Phytochemical study and anti-inflammatory activities of *Citrusaurantifolia* (Rutaceae) leaves collected in the region of Yamoussoukro, Ivory Coast.

Abstract: The aim of this work is to characterize the aqueous extract of *Citrusaurantifolia* leaves in order to identify the bioactive molecules present in the leaves and to evaluate the anti-inflammatory activity of the leaves of this plant. The phytochemical study was carried out using the tube staining test. The anti-arthritic activity of the plant has been studied on a model of chronic inflammation in vivo of inducing arthritis in the leg left rats by a full solution of Freund's adjuvant compared diclofenac used as a reference according to an oral treatment. The treatment of the arthritic rats with the extract at the doses of 250, 500 and 1000 mg/kg/bw and the diclofenac sodium (5 mg/kg/bw) started the day after the second injection and continued without interruption until the end of the experiment (28 days). Parameters such as edema and weight of the rats were evaluated during this experiment. The characterization of the aqueous extract of *Citrusaurantifolia* indicates that the leaves of this plant is rich in polyphenols, flavonoid, tannin, terpenoids, saponins, tannins and alkaloids. The extract inhibited CFA-induced paw edema. Furthermore, the comparative study performed on this animal model of chronic inflammation revealed that the activities of the extract were comparable to those of the reference molecule. As regards the weights, the rats treated with both the extract and the reference molecule showed a rapid recovery compared to untreated arthritic rats. These results show that the aqueous extract of *C. aurantifolia* prevented the pathological development of experimentally induced arthritis in rats. The active molecules present in the leaves of *C. aurantifolia* would be potential anti-inflammatory

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Introduction

Arthritis is an autoimmune disease characterized by chronic inflammation of the joints leading to destruction of cartilage. Rheumatoid arthritis affects more than 21 million people worldwide, three times more women than men (Chopra et AbdelNasser, 2008). It is associated with aging and affects mostly the elderly, although it is common in people aged 30 to 50 years (Lundkvist et al., 2008). Inflammation-relate disorders are manage through the implementation of various intervention strategiesaims at suppression pro-inflammatory mediators. The drugs currently available for the treatment of arthritis are analgesics, immunosuppressant, steroidal and non-steroidal anti-inflammatory drugs(Sarwar et *al*., 2011, Lundkvist et *al*., 2008). However, patients with rheumatoid arthritis experience unwanted side effects from the use of these products. These drugs are associated especially with long-term use with damage to the gastrointestinal tract, kidney, immune systems and even cardiac complications (Sarwar et *al*., 2011).

It is therefore necessary to find alternative sources of anti-inflammatory drugs, especially from plant or animal sources with no or minimal side effects.

*Citrus aurantifolia*belonging to the Rutaceae family. The leaves of this plant are widely used in the treatment of many pathologies because of its multiple therapeutic effects (Ibukun *et al*., 2007).

They are used in the treatment of ulcers, skin and oral infections, pain, nausea and fever (Khan, 2010 ;Kunow, 2003).

The objective of the present study is to evaluate the anti-arthritic activity of the aqueous extract of Citrus *aurantifolia* leaves on arthritic rats induced by Freud's complete adjuvant. Materials and Methods

Materials

It consists of the leaves of *Citrus aurantifolia*, harvested in Yamoussoukro in Côte d'Ivoire. The leaves were dried at room temperature in an airy, dark place.

Animal material

The study involved wistar rats raised in the ENS animal house weighing between 160 g and 170 g. The rats were acclimatized for one week at 25°C before the experiment.

Methods

Preparation of the crude extract:

The leaves were air-dried in the Laboratory at room temperature and then pulverized with a grinder. The powder obtained was sieved with a 0.4 mm sieve**.** During the extraction step, the Erlenmeyer flasks were completely covered with aluminum foil in order to be protected from light which could degrade the molecules.

Aqueous *extraction of Citrus aurantifolia* leaves

The aqueous extract of *Citrus lemon* (Rutaceae) leaves were prepared according to the method of Bagré*et al.* (2011). Thus, fifty grams (50 g) of plant powder were macerated in 500 ml of distilled water and then homogenized under

magnetic stirring for 24 hours at 50°C using an IKAMAG RCT magnetic stirrer**.** The homogenate obtained is successively filtered five times on hydrophilic cotton. The filtrate obtained is evaporated using a BUCHI 461 Water Bath rotary evaporator at 60°C to reduce the volume of the filtrate**.** The residue is then dried in an oven at 50°C. The dry extract obtained is stored in a refrigerator.

Characterization of the different metabolites present in the aqueous extract of *Citrus aurantifolia* leaves

The phytochemical screening allowed the detection of the presence or absence of essential chemical families such as phenolic compounds, heterosides including saponosides, nitrogenous compounds in particular alkaloids, isoprenoids which contain steroids, and reducing compounds. These examinations are based on staining and precipitation reactions.

The reagents used for the phytochemical screening of the different extracts were developed in the biochemistry laboratory at UFHB. They have been tested on other plants and gave results consistent with previous studies.

❖ **Alkaloid detection tests**

A quantity of 5 ml of 1% HCL mixed with 1 ml of each extract, are heated in the water bath, then the mixture is divided into two equal volumes.

One volume is treated with Mayer's reagent, the other with Wagner's reagent. The formation of a white or brown precipitate reveals the presence of alkaloids(Majob., 2003).

❖ **Polyphenoldetection tests**

One drop of 2% alcoholic ferric chloride solution is added to 2 ml of extract. The appearance of a more or less dark blue-black or green coloration indicates the presence of phenolic compounds (Harbone, 1998).

❖ **Flavonoiddetection tests**

Test 1: A few drops of concentrated HCL are added to 5 ml of extract more 3 chips of magnesium. The presence of flavonoids is characterized by red, orange or pink colorations (Karumiet *al*., 2004).

Test 2: A quantity of 5 ml of extract is evaporated. After cooling, 5 ml of hydrochloric alcohol diluted twice in a test tube is added to the residue obtained.

To this mixture are added some magnesium chips. The addition of 3 drops of isoamyl alcohol to this mixture intensifies the supernatant of the solution to an orange-pink color that characterizes the presence of flavones, or purplish-pink, those of flavanols**,** which indicates the presence of free flavonoids. In the case of flavonicheterosides, the colorations are less intense(Harbone, 1998).

❖ **Tests for leucoanthocyanins**

Leucoanthocyanins are characterized by the cyanidin reaction without addition of magnesium chips with heating for 15 minuites in a water bath. In the presence of leucoanthocyanins, a cherry-red or purplish coloration develops (Harbone, 1998).

❖ **Anthocyanindetection tests**

To 5 ml of 5% infusion showing a more or less dark coloration, were added 5 ml of sulphuric acid then 5 ml of ammonium hydroxide (NH4OH). If the coloration increases by acidification and then turns blue-violet in basic medium, there is the presence (Harbone, 1998).

- **Tests for free quinones**

5 ml of extract plus a few drops of soda (1% NaOH) turns yellow, red or purple in the presence of free quinones(Oloyede, 2005).

- **Anthraquinone detection tests**

To 10 ml of extract is added 5 ml of 10% NH4OH and the mixture is stirred. The purple coloration indicates the presence of anthraquinones(Oloyede, 2005).

- **Tests for coumarins**

A few milligrams of each extract are solubilized in 2 ml of hot water. The solution obtained is divided into two equal parts, the first representing a control and the second being treated with 0.5 ml of 10% NH4OH. The examination is carried out under ultraviolet light and the appearance of an intense fluorescence reveals the presence of coumarins(Benmehdi, 2000).

- **Tannin detection tests**

The presence of tannins is demonstrated by adding to 1 ml of each extract, 1 ml of water and 1 to 2 drops of 1% FeCl3 solution. The appearance of a green coloration indicates the presence of catechic tannins and the appearance of a blackish-blue color indicates the presence of gallic tannins(Karumiet *al*., 2004).

- **Sterol and TerpeneTesting:**

To 5ml of extract are added 2 drops of Acetic Anhydride (C4H6O3) and one drop of Sulfuric Acid (H2SO4). The purple or violet color indicates the presence of sterols and terpenes(Kablanet *al*, 2008).

- **Saponoside detection tests**:

A quantity of 10 ml of distilled water is added to 5 ml of extract and the solution is stirred for two minutes. The presence of saponosides is confirmed by the appearance of a persistent foam for more than 5 min, the result is positive when the height of the foam is greater than 1 cm (Karumiet *al*., 2004).

Chronic inflammatory study: Freund's complete adjuvant-induced arthritis

- **Method**

Arthritis was induced in rats by injecting 0.4 ml of Freund's Adjuvant Complete (AFC) into the left hind paw subplantar surface. The animals were divided into 7 groups of five rats each namely:

- The healthy control rats: the normal group which was orally administered with 1 ml of distilled water
- Arthritis control rats: Arthritis-induced control group is given 1 ml of distilled water orally;
- Diclofenac batch: arthritic rats receive orally 5 mg / kg of diclofenac sodium ;
- EAC250, EAC500 and EAC1000 : are the arthritis groups that received the extract solution at 250, 500 and 1000 mg/kg/pc, respectively.

After 24 H of DWI injection in their left hind paw subplantar region at day "0", the solutions were administered orally to the animals once a day from day [1] until day 28. The anti-arthritic effect of the drugs and the aqueous extract of *Citrus aurantifolia*(EAC) was evaluated by taking caliper measurements of the paw thickness on days 1, 4, 8, 12, 16 and 20.

The extent of arthritis was assessed by determining the average percent increase (PA) in rat paw volume according to the formula:

$$PA = \frac{V_t - V_0}{V_0} \times 100$$

Vt : Volume of the leg at time t

V0 : Initial volume of the leg

The anti-arthritic activity of the products was also evaluated by calculating the percentage of inhibition (PI) of edema according to the formula:

$$PI = \frac{PA_{AW} - PA_T}{PA_{AW}} \times 100$$

PI : percentage of inhibition

PA_{AW}: percentage increase in arthritis witnesses

PA_T: percentage increase in treatments

Results

Secondary metabolites of the different extracts

Characteristics of the extract

The characteristics of the extract are based on their yields as well as their appearance and color after oven drying at 50°C for three days. The yield of the total aqueous extract of *C. aurantifolia*was 16.36%. The dry extract of the plant leaves had a straw-like and oily appearance with a brown coloration.

Phytochemical screening

The phytochemical screening of the different extracts allowed to identify the families of secondary metabolites in the organs of this plant by methods of highlighting the metabolites by specific reagents. The results of the screening are shown in **Table I.** The phytochemical screening of the aqueous extract of *Citrus aurantifolia*leaves revealed the presence of catechic tannins, gall tannins, coumarins, sterols and terpenes, flavonoids and alkaloids and polyphenols. The flavonoids highlighted in the leaves belong to the flavonol and flavanol family because by the cyanidin reaction it was observed a pinkish-purple coloration of the supernatant of the solution contained in the test tubes.

Table I: Highlighting of secondary metabolites present in the aqueous extract of *Citrus aurantifolia*leaves

Metabolites	**Résults**
Polyphenols	+
Flavonoid	+
Leucoantocyan	-
Anthocyan	+
Gallic tanin	+
Catechitica tanin	+
Coumarin	-
Free quinone	-
Anthraquinon	-
Sterol et Terpene	+
Saponin	+
Alcaloid	+

Evaluation of the activity of *Citrus aurantifolialeaves* on paw edema of arthritic rats

The symptoms observed were redness, swelling and deformation of the paw. These signs were more pronounced in the untreated arthritic rats than in the treated ones. In contrast, none of these signs were observed in the healthy control lot that did not receive ACF injection. The changes in paw volume and percentage increase in edema are shown in Figure 1 below. In all batches which were injected by ACF, signs of arthritis appeared on the first day after ACF injection.

The edema of the arthritic rats reached its maximum level on the 8th day, the diameters of the measured edema was 6.77±0.33 mm on average with a percentage increase of edema of 147.4%. In arthritic rats treated by the diclofenac sodium, the peak of edema was also reached on day 8. The paw diameters were 5.59± 0.44 mm, which corresponds to a percentage increase of 95.26% (Figure 2). The arthritic rats treated with the different doses of the aqueous extract of *Citrusaurantifolia*, the diameters of the edemas were 5.44±0.69 mm, 5.12±0.06 mm and 4.7±0.145mm respectively for the different doses of 250, 500 and 1000 mg/kg/bw corresponding to the percentages of increase in the edemas of 96.79%, 86.03% and 74.88% respectively. The results show a reduction in the volume of the oedematous paw in rats treated with the extract and the reference molecules with a better reduction in the batches treated with the extract at doses of 500 and 1000 mg/kg/pc.

A significant difference ($p<0.05$) was observed on the ^{4th} day of treatment of arthritic rats at the doses of 500 and 1000 mg/kg/bw of the aqueous extract of *C. aurantifoliacompared* toarthritic controls. At the end of the treatment, there was a significant difference ($p<0.001$) in the percentages of inhibition of the arthritis rats treated with the different products compared to the untreated arthritis controls.

Evaluation of the inhibition of edema of the legs of arthritic rats by aqueous extracts of *aurantifolialemon*

The percentages of edema inhibition by the aqueous extract of *C. aurantifoliaare* shown in Figure 3 below. The aqueous extract of *C. aurantifoliaand* the diclofenac molecule significantly reduced edema of the rat paws. Arthritic signs were also reduced compared to untreated arthritis controls. The percentages of inhibition of paw edema in the diclofenac batch were 20.5%, 41.59% and 35.5% on days 4, 8 and 28 respectively.

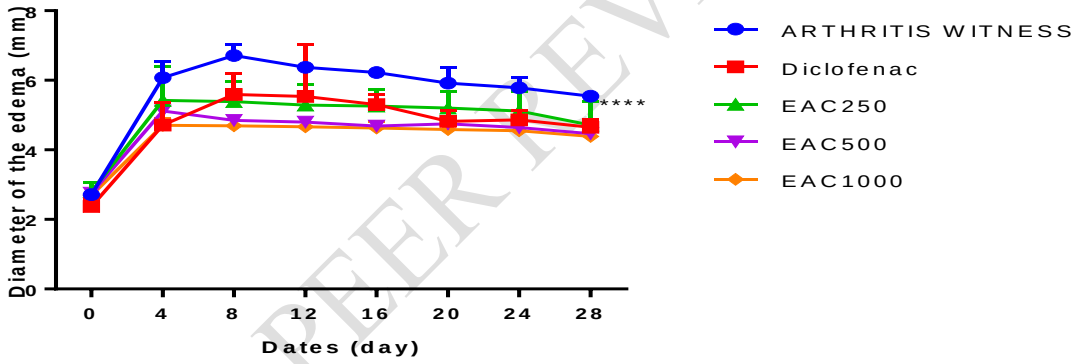
In rats treated with aqueous leaf extract, the percentages of inhibition of edema on the ^{4th} day were 25.3%, 29.67% and 37.67% on days 4, 8 and 28 respectively. The percentages of inhibition on day 8 were 35.5%, 48.35% and 49.55%. At the end of the experiment (28 days), the inhibition percentages were 34.01, 40.35 and 48.87 respectively.

No significant difference ($p>0.05$) was observed between the batches treated with the extract and the batch treated with the molecule.

Evaluation of the aqueous extract of *Citrus aurantifolia* leaves on the weight of arthritis rats

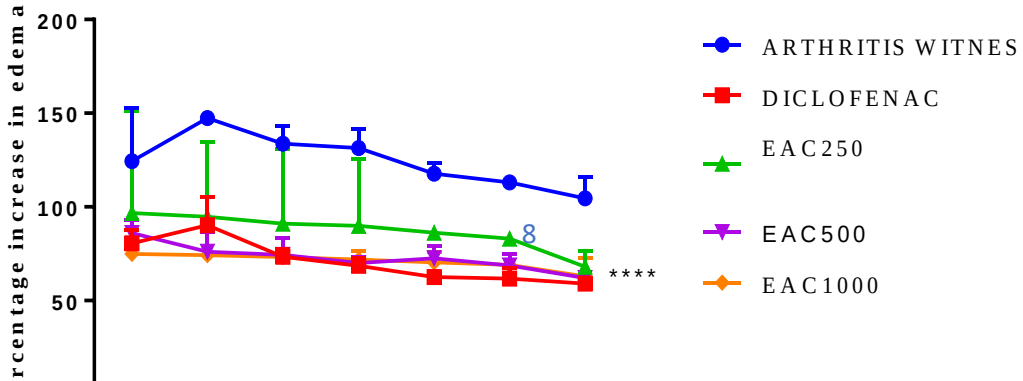
The weight gains of the rats during the course of the experiment are shown in Figure 4. During the first few days after injection of ACF under the plantar fascia of the rats' feet, the results show

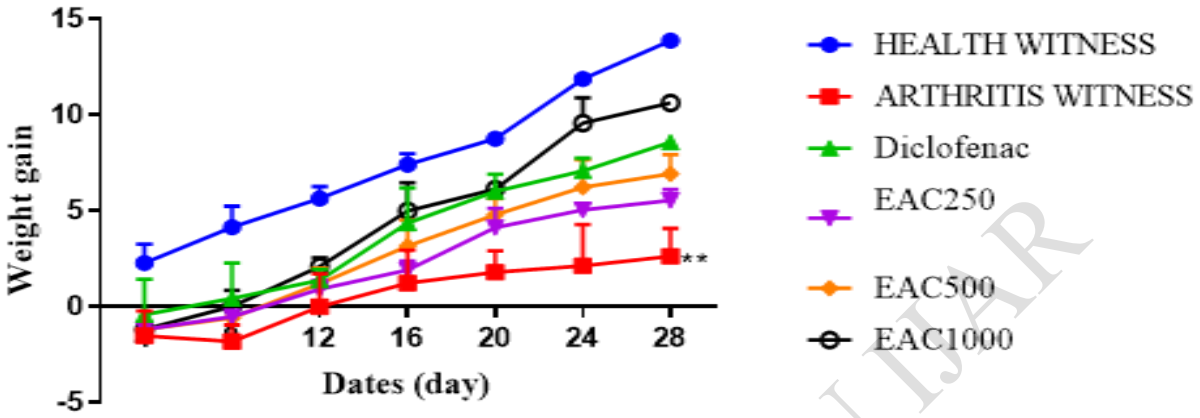
a decrease in the body weight of arthritis rats. The weight of the arthritis controls decreased significantly. The weight gains in grams of rats on the 4th day of the experiment were 2.27±0.69, -1.52±0.9, -0.42±0.1, -1.187±0.04, -1.19±0.01 and -1.19±0.02 respectively for the no control, arthritis control, diclofenac batch and the batches treated with the extract at doses of 250, 500 and 1000 mg/kg/bw. A clear recovery of the weights of the rats of the batches treated with diclofenac and the plant extract at the dose of 1000 mg/kg/bw with 0.429±0.13 g and 0.005±0.5 g respectively. For the other batches, recovery was observed on day 12 for the batches treated with *C. aurantifoliaextract* at doses of 250 and 500 mg/kg/pc with weight gains of 0.9±0.3 g and 1.18±0.5 g respectively. In the untreated arthritic rats, recovery in weight gain was observed on day 16 (1.22±0.11 g). No significant difference was observed between the weight gains of healthy control rats and arthritic rats treated with the extract and the reference molecule. However, there was a significant difference ($p<0.01$) between non-arthritic and untreated arthritic rats.



The results expressed are the means of the edema diameters of the arthritis rats untreated and treated with *Citrusaurantifolia* (standard error), with n=6. ****p<0.0001: statistically significant compared with arthritis witness group.

Fig. 1: Variation in the diameters of edematous legs





The results expressed are the average weight gains during the experiment (standard error), with n=6. **p<0.01: statistically significant compared with arthritis witness group.

Fig. 4: Effects of the extract on weight gain in arthritic rats

Discussion

The phytochemical screening has revealed the presence of tannins, sterols and terpenes, anthocyanins and flavonoids in the leaves of the lemon tree. These results allow to say that this plant can have very interesting medicinal and biological activities. The presence of alkaloids in the leaves indicates that they may have antibiotic activities (Sandrine, 2004).This could also justify the use of the decoction of the leaves for the treatment of some diseases such as Helminthics and malaria **(Béné et *al*, 2016).**The presence of the different metabolites in the different aqueous extracts could favor the use of leaf decoctions in traditional medicines. Moreover the massive presence of polyphenolic compounds, allows us to say that the plant could have anti-inflammatory, antioxidant, antiviral activities **(Ecra, 2001)**.

These results show us that the leaves of *Citrusaurantifolia*could have very interesting biological and medicinal activities.

CFA-induced arthritis in rats is a chronic inflammatory disease characterized by joint destruction, pain, swelling, tenderness and difficulty in movement. This inflammation is mediated by chemical mediators, chemotactic factors, leukocyte and phagocyte migration causing cartilage and tissue damage. The paw eodema is an index for measuring the anti-arthritic activities of various drugs in this model. The ACF-induced arthritis model in rats has many similarities to the arthritis model in humans (Tag et *al*., 2014). The ACF-induced arthritis model

is a model used to evaluate the anti-inflammatory efficacy of products for the treatment of arthritis (Jaijeshet *al*., 2008, Lin et *al*., 2017). ACF-induced arthritis showed chronic inflammation from day 1 and peaks on the 8 day of experimentation in arthritic rats. This inflammation persisted for the next few days until the end of the experiment. Indeed, according to RayhanaRayhana (2014)the ACF-induced arthritis showed a chronic inflammation, the first 2 to 4 days and this chronic inflammation persists the next weeks. However, the standard drug (sodium diclofenac) and aqueous extract of *C. aurantifolia*barks significantly suppressed the edema of the rats' paws. The reduction in edema could be due to the inhibition of leukocyte infiltration as well as inhibition of bone erosion by the diclofenac molecule and aqueous extract of *C. aurantifolia*leaves (NargarkaretJaglap, 2017; Bhatt et *al*., 2017).The weight of the animals was used to assess the physiological state of the animals, including the effect of the molecules and the lemon leaf extract on the weight of the animals with arthritis **(Winder et *al.*, 2005).**Moreover, the incidence and severity of arthritis caused body changes in the rats from the first day of induction by a considerable decrease in their body weight. The decrease in body weight of arthritis-induced animals would be related to either the systemic disease (BeutleretCerami, 1989).This decrease could also be associated with a decrease in locomotion supported by an increase in edema or, by a reduction in food consumption and metabolic changes (Alabarseet *al*., 2018 ;Filippinet *al*., 2013).In our study, from the 8th day of treatment, the weights of arthritic animals treated with diclofenac and *C. aurantifolia*leaf extract were stabilized. In contrast, the untreated arthritis rats had their weights stabilized from day 16 onwards. The resumption of the weight increase of the rats treated with the extract seems to be correlated to the anti-inflammatory action of the active principles present in the plant (Ramprasathet *al*., 2006).This effect testifies to the anti-inflammatory and anti-arthritic effect of the aqueous extract of the plant leaves.

Conclusion

This study is part of the research of new therapeutic sources from plant resources. This study confirms the anti-inflammatory virtue of the aqueous extract of the leaves of citrus *aurantifolia*since it significantly reduces the edemas of the legs of rats induced by the complete solution of Freud's Adjuvant. This experimentation allowed to evaluate the anti-arthritic activity of the plant. Further analysis will allow to quantify this anti-arthritic activity by establishing a precise dose-effect relationship and then to look for a stable galenic formulation that can be used to support this activity in order to make it a medicine against arthritis pathologies.

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