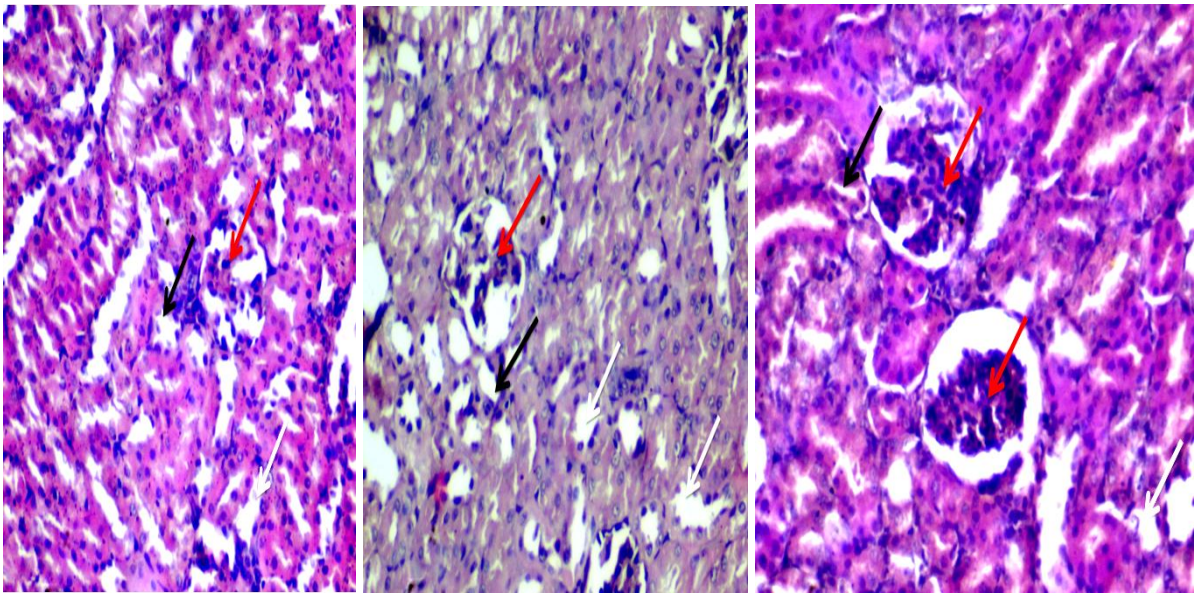
**Values are recorded as means±SEM (n=5). *Values are statistically significant ($p<0.05$) in relation to control. One way ANOVA follow by Newman-Keuls Multiple Comparison tests.

Histomorphological study

Effect of *E. ivorense* crude extract and fractions on kidney morphology

Figure 1 (a-c) shows the effect of sub-chronic administration of *E. ivorense* semi-purified fractions on the morphology of the rat kidney. Gross examination revealed normal kidneys taken from animals in all groups studied. Histological examination of kidney slides from animals in the control group (Figure 1 a) revealed well demarcated medulla and cortex, uniform sized Bowman's capsule and space, glomeruli with deeply staining nuclei, collecting ducts, renal tubules and blood vessels all in keeping with normal histology. In animals administered F3C5-MQ at 25 mg/kg (Figure 1 b) the kidneys had mildly shrunken glomeruli,

dilated Bowman'scapsule and space, oedematous tubular cells and dilated tubular lumen. In animals administered semi-purified fraction at 50 mg/kg (Figure 1c), the kidneys had mildly shrunken glomeruli and oedematous tubular cells.



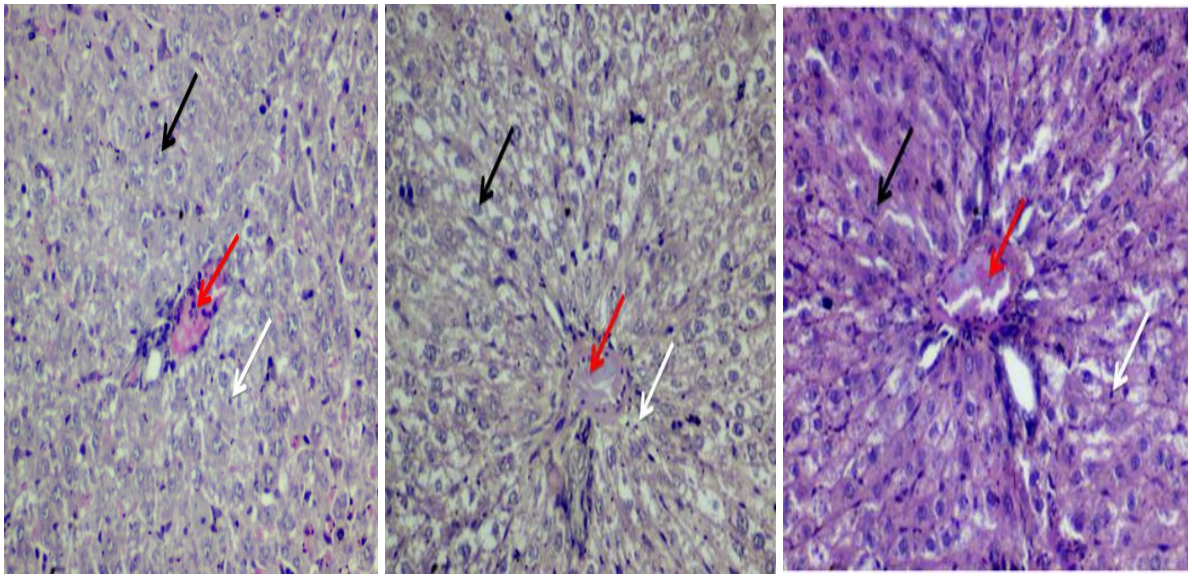
(a) **(b)** **(c)**

Figure 1 a, b, and c: Representative photomicrographs of hematoxylin and eosin (H&E) stained sections of the rat kidney showing glomerulus (red arrow), proximal tubule (black arrow) and distal tubule (white arrow). H&E stain (Mag×160).

Effect of F3C5-MQ on liver histology

Figure 2 (a-c) shows the effect of subchronic administration of *E. ivorense* semi-purified fractions on the morphology of the rat liver. Gross examination revealed normal liver taken from animals in all groups studied. Histological examination of liver slides from animals in the control group (Figure 2 a) revealed normal liver architecture showing radially arranged cords of hepatocytes with intervening sinusoids and normal central veins and terminal venules; features are in keeping with normal hepatic histology.

In groups administered F3C5-MQ at 25 (Figure 2 b) showing dilation of the central vein (red arrow), hepatocyte (black arrow) and Kupffer cell (white arrow) infilteration and 50 mg/kg (Figurer 2 c) dilatation of the central vein, and Kupffer cell were observed.



(a) **(b)** **(c)**

Figure 2 a, b, and c: Representative photomicrographs of hematoxylin and eosin (H&E) stained sections of the rat liver in the control group showing the central vein (red arrow), hepatocyte (black arrow) and Kupffer cell (white arrow). H &E stain (Mag×160).

DISCUSSION

Extraction is a fundamental step in drugs discovery of a new drugs from medicinal plant and is a process of separation of medicinally active constituents from their sources.[18] For extraction purposes classical techniques like maceration, decoction, percolation, soxhlet, microwave-assisted extraction, ultrasound extraction, and supercritical fluid extraction have been used.[19] Different solvent such as methanol, ethanol, acetone alone or with water, ethyl-acetate have usually been used for classical extraction.[19] The use of an appropriate extraction method, plant material and solvent ensure a good quality extract.[18] Appropriately 2.5kg of plant powder was extracted by maceration with 5litre of 75% methanol and then dried using rotary evaporator afforded 85.6g, 3.42%w/w. Methanol was used for extraction in this study because, it give the positive test for most of the constituents during phytochemical screening and also, it has very high extractive value as it can separate both the polar and non-polar compound present.

Some of the physical signs of toxicity (acute) observed in this study were reduced motor activity, ataxia, scratching, increased respiratory rate and death. Acute toxicity test gives clues on the range of doses that could be toxic to the animals, it could also be used to estimate the therapeutic index of drug and xenobiotics.[20] It is defined as dose that killed 50% of

population of experimental animals. The higher the values of LD50 for a substance, the relatively safer the substance assumed to be. The median lethal dose (LD50) of the extract was found to be 89.4mg/kg and this indicates that the extract is fairly toxic compared with toxicity classification.[21]

Acetic acid mouse writhing is widely used animal model for routing screening of compound with peripheral analgesic activity.[22,23,24] The writhing response is considered to be a visceral inflammatory pain.[25,26] Acetic acid is a chemical irritant that produces tissue necrosis of the peritoneal region accompanied by the release of chemical mediators such as bradykinin, prostaglandin, histamine, substance P, vasoactive polypeptide, which cause pain either by activation or sensitization of nociceptors that encode tissue injury[27,28] whilst the hot plate or tail immersion model of pains is generally used to detect centrally acting analgesics.[12] Fractions (BF, EAF, DCMF and EiF-1) and semi-purified fractions (F3C2 and F3C5-MQ) produced significantly ($p<0.05$) anti-nociceptive effects in chemically induced nociceptive pain stimuli in mice. The inhibitory effect exhibited by fractions (BF, EAF and DCMF) and semi-purified fractions (F3C2 and F3C5-MQ) against nociceptive action of acetic acid in mice may suggest the presence of phytochemically active substances with analgesic property indications suggesting peripheral action. This suggestion further supported by the finding that fractions (BF, EAF and DCMF) and (F3C2 and F3C5-MQ) inhibited the nociceptive (inflammatory pain) behavior produced by formalin in mice.

Formalin is used as chemical noxious stimuli to trigger pain. This test was normally used to study both central as well as peripheral analgesic activity.[29] Injection of formalin is associated with the neurogenic pain during early phase followed by the pain due to inflammation during the late phase.[12] The neurogenic pain is centrally mediated and is attributed to the direct stimulation of nociceptive primary afferents nerve fibers and the release of pain mediators such as kinin, histamine and serotonin. The inflammatory pain is peripherally mediated and it is due to peripheral release of chemical pain mediators that sensitize or activate nociceptors such as prostaglandin.[30] The peripherally analgesic drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) are only effective against inflammatory pain produced by formalin.[29,31] In contrast, the centrally acting analgesic drugs such as morphine inhibit both the neurogenic and inflammatory pains caused by formalin. The inhibitory effect demonstrated by fractions (BF, EAF and EiF-1) and F3C5-MQ against neurogenic and inflammatory pains may suggest peripheral and central analgesic

actions similar to morphine. The inhibitory effect demonstrated by DCMF and F3C2 against inflammatory (phase) pain may suggest peripheral analgesic effect similar to NSAIDs.

Hot plate is a transparent glass cylinder used to keep the animal on the heated surface of the plate.[12] The temperature of hot plate is set using a thermoregulated water-circulated pump. This hot plate test is also considered to be sensitive to drugs acting at the supraspinal modulation level of the pain response,[32] suggesting atleast a modulatory effect of the investigated extract and fractions. The time of latency or reaction time is defined as the time period between the zero point, when the animal is placed on the hot plate surface, and the time when the animal licks its paw or jumps off to avoid pain.[33,34] Hot plate test is normally used to evaluate the centrally acting analgesics.[35] Fractions (DCMF and n-HF) and (F3C2) did not show any promising analgesic activities, whilst fractions (BF, EAF and EiF-1) and F3C5-MQ did exhibit analgesic activities. Hence, the result of hot plate test supported the result of formalin-induced paw licks and affirmed the presence of centrally acting analgesic activity. However, it is not known whether the analgesic action is opioid-like in nature and or involves dopaminergic or other mechanism. The use of selective antagonist like Naloxone or metoclopramide might help in understanding the mechanism involved.

Inflammation is typically characterized by increased permeability of endothelial tissues and influxes of blood leuckocytes into the intestitium resulting in oedema. Many different biological mediators' influences each step of inflammation cascade and typically anti-inflammatory agents exhibit therapeutic properties by blocking the actions of synthesis of some of these mediators. [36] Carrageenin-induced paw oedema was taken as a prototype of exudative phase of inflammation. This oedema depends on the participation of kinins and polymorphonuclear leucocytes with their proinflammatory factors including prostaglandins.[37] The development of oedema in the rat paw after the injection of carrageenin has been described as a biphasic event.[38] The initial phase starts immediately after the injection and reduces within one hour, and is attributed to the release of histamine and serotonin.[39] The second phase of swelling which begin at one hour and remain through three hour is due to the release of prostaglandin-like substances.[40] It has been reported that the second phase of oedema is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents. Generally NSAIDs strongly inhibit the second phase of carrageenin-induced oedema while some inhibit both phases. Indomethacin seems to inhibit both phases.[41]

The effective anti-inflammatory activity was observed with fractions (BF, EAF, DCMF and EiF-1) and semi-purified fractions (F3C2 and F3C5-MQ) treated animals for three hours measurement. The ability of fractions (BF, EAF, DCMF and EiF-1) and semi-purified fractions to suppress acetic acid-induced nociceptive and carrageenan-induced inflammation suggests a peripheral analgesic effect similar to NSAIDs. The significant anti-inflammatory effect shown by fractions (BF, EAF, DCMF and EiF-1) and semi-purified fractions against pain associated with second phase of formalin test and reduced pain episodes elicited by acetic acid may suggest involvement of phytochemically active constituents with prostaglandin synthesis inhibitory properties.

Fraction EiF-1 shows powerful anti-nociceptive effect in all the nociceptive experimental models, while fractions (EiF-2, EiF-3, and EiF-5) produced nociceptive behavioural effect as in acetic acid-induced writhing (not shown). EiF-1 is oily and produced a clonic-tonic convulsion when 50mg/kg body weight was administered intraperitoneally. Clinical intoxications induced by essential oils were characterized by tonico-clonic or solely clonic convulsions,[42] these was in confirmation of effect of EiF-1 at 50mg/kg body weight. There are reports of such symptoms in adults and children who used essential oils for therapeutic purposes.[43] The difference in group of chemical constituents of the crude methanol extract and fractions especially alkaloids, flavonoids, saponins, and terpenes (found in essential oil) may be responsible for the observed differences in analgesic and anti-inflammatory. Flavonoid and saponin are known to inhibit pain perception as well as anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of chemical mediator of inflammation.[44] Initial separation of the ethyl-acetate fraction of methanol extract, when subjected to column chromatography, yielded a total of 174 fractions, using gradient elution of hexane: ethyl acetate (9.5-0.5 to 0.5-9.5). Similar fractions were pooled together according to TLC profile to yield a total of six sub- fractions (EiF-1- EiF-6).

Sub-fraction EiF-1 yielded yellow oily coloured and on thin layer chromatography showed a prominent single purple band with Rf value of 0.84 and fluorescence quenching under long UV (364nm). This single fluorescent band turned brown on spraying with anillin sulphuric acid and subsequent heating, indicating the presence of terpenoid compounds.

Studies of proposed analgesic-like activity mechanisms have been conducted with some essential oil constituents that are widely known because of their uses in the pharmaceutical, cosmetic, and food industries. For example, (-)-linalool, a major component of many plant

essential oils, such as *Aniba rosaeodora* (Lauraceae),[45] acts on several receptors, including opioids, adenosine A1 and A2, cholinergic M2, and produces changes in K+ channels.[46,47] In an attempt to establish the mechanism by which F3C5-MQ exerts its anti-nociceptive activity, F3C5-MQ was evaluated using antagonist of notable mediators of nociceptive pathway such as naloxone, atropine and theophylline. Formalin test was employed in determining the mechanism because of its biphasic control of pain reflective of the different pathological processes involved nociception, it also allows for the elucidation of the possible mechanism responsible for the induction of analgesia.[29] Anti-nociception provoked by F3C5-MQ was annulled by atropine a nonselective muscarinic receptor antagonist, which implicates the muscarinic cholinergic system in the actions of the compound. Studies have shown that the activation of the muscarinic receptors (M1-M4) induces anti-nociception in various pain paradigms including inflammatory, thermal and neuropathic pain.[48,49,50] Terpenoid and steroid compounds are widely distributed in the plant and exhibit distinctive pharmacological properties. Naturally occurring terpenoids were reported to have possessed anti-inflammatory and anti-nociceptive properties.[51] Therefore, the antinociceptive activity of F3C5-MQ may be due to the activation of one or more of the muscarinic receptors and may also be attributed to the presence of fatty acids and or triterpene (amyrin) compounds found in F3C5-MQ.

Toxicity studies in animals are used to evaluate the potential health risk that may result from the administration of chemical compounds present in plant extracts. The adverse effects may present as alterations in levels of biomolecules and metabolic products, or the alteration in the normal functioning and structure of organs.[52] The liver, being the primary organ for the detoxification and distribution of drugs, and the kidney which is the major excretory organ, have been used generally to investigate the toxicity potential of different substances.[53] Experimental screening of plant products are crucial in ascertaining the safety and efficacy, also in establishing the active constituents of the herbal products. It is therefore pertinent to establish the safety of Erythrophleum ivorense through toxicological assessments. Sub-acute oral toxicity studies have provided information on drugs that may have adverse health effects.[54,55] Orally administration of F3C5-MQ (25 and 50mg/kg) for twenty eight days did not show any significant changes in body weight gain when compared with control rats. The liver enzymes, such as alanine and aspartate aminotransferase (ALT and AST) are established markers of liver cell injury while alkaline phosphate (ALP) is a marker of cholestasis.[56] These enzymes are also involved in amino acid metabolism and are useful in assessing the

functional integrity of the liver. ALT which is produced within liver cells, was reported to increase in conditions connected with the inflammation of death of the liver cells. As the cells get injured, ALT leaks into the blood stream leading to a rise in the serum levels a very sensitive marker of liver cell injury. Semi-purified fraction (F3C5-MQ) did not exhibit a significant effect on alanine aminotranferase (ALT), alkaline phosphate (ALP) and aspartate aminotransferase (AST) levels at all treatment doses compared with the control. The non-significant changes in ALT, AST and ALP in rats at all doses suggested that sub-acute administration of the fractions did not affect hepatocyte function in rats or causes any significant damage to the liver. Urea and creatinine are waste products of protein metabolism that are excreted by the kidney. Therefore, marked increase in serum urea and creatinine are indicators of functional injury to the kidneys. The kidney is the major organ involved in the clearance and excretion of drugs and drug products from the body. Damage to the kidney may follow administration of plant extracts, although incidences of reported toxicity in local settings are few. In this present study, changes in plasma urea and creatinine levels in F3C5-MQ treated groups showed no significant differences on a dose dependent manner indicating a normal renal function. Although, histopathological analysis revealed the presence of mild shrunken glomeruli and oedematous tubular cells in groups administered with the fraction compared to control. This suggests that the mild effects seen may be due to the vehicle (DMSO) since, there was no alterations in the biochemical parameters related to kidney function. Meanwhile, crude methanol extract of Erythrophleum ivorense stem bark at a dose of 50mg/kg significantly increased serum AST and ALP levels in treated animals; with a significant increase in creatinine ($p<0.05$) and Urea ($p<0.05$) respectively.

The current study demonstrates that the semi-purified fractions obtained from the stem bark extract of *Erythrophleum ivorense* has anti-nociceptive and anti-inflammatory activities in chemical and thermal models of nociception. The studies shows that fraction (F3C5-MQ) may be mediated via cholinergic pathway and fairly nontoxic.

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Competing interests

The authors declare that they have no competing interest.

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