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Study of the analgesic effect of the aqueous extract of the leaves of *Citrus aurantifolia* (Rutaceae) in mice

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Abstract

Citrus aurantifolia (Rutaceae) is a plant used in traditional medicine in the treatment of inflammatory and analgesic pathologies. The objective of this study was to evaluate the analgesic activity of the aqueous extract of the leaves of *Citrus aurantifolia* in mice. The phytochemical study revealed flavonoids, tannins, polyphenols, coumarins, saponosides and polyterpene sterols and the absence of alkaloids. Concerning the acute toxicity study at the single dose of 2000 mg/Kg pc orally, it revealed that the aqueous extract of the leaves of *Citrus aurantifolia* is not toxic. Thus, according to OECD guideline 423, the oral LD50 of this extract is in the range of 2000-5000 mg/kg bw. The analgesic activity of the aqueous extract of *Citrus aurantifolia* orally was determined by evaluating the rate of inhibition of pain caused by acetic acid. The 250 mg/kg bw and 500 mg/kg bw doses of the aqueous extract administered orally all showed analgesic activity. The best analgesic activity was obtained with an inhibition rate of 88.64% for the 250 mg/kg bw dose. The best analgesic activity was obtained with an inhibition rate of 88.64% for the 250 mg/kg bw dose, while with the 500 mg/kg bw dose, the inhibition rate of pain sensation was 47.57%. Therefore, the extract has an analgesic effect at low doses.

Keywords: *Citrus aurantifolia*; Analgesic activity; Mouse

1. Introduction

The popular use of plants remains of great importance in Africa for the treatment of many diseases. According to data provided by the World Health Organization [1], 80% of the world's population treat their health problems with traditional remedies. On the one hand because they often do not have access to the drugs prescribed by modern medicine, and on the other hand because these plants have a real effectiveness [2]. Thus [3] asserted: "Very early in evolution, humans use the resources present in their natural environment to heal themselves". Indeed, natural substances of plant origin are endowed with several biological activities such as antioxidant, antimicrobial, anti-inflammatory and analgesic activity [4].

The analgesic treatment, among other things, is generally based on synthetic molecules. These molecules are drugs prescribed by modern medicine and are often expensive. As a result, they have side effects, sometimes serious, including toxicity on the renal and digestive systems [5]. In order to minimize these costs and undesirable effects, research is increasingly developing natural processes based on extracts and active ingredients of plant origin. These raw, natural extracts isolated from plants used in traditional medicine can be resources for new drugs [6].

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Thus, *Citrus aurantifolia,* (Christm Panzer) known as Limettier, belonging to the Rutaceae family, was used to study its analgesic activity in mice. It is a plant widely used in the treatment of many pathologies due to its multiple therapeutic effects [7 ; 8 ; 9].

2. Material and methods

2.1. Biological and technical material

2.1.1. Plant material

The fresh leaves of *Citrus aurantifolia* used for this study were collected from their natural habitats in Bonoua in the southern Comoé region (Côte d'Ivoire). The plant was identified and authenticated by the National Floristic Center of the Felix Houphouët-Boigny University of Cocody under the herbarium specimen N°. UCJ01608.

2.1.2. Animal material

The animal material used for the study of acute toxicity and analgesic activity is white mice (*Mus musculus*). A total of twenty-six (26) male and female mice of body weight between 20 and 25 g were used for the different tests. Six (6) nulliparous and non-pregnant females were used for the acute toxicity assessment according to the Organisation for Economic Co-operation and Development (OECD) guideline 423 [10]. Twenty (20) mice were used for analgesic activity. All animals were treated and handled according to the standards of the manuals on the care and use of experimental animals. Their diet consisted of corn, dried fish, bread and soybean. They were given tap water in bottles.

2.1.3. Chemical material

The chemical material was composed of Aspirin 500 mg (DU RHÔNE), which is a pharmaceutical analgesic, a 0.9% NaCl solution for dilution and 0.6% acetic acid to induce pain.

2.2. Methods

2.2.1. Extraction method

The leaves of *Citrus aurantifolia* were cleaned and then dried in the shade at room temperature, i.e. away from the sun. After drying, they were crushed with an electric mill of IKA A 10 Laboortechnik (Germany) to obtain a powder. Fifty grams (50 g) of powder were mixed in one liter (1 L) of distilled water. The mixture was mixed with a blender (NASCO BL-9295 A) for 3 min, twice with a one-minute pause interval, and then filtered three times through a clean poplin cloth and five times through absorbent cotton. The resulting filtrate was placed in an oven in Memmert (Germany) at 50 °C for forty-eight hours (48 h) according to the method of Zirihi *et al* [11].

2.2.2. Phytochemical screening

A qualitative phytochemical screening was performed on the powder samples obtained, following extraction with an aqueous solvent. The analytical techniques described in the work of [12 ; 13] were used to highlight the presence of the phytocompounds: flavonoids, polyphenols, coumarins, tannins, alkaloids, sterols, saponosides and polyterpenes.

2.2.3. Acute oral toxicity

The toxicity study was conducted according to the "dose adjustment" method of OECD Line 423 (OECD, 2008). It consisted of administering *Citrus aurantifolia* aqueous extract at a dose of 2000 mg/kg body weight (bw). The test was carried out on 2 batches containing 3 female mice (*Mus musculus*), their behaviour and the number of deaths were observed over a period of 30 minutes, 4 hours, 24 hours and 14 days. Thus, after 4 hours of fasting, they were distributed as follows: the control lot received distilled water, at a rate of 10 ml/kg bw and lot 1 received the aqueous extract, at a rate of 2000 mg/kg bw.

According to the method of [14], after fasting, they were weighed and the test substance distilled water or aqueous extract of *Citrus aurantifolia,* depending on the batch, was administered orally using a cannula syringe. Then, behavioural observation was regularly carried out 3 hours after administration of the aqueous extract of *Citrus aurantifolia*. Then the animals were again given food and water at will. For a period of 14 days, signs of toxicity including coat change, motility, tremor, body mass, grooming, respiration, stool appearance, mobility and death were observed.

2.2.4. Analgesic activity

In addition, the verification of the inhibitory action of the extract on the pain caused by acetic acid was made by following the method of Sigmund et *al,* [15]. It was carried out in mice by intraperitoneal (IP) injection of 0.01 mL of a dilute solution of acetic acid. Thus, the analgesic effect of the extracts was evaluated according to the number of abdominal contortions after the injection of acetic acid (0.6%).

Four batches of four mice were made up :

- lot 1 control, received orally 2 mL of distilled water
- the reference lot 2, received the reference molecule, aspirin (500 mg) at 100 mg/kg bw
- Lots 3 and 4, corresponding to the treated lots, received the aqueous extract of the leaves of *Citrus aurantifolia* orally at 250 and 500 mg/kg bw respectively.

One hour after the different treatments, an injection of 10 µl of a solution of acetic acid diluted in liquid Nacl was made in the peritoneum of each mouse according to the method. The pain syndrome was characterized by stretching movements of the hind legs and torsion of the dorso-abdominal musculature.

Five minutes after the injection of acetic acid, the number of contortions was counted in each mouse for 20 minutes.

Analgesic activity was expressed as a percentage of pain inhibition for each group treated with extracts, aspirin and distilled water.

The results were expressed as the mean (M) of the twists performed in each group ± standard deviation (SD). The percentage of pain inhibition was calculated according to the following formula:

$$\% \text{ INH} = \frac{\text{Mb} - \text{Mt}}{\text{Mb}} \text{ X } 100$$

Mb: Average number of twists in control group

Mt: Average number of twists treated group

% INH: percentage of pain inhibition

2.2.5. Statistical analysis

The data were analyzed with the Graph Pad prism 7 software, the results were expressed as mean±error on the mean (ESM). The variance (ANOVA) followed by the Turkey test allowed the different comparisons to be made. The significance level was set at $p<0,05$.

3. Results and discussion

3.1. Results

3.1.1. Phytochemical screening

The qualitative analysis of the phytochemical characteristics carried out showed a variability in the chemical composition of the aqueous extract of the leaves of *Citrus aurantifolia*. (Table 1). Indeed, EAqCA contains polyphenols, flavonoids, tannins, coumarins, polyterpene sterols, saponosides and an absence of alkaloids. Coumarins, polyterpene sterols, saponosides are strongly present (Table 2). This presence resulted in the appearance of coloration with denser intensities in each test. On the other hand, the absence of alkaloids is characterized by the absence of the appearance of intense colored precipitates in the Dragendorff test.

3.1.2. Acute toxicity

The acute toxicity study with the single dose of 2000 mg/kg bw of EAqCA resulted in a small ($p<0.05$) significant reduction in body weight of mice during the 14 days post-treatment compared to control weights (Figure 1). Thereafter, examination of clinical signs of toxicity during treatment revealed no physical and behavioural changes in mice given

the extract at 2000 mg/kg bw compared to controls. No mortality was also observed during the experiments (Table 3). The lethal dose is in the range of 2000-5000 mg/kg bw (2000 mg/kg from pc< DL50<5000 mg/kg bw).

3.1.3. Effect of different treatments on acetic acid-induced pain

One hour after injection of acetic acid, the dose of 250 mg/kg bw of the aqueous extract of the leaves of *C. aurantifolia* showed a pain-inhibiting effect (88.64%) similar to that of Aspirin at a dose of 100 mg/kg bw (89.63%). The lot treated with the extract at 500 mg/kg bw produced less effective inhibition (47.57%) compared to Aspirin (Figure 2).

Table 1 Test for the detection and observation of phytochemical compounds.

Phytochemical compounds	**Highlighting test**	**Comments (positive or negative results)**
Polyphenols	Ferric chloride	blue-black or green-black colouring
Tannins	Ferric chloride	blue-black or green-black colouring
Flavonoids	basic lead acetate	orange-yellow colouring
Coumarines	potassium hydroxide	a disorder
Alkaloids	Dragendorff reagent	Absence
Sterol polyterpenes	Liebermann's reaction	Blue, green or violet colouring
Saponosides	Foam index test	High presence of foam of at least 1 cm

Table 2 Phytochemical characterization of aqueous extracts of *Citrus aurantifolia* leaves.

	Polyphenols	**Tannins**	**Flavonoids**	**coumarins**	**Alkaloids**	**Sterol polyterpenes**	**Saponosides**
Aqueous extract of *Citrus aurantifolia*	++	++	++	+++	-	+++	+++

(++) strong presence; **(+)** weak presence; **(-)** absence; **(+++)**: very strong presence

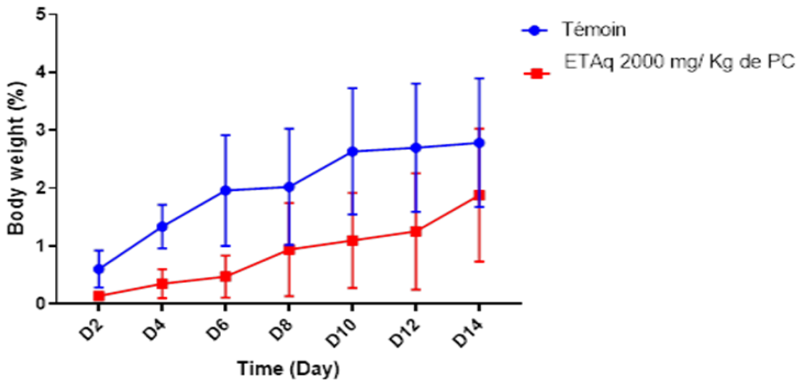

Figure 1 Effect of the aqueous extract of the leaves of *Citrus aurantifolia* on weight.

Table 3 Observation of clinical signs of rabies at a dose of 2000 mg /kg of body weight. Corporel.

Comments	30 min		4h		24h		7 days		14 days	
	C	T	C	T	C	T	C	T	C	T
Fur	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N
Mucosa	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N
Lethargy	N	N	N	N	N	N	N	N	N	N
Sleep	-	-	-	-	-	-	-	-	-	-
Coma	-	-	-	-	-	-	-	-	-	-
Convulsion	-	-	-	-	-	-	-	-	-	-
Trembling	-	-	-	-	-	-	-	-	-	-
Diarrhea	-	-	-	-	-	-	-	-	-	-
Morbidity	-	-	-	-	-	-	-	-	-	-
Food intake	N	N	N	N	N	N	N	N	N	N
Mortality	-	-	-	-	-	-	-	-	-	-

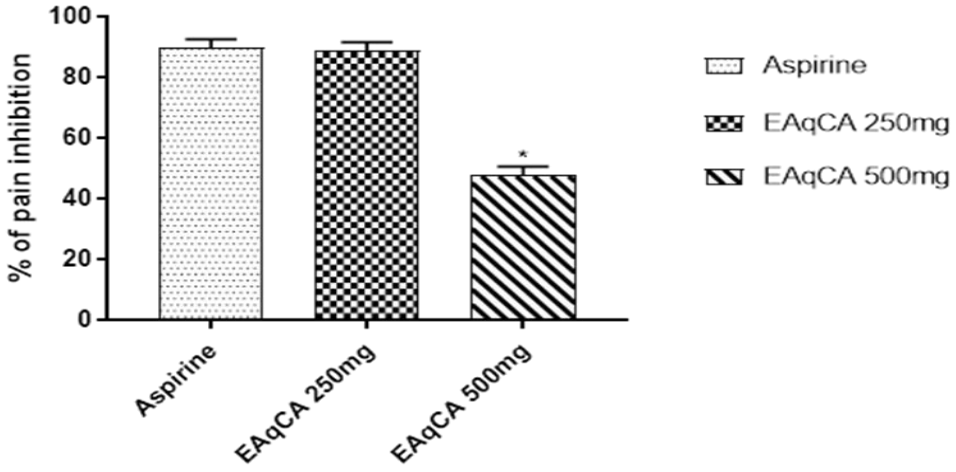

Figure 2 Percentage of EAqCA pain inhibition caused by acetic acid.

3.2. Discussion

The phytochemical study of the total aqueous extract of *Citrus aurantifolia* showed that the leaves of this plant contain polyphenols, flavonoids, saponosides, tannins, coumarins, sterols/polyterpenes but no alkaloids. The richness of the leaves of *Citrus aurantifolia* in secondary metabolites justifies their great effectiveness in traditional therapeutic use. Flavonoids and polyphenols are very present in these leaves, they are known for their antioxidant, anti-inflammatory and analgesic properties [16]. In addition, saponosides and coumarins are known for their anti-inflammatory and anti-oedematous effects. They also exert a protective action on the nervous system [17]. These results are similar to the studies conducted by Ali [18]. Indeed, it was found that the methanoic extracts of the leaves and essential oils of the fruits of *Citrus aurantifolia* contain secondary metabolites such as polyphenols, flavonoids, saponosides, tannins, coumarins, sterols/polyterpenes and no alkaloids. This richness of this aqueous extract of *Citrus aurantifolia* in active

chemical compounds could explain its traditional use in the treatment of inflammation, pain, malaria, allergy, diarrhea, gonorrhea and diabetes.

Evaluation of the acute toxicity of aqueous extracts from the leaves of *Citrus aurantifolia* in mice showed that this extract, administered orally, did not cause mortality or clinical signs at the single dose of 2000 mg/kg bw. With regard to body weight changes, monitoring of the change in body weight of the animals during the acute toxicity experiment with EAqCA at 2000 mg/kg bw showed no significant change during the 14 days of treatment of the treated lot compared to the control lot. Thus, according to OECD guideline 423, the LD50 of EAqCA is in the range of 2000-5000 mg/kg bw. These results are consistent with the work of Ali [18] whose study of the toxicity of Citrus limon extract administered orally at 2000 mg/kg bw did not result in death or behavioral change during the 14-day test period.

In terms of analgesic activity, injected acetic acid caused tissue damage that acted indirectly by stimulating the activation of various endogenous mediators: serotonin, histamine, substance P and prostaglandins [19]. These chemical mediators stimulate peripheral nociceptive neurons and induce an increase in vascular permeability, which leads to the sensation of pain in the abdomen [20]. The analgesic activity the leaves of *Citrus aurantifolia* have a protective effect, i.e. analgesic against the chemical pain induced. At a dose of 250 mg/kg of EAqCA, 88.64% inhibition of acetic acid-induced torsion was noted. This result is not significantly different from the reference molecule Aspirin, which has 89.63% pain inhibition. On the other hand, at the higher dose, i.e. 500 mg/kg EAqCA, there was a low level of 47.57% pain inhibition compared to the reference solution Aspirin. These results would explain a strong inhibition of prostaglandin synthesis at 250 mg/kg bw and a weak inhibition of this synthesis at 500 mg/kg bw. The dose of 250 mg/kg bw would therefore be the recommended therapeutic dose for the treatment of pain. The analgesic effect of *Citrus aurantifolia* aqueous extract could be due to the presence of phenolic compounds and coumarins. These compounds are indeed known for their analgesic properties in medicinal plants. They inhibit the release of cytokines and the biosynthesis of prostaglandins [21]. These results are in agreement with the work of [22] which attests that the analgesic activity of essential oils from different parts of the bitter orange: Citrus aurantium var. amara Link could be due to the presence of polyphenols and coumarins that inhibit prostaglandin synthesis.

4. Conclusion

The phytochemical study of the aqueous extract of the leaves of *Citrus aurantifolia* showed the presence of polyphenols, flavonoids, tannins, saponins, sterols and polyterpenes. In addition, alkaloids were found to be absent.

Acute toxicity in female mice showed no mortality or signs of special toxicities after oral administration of the aqueous extract of the leaves of *Citrus aurantifolia* at the dose of 2000 mg/kg bw tested. The LD50 therefore ranged from 2000 mg/kg bw to 5000 mg/kg bw.

Analgesic activity was also studied according to the peripheral component. In the twist test that was used to evaluate peripheral analgesic compounds, the aqueous extract from the leaves of *Citrus aurantifolia* showed good activity at the low dose.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors have seen and approved the manuscript as submitted. All authors participated in the work in a substantive way and are prepared to take public responsibility for the work. All authors of the manuscript have no conflict of interests to declare. The manuscript submitted to the journal is not copied or plagiarized version of some other published work. All the data taken from other sources is written in authors own language and properly cited. The text,

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Statement of ethical approval

The animal material used for this study, were used according to the Organisation for Economic Co-operation and Development (OECD) guideline 423.

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