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Acute toxicity, anti-inflammatory and analgesic effects of aqueous extract of *Tetracera alnifolia* Willd. (Dilleniaceae)

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ABSTRACT

This work is part of the valorization of the traditional use of *Tetracera alnifolia* (Dilleniaceae). In this study we evaluated acute toxicity, anti-inflammatory and analgesic effects in rodent, and chemically screened its aqueous leaves extract. The phytochemical classic tests allowed to identify his various chemical families. Standard procedures by using OECD Guideline were followed to evaluate toxicity in mice. To assess anti-inflammatory the carrageneen induced paw oedema and the pellet of cotton were used. Acetic acid and rat paw pressure methods served for the evaluation of the analgesic. This plant is very rich in flavonoids, cardiotonic heterosids and saponosides, and rich in alkaloids, steroids, terpenoides and tannins. Leaves aqueous extract is considered low-toxic with an LD50 greater than 2000 mg / kg. At this dose the extract exhibits signs of toxicity inclunding dyspnea and motor impairment. At the doses of 200 and 400 mg/kg the extract showed a significant effect on carrageenan-induced edema ($p< 0.001$), acetic acid induced abdominal cramps ($p< 0.001$) and on the nociceptive freshold($p< 0.001$) and latency response of paw pressure ($p< 0.001$). These effects are due to presences of its secondary metabolites. This pharmacological results confirm the traditional uses of this plant underlined on stomach pain, swelling and gout.

Key words: Traditional medicine, *Tetracera alnifolia*, Aqueous extract, Phytochemistry, Acute toxicity, Antiinflammatory, Analgesic.

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Introduction

Plant treatment is a major alternative for populations in developing countries because it is cheaper, very often, well tolerated, accessible and available. Plant-based medicine has a socio-environmental and cultural integration in the countries of Africa. Plants that humans use in Africa can be found in household or in the geographical areas where their activity occurs daily and are part of their ethnic or tribal culture. It can therefore be said that the socio-cultural and geographical environment is the local pharmacy in sub-Saharan Africa. However, scientific validation of these treatments is necessary in order to guarantee safety and assurance on their effectiveness among users.*Tetracera alnifolia* Willd is used in traditional medicine to treat malaria, diarrhea, stomach pain, gonorrhea, inflammation, pain, cough, sexually transmitted infections and to purify breast milk [Arkinstall, 1979 ; Adjanohoun, 1988 ; Nsonde Ntandou et *al*., 2005 ; Kayode, 2008 ; Betti et *al*., 2013 ; Baldé et *al*., 2014]. *Tetracera alnifolia* Willd is one of the herbs included in the recipe of the improved Congolese drug, Tetra®. It is from this plant that the commercial name of Tetra comes. Tetra® is indicated for inflammation and pain (Abena et al., 2002). This work is part of the valorization of the traditional use of *Tetracera alnifolia* (Dilleniaceae). In this study we evaluated acute toxicity, anti-inflammatory and antalgic effects in vivo, and perform chemical screening of the aqueous extract of *Tetracera alnifolia* (Dilleniaceae) leaves.

Material and methods

Plant material

The leaves of *Tetracera alnifolia* Willd (Dilleniaceae) were collected March 25th, 2015 from Makana village (Pool, Republic of Congo). The plant was identified by Professor Jean-Marie MOUTSAMBOTE a voucher specimen was deposited and registered at the National herbarium under the noumero B. Descoings № 5606. To dry, these leaves were spread on a bench for three weeks in the Laboratory of Physiologie et Physiopathologie Animales of the Faculté des Sciences et Techniques at the Université MarienNgouabi, Brazzaville, in the Republic of Congo.Then they were sprayed with a mortar. The powder obtained after spraying was used as the final vegetable material for the study.

Animals

Wistar male and female rats 18-20 weeks old, weighing between 130 and 260 g, and male and female albino mice weighing between 17-30 g were used. They were obtained from the animal house of the Faculté des Sciences et Techniques of the UniversitéMarien NGOUABI of Brazzaville, Republic of Congo. These animals were kept under standard conditions (25 ± 5° C, 40-70 % RH, 12h light/dark cycle) and had free access to standard water and food. The ethical rules published by International Association for the Study of Pain (Zimmermann, 1983) were respected.

Extract preparation

50 g of *Tetracera alnifolia* Willd powder was dissolved in 500 ml of distilled water. The resulting mixture was boiled at 100 ° C in a thermostatically controlled water-bath for 25 minutes. The resulting solution was filtered, and the collected filtrate was concentrated to half in a water- bath set at 55 ° C.

Acute toxicity

The acute toxicity class method described in the OECD guideline 423 (2001) was used. This method is used to classify substances in order of toxicity in a similar way, and is not intended to calculate a specific LD50 value. The absence or manifestation of the mortality associated with the substance at any dose at a group stage determines the next step. These may include: stopping the test, administering the same dose to three additional animals and administering a dose immediately above or below to three additional animals. Two hours before the test, mice with free access to water were deprived of food. Five (5) groups of 3 females each were formed. Each mouse was labeled for individual identification. Each group was placed in a cage. The 1st group (control) received 10 ml / kg of distilled water orally per mouse, the 2nd and the 3rd groups (tests) were treated respectively with doses of 300 and 2000 mg / kg per mouse with the extract orally. After administration of the products, mice were observed individually at 1 /2h, 1h and 2h the first day, in order to note the immediate signs of intoxication, namely dyspnea, salivation, motility disorders, diarrhea, convulsion and ptosis . Mortality was assessed over 72 hours. The

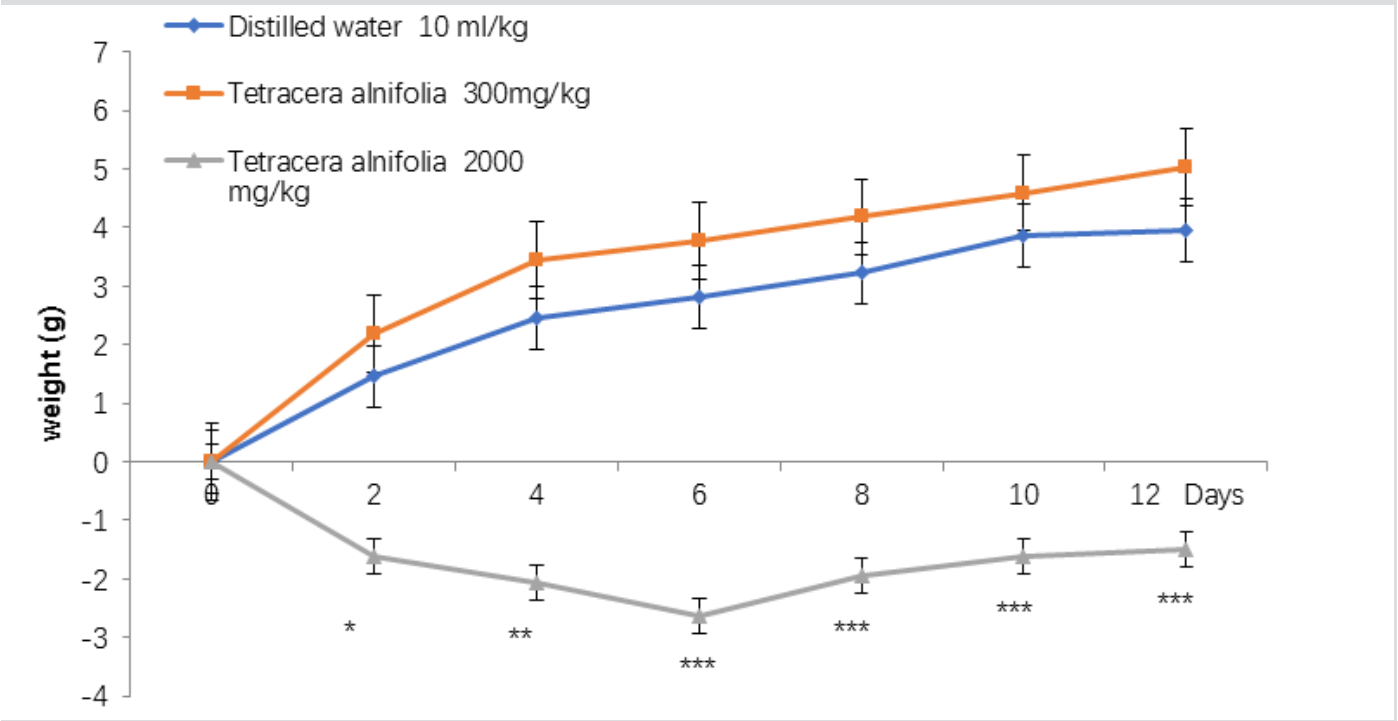


Figure 1. Effect of *Tetracera alnifolia* Willd leaves aqueous extract on the evolution of body weight in albino mice. The results are expressed as mean ± standard error, n = 3 rats per group, * p <0.05; ** p <0.01; *** p <0.001 compared to the control group.

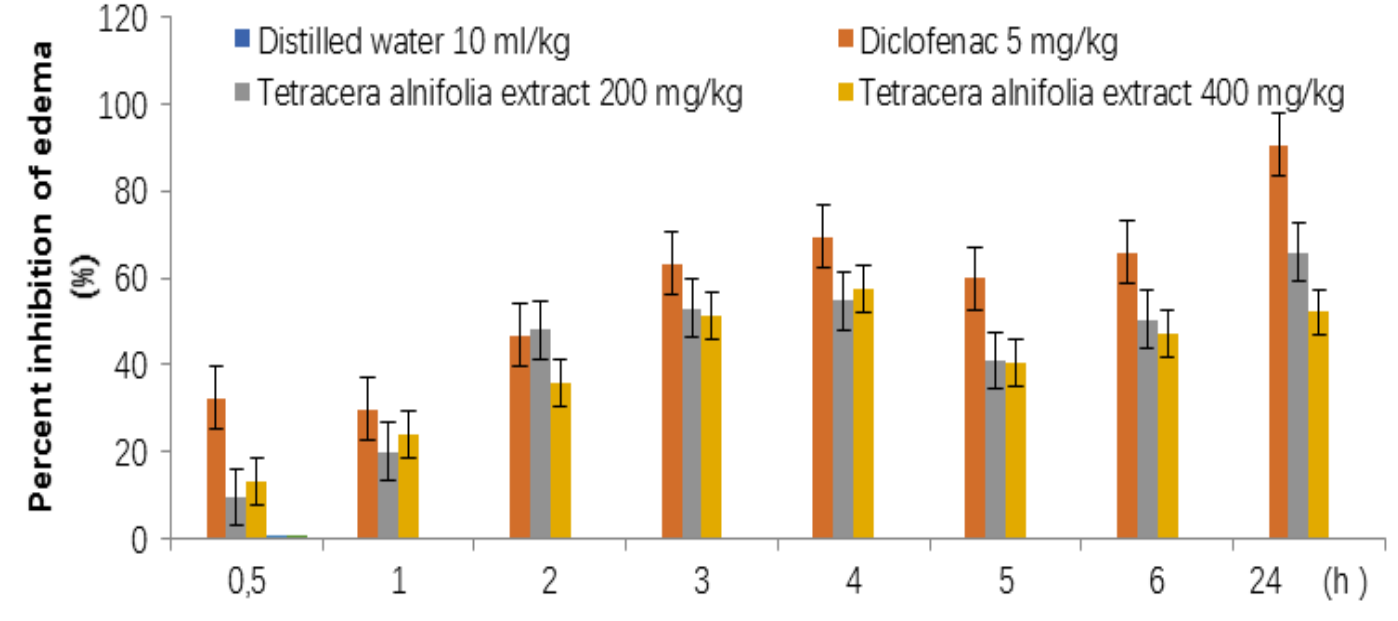


Figure 2. Percent of anti-inflammatory effect of the aqueous extract of the leaves of Tetracera alnifolia Willd on carrageenan-induced edema in the Wistar rat. The results are expressed as mean ± standard error, n = 5 rats per group.

other observations were made during 14 days following the administration of the products, and covered: mobility, nature and frequency of faeces, sleeping, duration and frequency of sleep, condition of the hair, skin condition, convulsions, eye condition or form and respiratory activity. The weight of each mouse was measured every 2 days for a period of 14 days.

Anti-Inflammatory Activity

Study of acute inflammation

The effect of the aqueous extract on acute inflammation was evaluated according to the carrhagenin-induced paw edema method described by Nsonde Ntandou et al. (2010). An anti-inflammatory substance inhibits this increase compared to the control which receives distilled water.Four (4) groups of five (5) rats each were formed. The 1st group (control) received 10 ml / kg of distilled water orally per rat. The second group received 5 mg / kg diclofenac, used as a reference molecule, orally per rat. The 3rd and 4th groups (tests) received respectively 200 and 400 mg / kg of extract orally per rat.One (1) hour after oral administration of the products, animals received a subplanar injection of 0.05 ml of 1% carrageenan (dissolved in 0.9% NaCl) on the right hind paw. After the injection of carrageenan, the evolution of the edema is monitored by measuring the volume of the paw at ½ , 1, 2, 3, 4, 5, 6 and 24h using a plethysmometer 7140 Ugobasile, Italy. The anti-inflammatory effect was determined by the percent inhibition (% PI) of the inflammation.

Study of chronic inflammation

The effect of the extract on chronic inflammation was studied using the cotton pellet method described by Mossa et al. (1995). Indeed, the cotton pellet introduced into the animal's body is considered a persistent foreign body. This cotton pellet with the defense cells of the organism will form fibrosis. An anti-inflammatory substance reduces the granuloma mass compared to the control that receives distilled water.100 mg of cotton pellet was sterilized at 60 ° C for 24 hours. The sterilized cotton pellet was introduced by incision into the subcutaneous area of the shoulder of each rat after anesthesia with ether. Then the incision was closed by suturing with needles. The animals were treated with oral administration of the products for seven (7) days. Three (3) groups of five (5) rats each were formed. The first group considered as control received 10 ml / kg of distilled water per rat; the second group considered as a reference received 5 mg / kg of diclofenac per rat and the third group considered as a test received 200 mg / kg of the extract per rat. For ethical reasons, we used the 200 mg / kg dose to evaluate the chronic anti-inflammatory effect.On the eighth day, the cotton pellet is removed, these tissues are excised, carefully trimmed of excess adhering tissue and dried in an oven at 60 ° C, during 24 h and then weighed. The anti-inflammatory effect is determined by the percent inhibition (% PI) of granulomatous tissue formation.

Analgesic effect

Rat hind paw pressure test

The pain was induced by the pressure exerted on the right hind paw of rat using a Cat.no analgesimeter. 37215, Ugo Basile, Italy [Kissin et al., 2000; Nsonde-ntandou et al., 2010]. An analgesic substance increases both intensity and duration of reaction as compared to the control which receives distilled water. Four (4) groups of five (5) rats each were formed. The 1st group (control) received 10 ml / kg of distilled water per rat, the second group received 30 mg / kg of tramadol, used as reference molecule, per rat, the 3rd and 4th groups (tests) were Received respectively 200 mg / kg and 400 mg / kg of the extract per rat. One (1) hour after the oral administration of the products, the pain was induced by pressure of the right hind paw. The rat is immobilized and its right hind leg (at the level of the plant) is placed on the base. Then, the threshold of sensitivity of the animal is noted, which corresponds to the mass at which it removes its paw. This makes it possible to determine the reaction time that the animal takes to remove its paw.

Acetic acid test

The effect of the extract on the pain induced by 0.6% acetic acid was evaluated according to the method described by Koster (1959). It consists in causing visceral pain in the mouse by intraperitoneal injection of 0.6% acetic acid. In fact, acetic acid causes five (5) minutes after injection, abdominal pain which manifests itself by torsions of the dorso-abdominal muscles or cramps. An analgesic substance reduces the number of

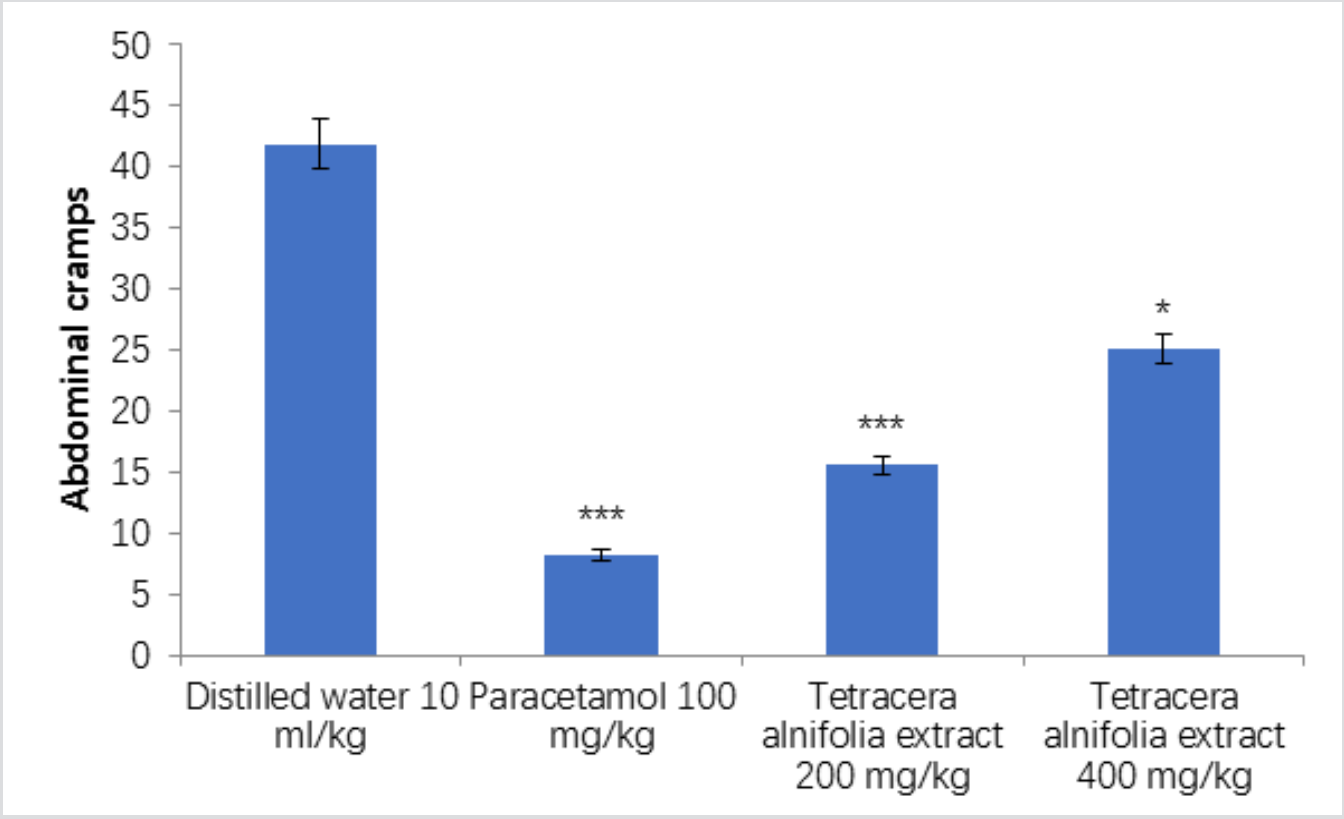


Figure 6-a. Effect of *Tetracera alnifolia* Willd leaves aqueous extract on the pain induced by the acetic acid 0.6% in the albino mouse. The results are expressed as mean ± standard error with * p <0.05; *** p <0.001 compared to control, n = 5 rats per group.

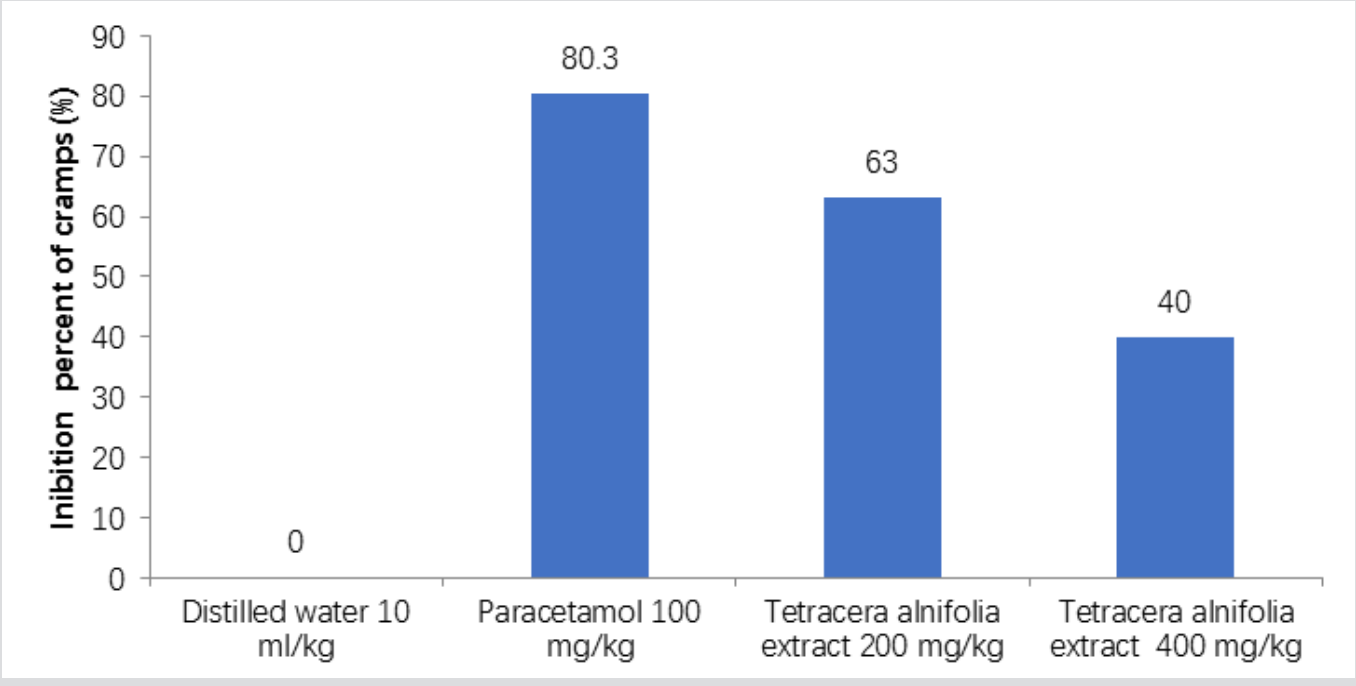


Figure 6-b. Percent inhibition of cramps of *Tetracera alnifolia* Willd leaves aqueous extract of on the pain induced by the acetic acid 0.6 % in the albino mouse.

cramps compared to the control group that received distilled water. The 1st group (control) received 10 ml / kg of distilled water per rat, the 2nd group received 30 mg / kg of tramadol, used as reference molecule, by rat, the 3rd and 4th groups of the tests received respectively 200 and 400 mg / kg of the extract per rat. 1 h after oral administration of the products, acetic acid was injected at 0.6% (0.1 l / 10 g weight of animalip) and the cramps performed by each mouse were immediately counted for ten 10 minutes [Fábio et al., 2001]. The analgesic activity was expressed as percent inhibition (% PI) of the pain.

Results

Acute toxicity

General comment

The results of the acute toxicity study are shown in Table I. The extract (300 mg / kg) has not caused mortality but alters the general behavior of the animals on the day of treatment. One death in three mice was recorded in the group of mice treated with the extract at a dose of 2000 mg / kg. At this dose, the extract exhibits signs of toxicity, including dyspnea and motor impairment. The LD50 of these extracts is estimated to be greater than 2000 mg / kg according to the SCGH [OECD 423, 2001].

Effect of extract on body weight of mice

The effect of the extract on the body weight of the mice is shown in figure 1. At 300 mg / kg of the extract, a not significant increase in body weight was observed the 4th, 6th and 12th days. The aqueous extract of *Tetracera alnifolia* Willd (2000 mg / kg), causes a significant loss of body weight, remarkable the 2nd(p< 0,05), 4th (p< 0,05), 6th (p< 0,001) and 12th (p< 0,001) days; followed by progressive weight gain the following days.

Anti-inflammatory activity

Effect on the ratpaw edema induced by carrageenan

The extract at 200 and 400 mg / kg showed a significant anti-inflammatory effect on carrageenan-induced edema (Table II). This effect is very marked from the 4th hour after induction of the edema (figure 2).

Effect on the formation of granuloma

The inhibition of the formation of cotton pellet granuloma by extract at the doses of 200 mg/kg is not important in comparison with the control (Figures 3 and 4)

Analgesic activity

Effect on the mechanical pressure of the rat's hind paw

The figure 5 shows the effect of the extract at doses of 200 and 400 mg / kg on the pain induced by the pressure of the paw. The results are presented on the one hand as a function of the threshold intensity (figure 5-a) which corresponds to the mass at which the animal withdraws its paw and on the other hand as a function of the threshold time (Figure 5-b) that the animal puts to react to the stimulation. This extract significantly inhibits pain induced by mechanical stimulation.

The results of the effect of the extract on the abdominal cramps induced by acetic acid are presented in figures 6-a and 6-b. This extract at 200 mg / kg significantly protects the mice against pain induced by 0.6% acetic acid (p <0.001) (Figure 6-a), with a percentage inhibition of 63% (figure 5-b). The paracetamol (reference) reduce significantly (p<0,001) the number of abdominal crampswith 80,3% (figure 6-b).

Chemical screening

The results of the chemical screening of the extract are recorded in Table III. In this extract there are more saponosides, cardiotonic heterosides and flavonoids but not anthocyanins.

Discussion

The objective of the first part of the study was to evaluate the acute toxicity of aqueous extracts of *Tetracera alnifolia* Willd (Dilleniaceae) leaves in mice.Indeed, the study of the acute toxicity of an extract is indispensable to determine the LD50 which helped to adapt the treatment to the limits of tolerance, and therefore to fix the therapeutic dose. The aqueous extract of *Tetracera alnifolia* Willd (300 mg / kg) caused no mortality, whereas at the 2000 mg / kg dose, it caused one death. However, this extract is considered to be low toxic with the LD50 greater than 2000 mg / kg according to the SCGH [OECD 423, 2001]. This makes it possible to classify it in category 5 of low toxic substances. Doses of 200 and 400

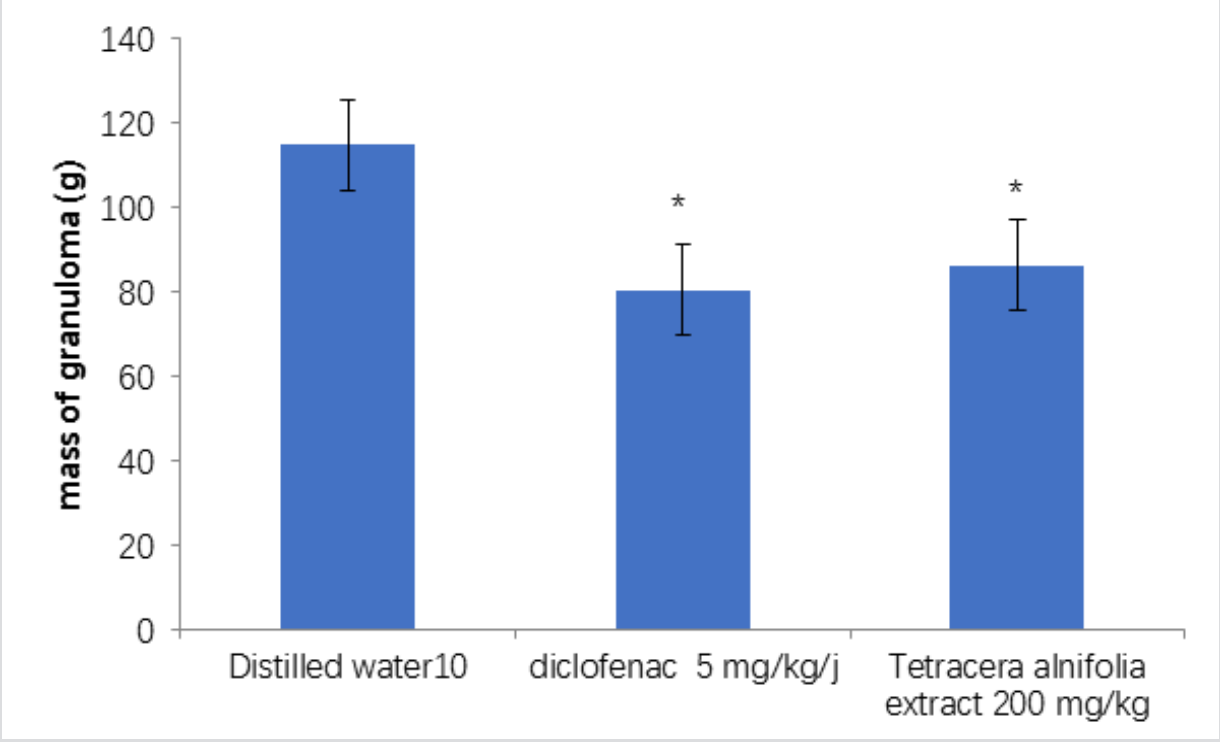


Figure 3. Anti-inflammatory effect *Tetracera alnifolia* Willd leaves aqueous extract on the formation of granulomatous tissue in the Wistar rat. The results are expressed as mean ± standard error, n = 5 rats per group. * P <0.05 and ns: not significant compared to the control.

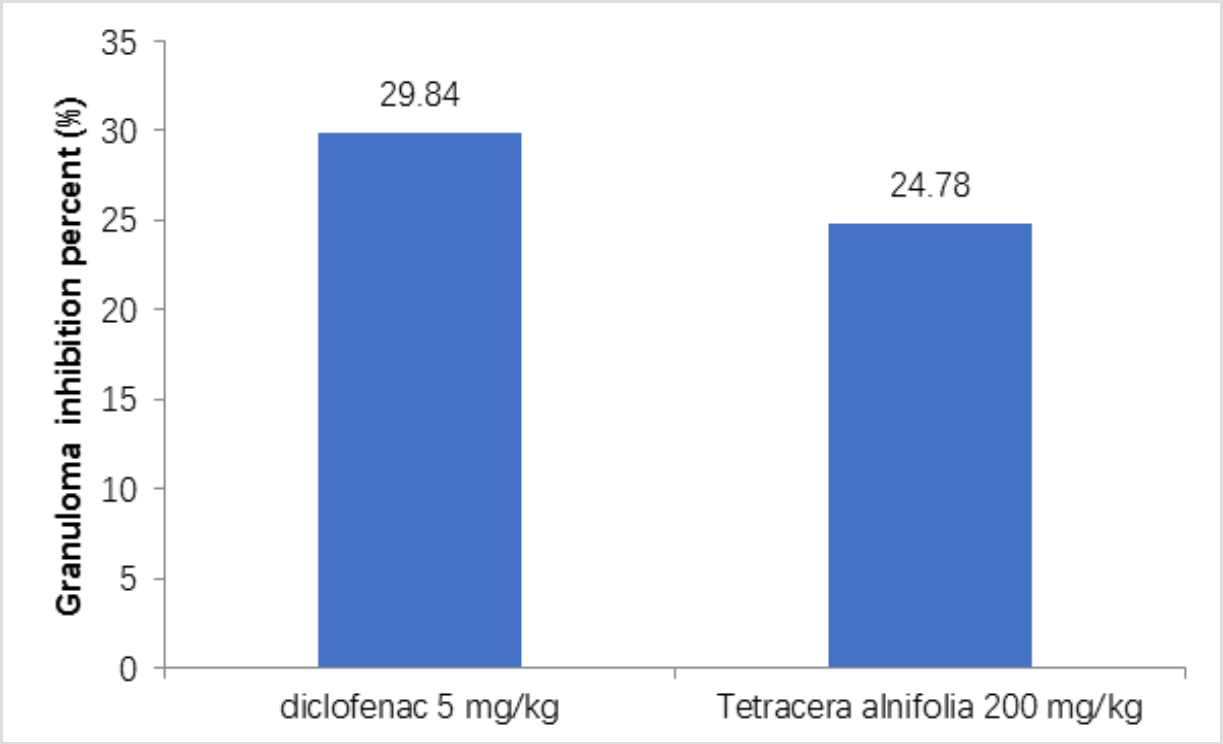
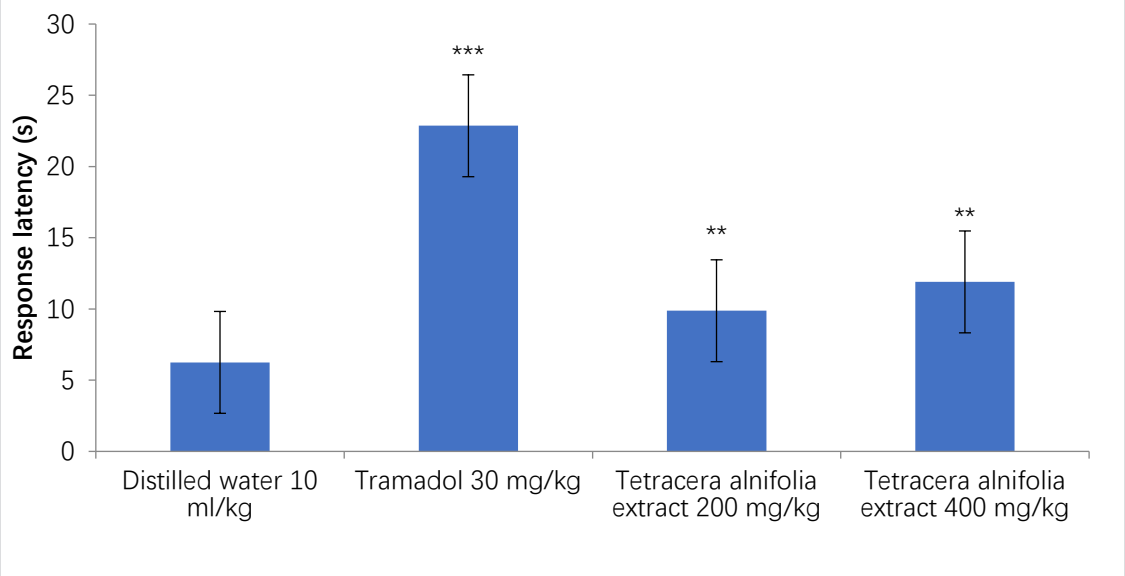


Figure 4. Inhibition percents of *Tetracera alnifolia* Willd leaves aqueous extract on the formation of granulomatous tissue in the Wistar rat. The results are expressed as mean ± standard error, n = 5 rats per group. * P <0.05 and ns: not significant compared to the control.



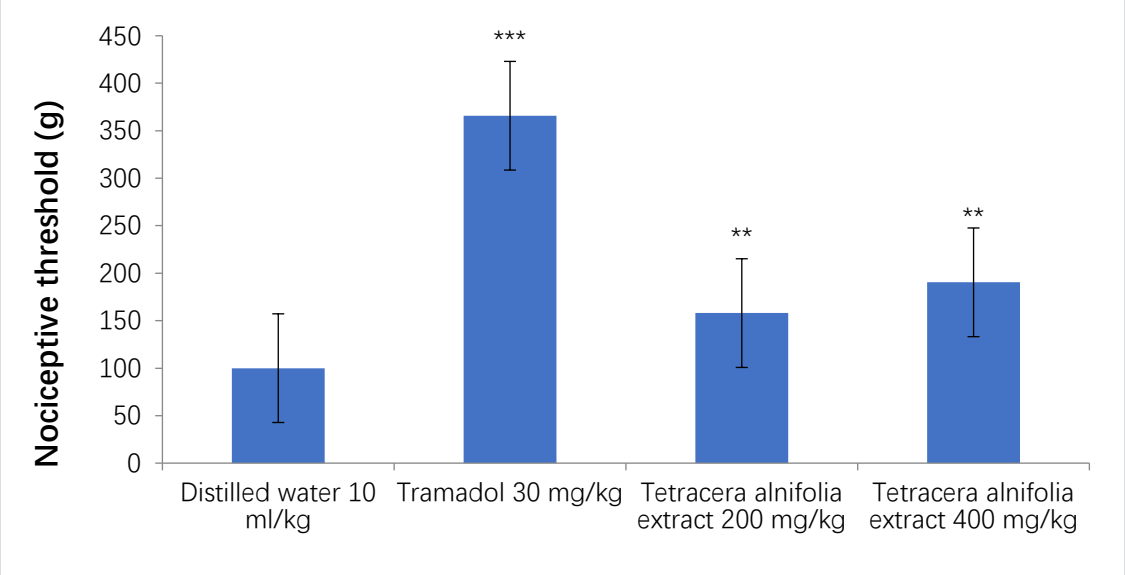


Figure 5 (a-b). Effects of leaves aqueous extract of *Tetracera alnifolia* Willd on pain induced by the rat paw pressure. The results in grams (a) and second (b) are expressed as mean ± standard error with ** p <0.01, *** p <0.001 compared to control, n = 5 rats per group.

Table I Effets of *Tetracera alnifolia* Willd leaves aqueous extract on acute toxicological parameters

Doses of extract (mg / kg)	Number of animals at start of study	Signs of toxicity	Signs of Toxicity	Number of death	Limit of LD50	OECD Extract Class
300	3	Little mobile, existence of sleep after 1 hour, half-closed eyes (ptôsis)	3	0	> 2000 mg/kg	Category 5 LowToxicity
2000	3	Immobile, insensitive to noise and touch, long and frequent sleep, erect hairs, ptôsis, dyspnea	3	1	> 2000 mg/kg	Category 5 LowToxicity

mg / kg, well below 2000 mg / kg were therefore selected for pharmacological studies. The aqueous extract of *Tetracera alnifolia* Willd at a dose of 2000 mg / kg causes significant body weight loss.Moreover, during the observation period on the behavior of the mice, there was a refusal to feed even when they were brought close to the food, dyspnea, insensitivity to noise and touch, reduction in mobility, as well as the complete immobilization of animals. These results suggest that the extract at a dose of 2000 mg / kg alters traction and hence prevents mice from gaining access to the feed. This suggests that the decrease in weight could be due to a decrease in consecutive food intake, either to the inability to move towards the food, or to the impairment of digestive functions. This extract also has inhibitory influences on respiratory function and sensitivity. These results are in agreement with those of Nwabueze (2013) which showed a toxicity of this same extract in the leech. Indeed, any biologically active substance is capable, at high or low dose, of producing adverse effects [Ouedraogo et al., 2001]. In addition, these secondary metabolites produced by the plant to protect themselves from herbivores may be harmful to any animal [Chitra et al., 2009].

The second part of the study aimed to evaluate the anti-inflammatory effect of the extract in the rat. To do this, anti-inflammatory activity was performed using two models of inflammation induction: carrageenan edema in the study of acute inflammation and cotton pellet in the study of chronic inflammation. Carrageenan edema is an excellent representative model of acute inflammation [Nsonde-ntandou et al., 2010; Elion-itou et al., 2014]. Indeed, carrageenan is a polysaccharide which injects into the animal induces local inflammation characterized by increased vascular permeability, edema and extravasation of neutrophils [Gamache et al., 1986]. The action of carrageenan is biphasic. The first phase (1/2 to 1 hour) involves the release of the amino-vasoactive mediators (histamine, serotonin); the second phase (1 hour after) would be mediated by prostaglandins. The kinins intervene between the two phases [Perianayagam et al., 2006].By this method, this extract at the doses used has an anti-inflammatory effect which is very interesting and virtually identical to that of diclofenac, used as a reference drug. The extract at 400 mg / kg per os is effective from the first hour. This suggests that they will affect the effect of histamine and / or serotonin. This extract (200 and 400 mg / kg also significantly inhibits the evolution of edema until the last phase of carrageenan-induced inflammation. This effect could be explained by the inhibition of the liberation of the prostaglandins and / or the reduction of their effect. These results are in agreement with those of Oyebanji which has demonstrated an anti-inflammatory effect of *Tetracera potatoria* which has the same genus and family with*Tetracera alnifola* [Oyebanji et al., 2013].Acute inflammation may be complicated by the persistence of the pathogen or by an exaggeration of cell functions, hence the need to study chronic inflammation. Cotton pellet granuloma is an excellent model of chronic inflammation to study the proliferative phase of inflammation [Abena et al., 1997;Mahadi Hasan et al., 2014]. The inflammatory response such as extravasation, granuloma formation, and cotton pellet exudate could be easily detected through this technique [Singh et al., 2012].The extract (200 mg / kg) did not show a significant effect on the cotton pellet test, but on carrageenan-induced edema the inhibition was significant. This result suggests that the aqueous extract of *Tetracera alnifolia* Willd has an effect against acute inflammation. It would oppose the release of pro-inflammatory mediators thus preventing vasodilation and increasing vascular permeability thus the evolution of edema. The dose of 200 mg/kg does not have a significant inhibition effect on granuloma because it is too low. The active principle provided at this dose is not sufficient to induce a significant effect, because the effect depends on the dose.

Our findings are consistent with those found with Tetra®, a traditional Congolese recipe combining some 20 plants, including *Tetracera alnifolia* Willd [Abena et al., (2002)]. A clinical study of the anti-inflammatory properties of this improved traditional drug has shown comparable effects to ketoprofen, characterized by a decrease in swelling and plasma levels of inflammatory proteins [Abena et al., (2002)]. This would justify a mechanism of action by inhibiting cyclooxygenase.The results obtained in this study suggest that *Tetracera alnifolia* Willd is partly responsible for the anti-inflammatory activity of Tetra®. They confirm the therapeutic indications of this medicine and consequently constitute a support for its use.

Table II. Effect of the aqueous extract of the leaves of *Tetracera alnifolia* Willd on carrageenan-induced edema in the Wistar rat. The results are expressed as mean ± standard error, n = 5 rats per group, * p <0.05; ** p <0.01; *** p <0.001 and ns: not significant compared to the control group.

Treatment	Dose mg/kg	Volume of edema in ml							
		1/2h	1h	2h	3h	4h	5h	6h	24h
Distilled water	10 ml/kg	0,664±0,08	1,006±0,079	1,576±0,060	2,37±0,061	2,87±0,072	2,208±0,086	1,976±0,091	1,228±0,082
Diclofenac	5	0,448±0,06ns	0,706±0,06*	0,836±0,07***	0,868±0,07***	0,872±0,07***	0,882±0,08***	0,672±0,09***	0,114±0,01***
Extract	200	0,6±0,07ns	0,804±0,08ns	0,816±0,07***	1,112±0,05***	1,292±0,08***	1,302±0,08***	0,98±0,05***	0,418±0,08***
Extract	400	0,576±0,06ns	0,764±0,03*	1,008±0,06***	1,15±0,09***	1,212±0,07***	1,314±0,05***	1,046±0,08***	0,588±0,04***

Table III. Secondary metabolites contained in the aqueous extract of *Tetracera alnifolia* Willd.

Chemical Families	Plant
	Tetracera alnifolia
Alkaloids	+
Anthocyanins	-
Flavonoids	++
Cardiotonicheterosides	++
Saponosides	++
Steroids and Terpenoids	+
Tannins	+

++ Very present, + present, - absent.

The aim of the last part of this study was to evaluate the analgesic effect of the extract in rodents (rats and mice) using acetic acid and paw pressure. The acetic acid test is well known for the evaluation of an analgesic effect with a mechanism of action of the peripheral type [Oyebanji et al., 2013; Makambila-koubemba, 2014]. Indeed, acetic acid sensitizes local peritoneal receptors to prostaglandins PGE2 and PGF2**α** [Deraedt et al., 1980]. The paw pressure test using the analgesimeter is one of the reliable methods for evaluating an analgesic effect with a central mechanism of action [Nsonde-Ntandou et al., 2016]. In this model, opioid analgesics are very effective [Nkeh et al., 2002].The extract at doses of 200 and 400 mg / kg significantly inhibits pain caused by pressure on the rat paw. These results suggest that this extract could inhibit the release of algogenic mediators. At the dose of 200 mg / kg, this extract significantly inhibits pain caused by acetic acid. This effect could be explained by the inhibition of prostaglandin synthesis. These results are in agreement with those found Oyebanji et al. (2013) which demonstrated an analgesic effect of *Tetracera potatoria* (Dilleniaceae).The classical chemical analysis of the extract has shown that this plant contains saponosides, flavonoids, tannins, alkaloids, steroids and terpenoids. Our results are therefore in agreement with those of these authors [Lawal et al.,2011 and Lima et al., 2014].The toxicity, the anti-inflammatory ant the analgesic effects could be explained by the presence in this plant of flavonoids, anthocyanins, saponosides, tannins, steroids and triterpenoids, which are known for their anti-toxicity, analgesic and inflammatory properties [Thongsaard et al., 2001 ; Bruneton, 2009]. The pharmacological results obtained confirm the traditional uses of *Tetracera alnifolia* Willd underlined by Adjanohoun (1988) and Burkill (1985) on stomach pain, swelling and gout.

Acknowledgments

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