



The Impact of the Recipe from *Zanthoxylum leprieurii* Bark, *Harungara madagascariensis* Leaves and *Xylopia aethiopica* Fruits on Nociception and Inflammation in Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Inflammation has a major role in the pathophysiology of sickle cell disease, in fact when it is recurrent, it contributes more to the obstruction of blood vessels through several biochemical mechanisms by provoking vaso-occlusive crises. In order to mitigate these adverse effects in sickle cell patients, the analgesic and anti-inflammatory potentials of extracts of a combination of organs of three plants were evaluated in this study. The analgesic and anti-inflammatory activities of the aqueous (DZHm) and hydroethanolic (EZHm) extracts (200, 400 and 800 mg/kg) were evaluated in rats as models of acetic acid-induced torsion and Carrageenan-induced hind paw edema, using Diclofenac and Ibuprofen as reference molecules. Oral administration of ZHm extracts at doses of 200, 400 and 800 mg/kg produced anti-inflammatory and analgesic effects. These extracts significantly reduced edema and pain. These effects are time and dose dependent. In terms of analgesic effect, the dose of 800 mg/kg bw generated a high inhibition rate of 84.51% and 73.06% for DZHm and EZHm respectively, while the inhibition percentage of Ibuprofen was 78.3 ± 2.09 at the dose of 100 mg/kg bw. The anti-inflammatory effect was 60, 55% for DZHm, 56.08% for EZHm and 62.91% for Diclofenac at 10 mg/kg Pc. Diclofenac and Ibuprofen used for these two tests generated similar activities with ZHm extracts at the dose of 800 mg/kg Pc. The tested extracts (DZHm and EZHm) have analgesic and peripheral anti-inflammatory potentials justifying the use of this recipe in the traditional environment.

Keywords: Anti-inflammatory; analgesic properties; medicinal plants.

ABBREVIATIONS

DZHm: Decocted extract of combination Zanthoxylum leprieurii, Xylopi aethiopia, and Harungara madagascariensis

EZHm: Ethanolic extract of combination Zanthoxylum leprieurii, Xylopi aethiopia, and Harungara madagascariensis

1. INTRODUCTION

Inflammation is a reactionary phenomenon implemented by the organism whenever the integrity of its morphological and biological constants are threatened [1]. It is therefore not synonymous with infection, but infection can be the cause of inflammation [2]. Inflammation has multiple origins among which are infections, physical and chemical agents [3]. Inflammation can be manifested by various symptoms such as edema (swelling or tumor), pain, fever often accompanied by anemia. Anemia is the main cause of hospitalization in sickle cell disease because it leads to painful vaso-occlusive crises. These painful attacks and anemia often dominate the symptomatology of the sickle cell patient under 5 years of age. Abdominal pain crises sometimes correspond to spleen infarctions [4]. However, sickle cell disease does not currently have sufficient curative treatment [5]. Thus to improve the quality of life or relieve patients, clinicians prescribe purely symptomatic treatment. Only bone marrow transplantation currently provides a curative solution, while gene therapy is in full development with the new

Crisper-Cas9 technology [6]. However, these highly specialized therapies require appropriate infrastructures, qualified personnel with high financial means and a compatible donor, which generally explains their unavailability in Black Africa where the disease is more frequent. Natural substances could be a source of new types of drugs that could fight against these biological phenomena related to sickle cell disease. Several experimental studies have shown the anti-inflammatory, analgesic and antioxidant activities of *Zanthoxylum leprieurii*, *Harungara madagascariensis* and *Xylopi aethiopia* *in vitro* and *in vivo* [7,8,9]. In the context of the development of a phytomedicine for the management of sickle cell disease in Côte d'Ivoire and the sub-region, the present study was initiated to investigate the analgesic and anti-inflammatory activities of a recipe derived from different organs of *Zanthoxylum leprieurii*, *Harungara madagascariensis* and *Xylopi aethiopia*. Thus, in this study, it is important to test the hypothesis that the different parts of these collected species would act synergistically to inhibit Carrageenan and acetic acid-induced edema and spasm in rats.

2. MATERIALS AND METHODS

2.1 Plant Materials

The plant material consists of fruits of *Xylopi aethiopia* (Dunal) A. Rich, barks of *Zanthoxylum leprieurii* (GUILL), and leaves of *Harungara madagascariensis* (LAM). These plants were

inventoried and collected from December 2017 to February 2018 in the Abengourou region (East of Côte d'Ivoire). They were then identified at the National Center of Floristics of the University Félix Houphouët-Boigny of Cocody in February 2018 by the late Mr. Assi Jean. The identification numbers are 14787 for *Zanthoxylum leprieurii* (GUILL), 17684 for *Harungara madagascariensis* (LAM) and 16073 for *Xylopiya aethiopica* (Dunal) A. Rich. On the basis of the information collected from the tradithérapeutes the recipe was constituted. The powders obtained after pulverization of these plants were assembled in the quantities of 33.33 ± 1 g per plant to form a total composition of 100 g. The new composition obtained named ZHm, was used for the various tests.

2.2 Animal Materials

The animal material for the study consisted of male and female laboratory rats of the Wistar strain of the species *Rattus norvegicus*. These rats were 60 to 90 days old and weighing between 104 and 130 g were used. These animals were fed with FASCI[®] pellets and stale bread. The tap water used for watering them in washed bottles was constantly renewed. 3h before the tests, apart from the water, the bed of shavings was renewed in order to remove the pellets and the bread from the plastic cages. All procedures and techniques used in this experiment were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals [10].

2.3 Preparation of Extracts

2.3.1 Preparation of the hydroethanol extract

The hydroethanol extract was prepared according to the method of Zirihi [11]. One hundred grams (100 g) of plant powder was dissolved in one liter of hydroalcoholic solvent comprising 70% ethanol and 30% distilled water.

The mixture was then homogenized 10 times at a rate of 2 minutes per revolution using a Severin[®] brand blender. The homogenate obtained was wrung out in a cloth square and then filtered three times on hydrophilic cotton and then on whatman paper (3 mm). The filtrate was evaporated at 45°C using a Venticell[®] type oven for 24 hours. The dry powder obtained was the 70% ethanolic extract codified EZHm.

2.3.2 Preparation of the aqueous extract

Following the method of Konkon [12], one hundred grams (100 g) of plant powder was boiled for 20 min in 2 L of distilled water. The decoctate cooled to room temperature (25°C) was filtered three times on absorbent cotton and once on Whatman 3 mm filter paper. The filtrate was then dried at 50°C using a Venticell[®] type oven. The powder obtained is the total aqueous extract codified DZHm.

2.4 Phytochemical Screening

The phytochemical screening was performed according to the method used by Nemlin and Brunel [13]. The phytochemicals sought and the reagents used are presented in Table 1.

2.5 Determination of Peripheral Analgesic Activity

The method used is that described by Koster [14] and modified by Collier [15]. It consists of testing the inhibitory action of extracts on pain induced in rats by intraperitoneal injection of a dilute solution of acetic acid. The pain is manifested by a stretching movement of the hind legs and twisting of the dorso-abdominal muscles (spasms). Induction of the twists was performed by intraperitoneal injection of rats with a 1 % acetic acid solution at a rate of 10 ml/kg body weight (BW).

Table 1. Reagents and tests for characterization of chemical groups

Chemical groups	Reagents	Characteristic reactions
Alkaloids	Dragendorff Bouchardât	Precipitate or orange color Reddish-brown precipitate
Polyphenols	Ferric chloride	Blackish blue coloring
Flavonoids	Cyanidine	Pink-orange precipitation
Sterols and polyterpenes	Dear man	green ring
Tannins	Stiasny	precipitated in large flakes
Substances quinoniques	Bornstraëgen	Red or purple coloring
Saponines	Agitation	Persistent moss with a height of 10 cm

Eight batches of 5 rats were formed.

Lot 1 or control lot (TM) received distilled water.
Lot 2 received Ibuprofen (10 mg/kg/pc).
Lots 3, 4 and 5 received ZHm decoctate (DZHm) at 200, 400 and 800 mg/kg/pc.

Batches 6, 7 and 8 received ZHm hydroethanol extract (ZHmHE) at doses of 200, 400 and 800 mg/kg/pc.

One hour after the injection of the different solutions, 0.6% acetic acid at the dose of 10 mL/kg was injected into the rats [16]. Five minutes (5 min) after the injection of acetic acid solution, the number of abdominal twists was observed for 20 min. The analgesic activity which is the percentage reduction in the means of abdominal twists was calculated according to the equation:

$$\text{PIC}(\%) = \frac{M_0 - M_1}{M_0}$$

PIC: Percent of inhibition of twists,
M₀: Average of the twists of the control animal,
M₁: Average of the twists of the treated animal.

2.6 Evaluation of Anti-Inflammatory Activity

2.6.1 Test for inhibition of oedema of the rat paw with carrageenan

The test for anti-inflammatory activity was performed according to the method of Winter [17]. Injection of carrageenan under the plantar fascia of the mouse or rat hind leg results in edema (clinical sign of inflammation) of the metatarsal region. The intensity of this edema, which reaches its maximum development in 5 hours, is assessed by the increase in the volume of the paw (compared to the initial volume). Preventive oral administration of an anti-inflammatory product significantly reduces the development of edema.

Rats weighing between 120 g and 150 g were fasted for 24 h. They were divided into 9 batches of 6 rats each. The diameter at time T₀ of the right hind leg of each animal was determined using a digital caliper [18].

Lots 1 and 2 (normal and edematous rats) received distilled water. Rats in lot 3 (reference)

received Diclofenac Sodium (5 mg/kg bw). The other six batches received decoctate and hydroethnolic extract at 200, 400 and 800 mg/kg bw, respectively.

One hour after gavage administration of the different solutions, 0.1 mL of the 1% carrageenan solution, was injected dermally into the plantar fascia of the right hind paw of each rat in batches 2, 3, 4, 5, 6, 7, 8, and 9. The course of right edema was determined at 1h, 2h, 3h, 4h, 5h, and 6h [19].

The paw volume of all animals was measured with a caliper at 1h, 2h, 3h, 4h, 5h and 6h. The extent of edema was assessed by determining the average percent increase (% AUG) in rat paw volume according to the formula:

$$\%AUG = \frac{V_1 - V_0}{V_0} \times 100$$

V₁: Time *t* of paw diameter
V₀: Initial diameter of paw

The anti-inflammatory activity was also evaluated by calculating the percentage of inhibition (%INH) of edema according to the formula:

$$\%INH = \frac{\%AUG_{TM} - \%AUG_{TT}}{\%AUG_{TM}} \times 100$$

%AUG_{TM}: percent increase in paw of negative controls

%AUG_{TT}: percent increase in paw treated rats

2.7 Statistical Analysis

Statistical tests and graphs were made with the software Grap Phad. Prism.V 9.00 software (San Diego, California, USA). The results were presented as mean ± errors on the mean. The data were evaluated by the one-way ANOVA analysis method followed by Tukey's multiple comparison test at the 5% threshold to assess the significance of the observed differences.

3. RESULTS

3.1 Phytochemical Study

The qualitative phytochemical analysis performed on DZHm and EZHm revealed the presence of different groups of secondary metabolites. These results reveal the presence of polyphenols, tannins, flavonoids, alkaloids, steroids, triterpenes and quinone substances in

DZHm and EZHm; in contrast, saponosides were absent in EZHm (Table 2).

3.2 Peripheral Analgesic Power of the Extracts of the ZHm Recipe

3.2.1 Peripheral analgesic effect of DZHm on contorsions

Analgesic activity was assessed according to the acetic acid test (chemical stimulus). Fig. 1A summarizes the response of rats to pain induced by 1% acetic acid. During the 20 min after injection of the 1% acetic acid solution, the number of abdominal cramps observed in control rats was 53.33 ± 2.40 . For the other substances tested after acetic acid injection, the number of twists was 11.67 ± 1.66 ; 18.33 ± 1.66 ; 8.66 ± 3.18 and 8.33 ± 1.66 for Ibuprofen and DZHm at 200, 400 and 800 mg/kg/pc, respectively. Treatment with Ibuprofen significantly reduced the number of twists with a percentage inhibition of 78.3 ± 2.09 . As for the rats treated with 200, 400 and 800 mg/kg/pc of DZHm, the percentage of inhibition was 56.89 ± 0.64 ; 83.28 ± 6.69 and 84.51 ± 2.80 respectively. The analgesic action of DZHm at 400 and 800 mg/kg is similar to that of Ibuprofen at 100 mg/kg (Fig. 2A).

3.2.2 Peripheral analgesic effect of EZHm on contorsions

The results of this study are summarized in Figures 1B and 2B. The mean number of contortions in untreated control rats was 53, 33 ± 2.40 following intraperitoneal injection of acetic acid. In general, the number of contortions in rats treated with Ibuprofen and EZHm were decreased compared to that of control rats. In the presence of Ibuprofen (100 mg/kg), the number of stretches in rats was 11.67 ± 1.66 during the 20 min of observation, which corresponds to a percentage of protection of 78.3% compared to control rats. For rats treated with 200, 400 and 800 mg/kg/pc of EZHm, the mean number of stretches was 23.33 ± 1.66 ; 12.33 ± 1.45 and 14.33 ± 0.66 or a percentage of inhibition of 55.81%; 77.02%; 73.06% respectively.

The analgesic effect of DZHm and EZHm is comparable to that of Ibuprofen at 400 and 800 mg/kg/pc. The reduction in the number of contortions is dose-dependent for both extracts.

3.3 Anti-Inflammatory Potential of the Extracts of the ZHm Recipe

3.3.1 Effect of extracts on acute inflammation

The results obtained show that all rats that received Carrageenan developed paw edema. The volume of paw edema varied over time.

For animals that received NaCl (9%), edema increased to a peak characteristic of an inflammatory reaction at 4th hours with a maximum of $56.66 \pm 4.33\%$. Then, edema decreased at 5e and 6e hours with a percentage of 45.87% without reaching the initial volume (normal state). A significant ($p < 0.01$) reduction in edema by the plant extracts and in a dose-dependent manner was observed after 3 hours (Fig. 3A and B). The reference molecule also significantly reduced the edema induced by Carrageenan.

Oral administration of the aqueous extract (DZHm) at the doses of 200, 400 and 800 mg/kg bw significantly ($p < 0.01$) reduced edema compared with that of untreated rats 3, 4, 5 and 6 hours after Carrageenan injection. The percentage inhibition of DZHm at 200, 400, and 800 mg/kg Pc at 4e hours was 31.76%, 52.45%, and 62.25%, respectively, whereas that of Diclofenac was 62.91%. At the end of the 6th hours of experiment, the results show that Diclofenac at the dose of 10 mg/kg had better anti-inflammatory activity, with a percentage of inhibition of 82.82% and those of the aqueous extract were 28.44%; 45.44% and 60.55% at the different doses tested (Fig. 4A). The reduction of edema by the aqueous extract of ZHm at the dose of 800 mg/kg was similar to that of Diclofenac at 4th hours of observation.

Hydroethanol extract (EZHm) at the doses of 200, 400 and 800 mg/kg Pc also significantly ($p < 0.01$) reduced the edema of treated rats compared to untreated rats from 4e, 5e and 6e hours after Carrageenan injection. Compared with the percentage of inhibition of Diclofenac which was 62.91% at 4th hours and 82.82% at 6e hours, the percentages of inhibition of ZHm hydroethanol extract were 28.43%, 53.97 and 56.08% at 4th hours and then 28.26%, 50.45 and 53.27% at 6th hours for the doses of 200, 400 and 800 mg/kg Pc, respectively (Fig. 4B).

Table 2. Results of the phytochemical study of the extracts of the ZHm recipe

Decocted (DZHm)							Hydroethanol extract (EZHm)						
S/T	POL	FLA	TAN	SQ	ALC	SA	S/T	POL	FLA	TAN	SQ	ALC	SA
+	+	+	+	+	+	+	+	+	+	+	+	+	-

(+) : Presence; (-) : Absence

ALC: Alkaloids; TAN: Tannin, FLA: Flavonoid; POL: Polyphenols; S/T: Sterol / Triterpenes; SQ: Quinones; SAP : Saponosides

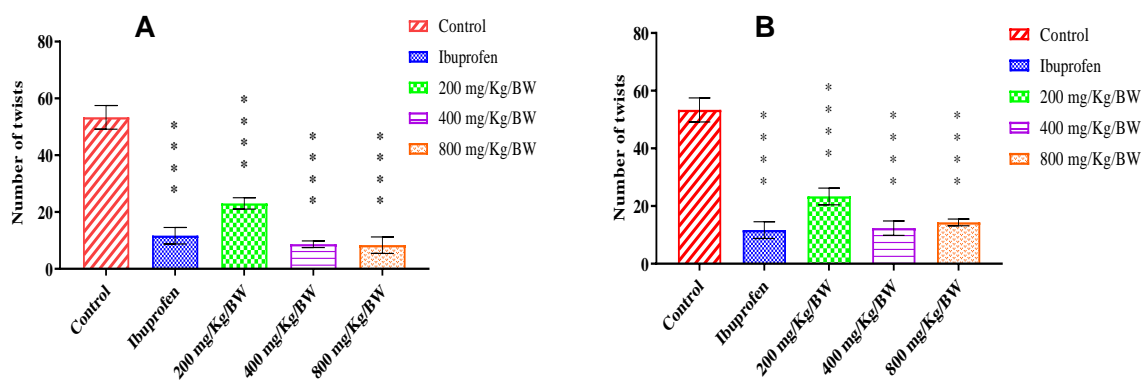


Fig. 1. Number of twists in the presence of Ibuprofen and different doses of the ZHm recipe extracts

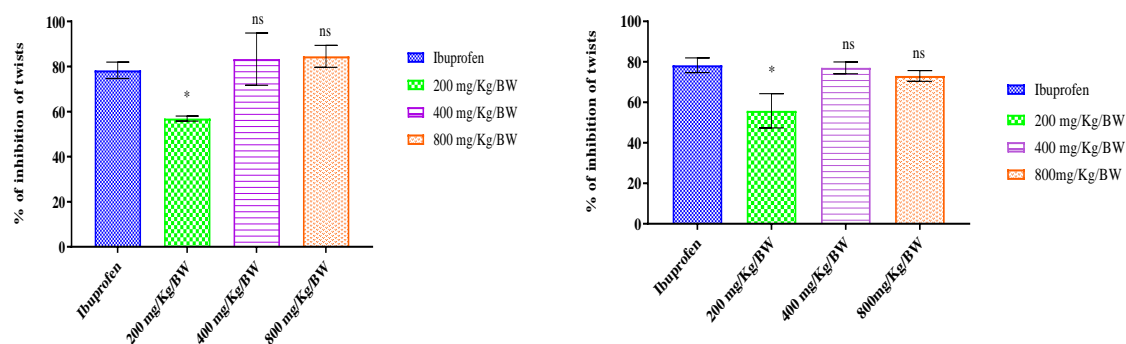


Fig. 2. Pain inhibitory effect of rats treated with Ibuprofen and ZHm extracts after acetic acid injection

Values are presented as mean ± standard deviation

(*) ($p < 0.05$); (****) ($p < 0.0001$) represents significant differences between anemia controls and treated rats

During the six-hour experiment, both aqueous and hydroethanol extracts of ZHm significantly inhibited paw edema in rats from 4e hours at the dose of 800 mg/kg, compared with untreated rats. The inhibitory power of ZHm extracts was more pronounced with the aqueous extract after 4e hours.

4. DISCUSSION

Many conditions cause pain and fever in humans. Some of these conditions often result in death. Although synthetic analgesics and anti-

inflammatory drugs are available in pharmacies, most synthetic products are expensive and also have many adverse effects [20]. In the sickle cell disease, Inflammation plays a major role in the pathophysiology, indeed, when recurrent, it further contributes to blood vessel obstruction through several biochemical mechanisms by causing vaso-occlusive crises. Thus, to mimic the painful attacks of sickle cell patients in rats, 1% acetic acid was injected intraperitoneally, a method used to study the peripheral analgesic effect of a substance. The pain elicited by injection of 1 % acetic acid is due to the release

of serotonin, histamine, bradykinin, and prostaglandins (PGE₂, PGF₂) [21]. Without analgesic, acetic acid administration generated an average of 53, 33 contortions in rats. A similar result was observed by Traoré [22]. These chemical mediators stimulate peripheral nociceptive neurons and induce increased vascular permeability. The result is a later, diffuse pain that manifests in rats as stretching of the hind legs and twisting of the dorso-abdominal musculature [23]. These seizures are the most disabling syndrome of the disease, moreover, they have an unpredictable and random nature [4]. Most often osteoarticular in nature in adults, they are associated with intense pain and variable frequency. Vaso-occlusive crises are the most frequent complications of sickle cell disease, so analgesics are the most commonly used medications by sickle cell patients for pain relief [4].

Administration of the extracts at different doses of 200, 400 and 800 mg/kg bw generated a reduction in the number of abdominal cramps caused by acetic acid. EZHm and DZHm at doses of 400 and 800 mg/kg bw, had an effect comparable to that of Ibuprofen. Indeed, the percentage of pain inhibition at the 400 mg/kg bw dose was 77.02%; 83.28% for EZHm and DZHm respectively and 73.06% and 84.51% at the 800 mg/kg bw dose while that of Ibuprofen was 78.3%. This activity of the extracts is dose-dependent. The analgesic effect of the extracts could be related to the inhibition of the release of pro-inflammatory mediators by the presence of alkaloids. Indeed, according to Allegra [24]; Eom [25]; alkaloids are well known for their ability to inhibit pain perception. Furthermore, Koua [26], showed that the presence of flavonoids in *Crinum scillifolium* extracts would have inhibited acetic acid injection-induced spasms. The presence of these chemical compounds in ZHm extracts would therefore be responsible for its peripheral analgesic activity.

In an attempt to alleviate vaso-occlusive crises in sickle cell patients, inflammation was induced in rats. The Carrageenan-induced edema test has characteristics similar to those of acute inflammation, namely increased extravasation of cells and fluid into tissues. This test typically evaluates compounds for acute anti-inflammatory activity. This test is a classic model of inflammation and increased pain sensitivity, which has been widely used over the years to evaluate the anti-inflammatory properties of NSAIDs and selective COX-2 inhibitors [27]. The

progression of edema after Carrageenan administration is a biphasic event, dependent on age and weight. Histamine, serotonin, and bradykinin are the first mediators to be detected in the early phase [28] and a late prostaglandin-supported phase is mediated by bradykinin, leukotrienes, and polymorphonuclear cells [29]. The significant inhibition of inflammation by ZHm extracts after 2 hours of edema induction, suggests the ability of the extracts to act on both the initial phases of acute inflammation. Indeed, ZHm may have inhibited the release of vasoactive mediators that induce inflammation and, thus, protected the rats to a greater extent. ZHm significantly and dose-dependently inhibited edema formation by Carrageenan in rats. The inhibition of paw edema by ZHm was more pronounced in the presence of decoctate.

In the case of acute inflammation, the results obtained show that the aqueous extract of ZHm at doses of 400 and 800 mg/kg bw significantly inhibits the formation of rat paw edema like the reference drug, Diclofenac (10 mg/kg bw). It is a non-steroidal anti-inflammatory drug (NSAID) that exerts anti-inflammatory activity through inhibition of cyclooxygenase (COX1 and COX2) and suppresses the production of prostaglandin, histamine and serotonin. These substances are involved in increasing vasodilation and vascular permeability [30,31]. The decrease in the volume of rat paw edema by ZHm extracts at 400 and 800 mg/kg bw could be explained by the presence of polyphenols in these extracts. These would have acted as substances with antagonistic action on the effects of histamine, serotonin, bradykinin and prostaglandin biosynthesis [26].

The extracts of ZHm having analgesic and anti-inflammatory effects could upstream attenuate the vaso occlusive crises in sickle cell patients. These different activities observed with ZHm suggest that there would have been an addition of molecules with anti-inflammatory and analgesic activity of the different plants from the ZHm recipe. Indeed, according to Johnson-Ajinwo and Nyodee [8]; Njan [32], *H. madagascariensis* has an anti-inflammatory, antipyretic and analgesic activity, moreover the work of Oso [33]; Macebo [7] on *X. aethiopica* show that these extracts inhibit proinflammatory molecules and spasms caused by formalin and acetic acid. Furthermore; the work of Zondegoumba [34] and Evelyne [35] also showed that *Z. lepreurii* has anti-inflammatory activity *in vivo* and *in vitro*.

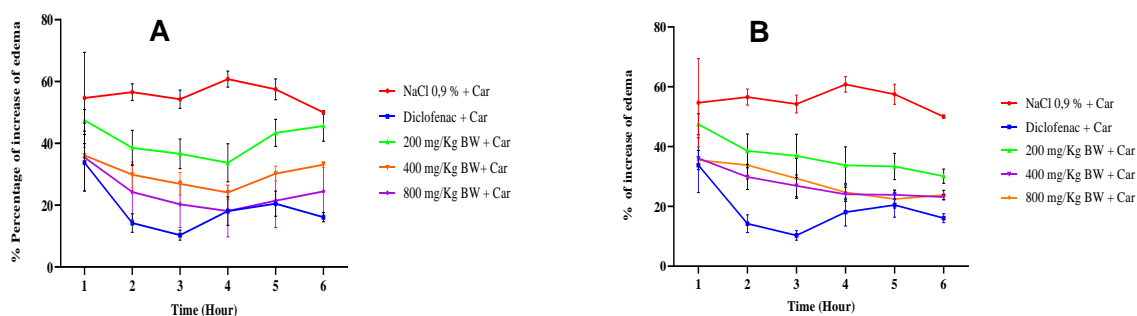


Fig. 3. Variation in the percentage increase in edema of rats treated with ZHm recipe extracts and diclofenac

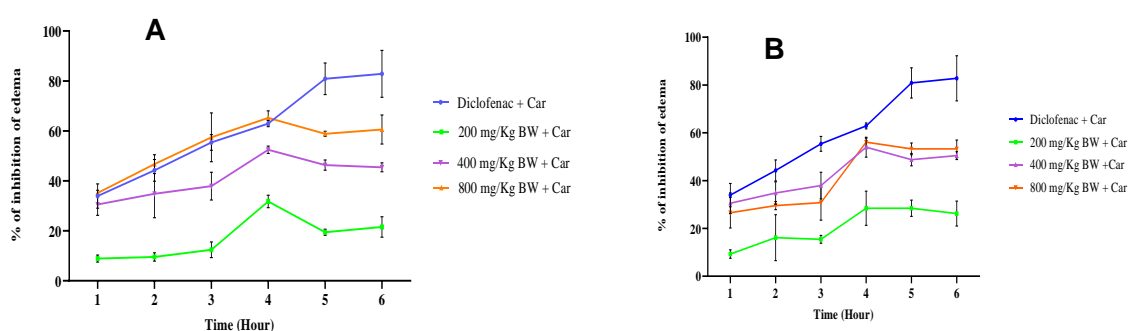


Fig. 4. Variation in the percentage of edema inhibition of rats treated with ZHm recipe extracts and Diclofenac

These observed effects were possible because of the therapeutic properties of the secondary metabolites identified during phytochemical sorting. Indeed according to the literature, alkaloids have antimalarial, antibacterial, analgesic and cardiotoxic effects [36]. Flavonoids are known for their antioxidant, anticancer and anti-inflammatory activities [37]. As for terpenoids, they also possess antimicrobial, antiparasitic, antiviral, antispasmodic, anti-inflammatory, and immunomodulatory properties [38,39]. As for saponins, they have antioxidant and anti-inflammatory properties and also participate in the delay of neural aging [40]. According to Gandonou [41] and Mpondo [42], tannin plants would be used as anti-inflammatory, anti-parasitic and analgesic.

Analysis of the phytochemical study results revealed the presence of alkaloids, catechic tannins, flavonoids, polyphenols, sterols, terpenes, and quinone substances in the aqueous and hydroethanolic extracts of the recipe (ZHm). The presence of these secondary metabolites in both ZHm extracts has been confirmed by different authors. Indeed, the phytochemical screening performed by Benson [9]; Tine [43] and Gnamien [44] on *Zanthoxylum leprieurii* revealed the presence of tannins,

flavonoids, terpenoids, alkaloids. The work of the following authors Oso [33]; Ehigiator and Adikwu [45] revealed the presence of tannins, flavonoids, leucoanthocyanins, terpenoids, saponosides, and steroids in the fruits of *Xylopia aethiopic*. These same compounds were found in extracts of *Harungara madagascariensis* [46,47]. Although these plant extracts in the recipe are rich in secondary metabolites and show promising biological activities, toxicological studies conducted by our team revealed that this recipe did not alter the biochemical and hematological parameters of test rats. These different results could therefore justify the use of this plant recipe in a traditional environment.

5. CONCLUSION

Oral administration of ZHm extracts at doses of 200, 400 and 800 mg/kg generated anti-inflammatory and analgesic effects. These extracts significantly reduced edema and pain. These effects are time and dose dependent. Diclofenac and Ibuprofen used in these two tests had similar activities with ZHm extracts at 400 and 800 mg/kg/Pc. These results give pharmacological credibility to the use of this herbal recipe as a natural remedy in the management of inflammatory pain.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s)

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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