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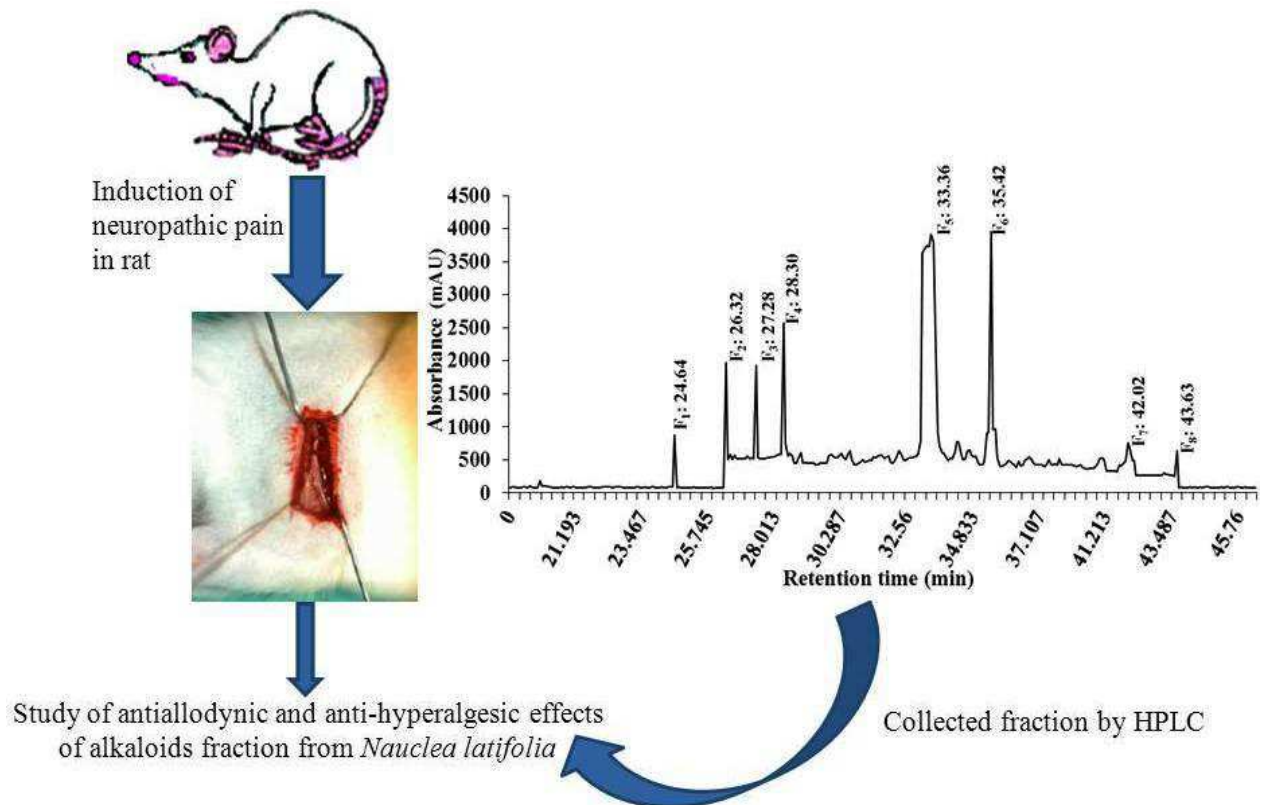
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# Graphical abstract

*Nauclea latifolia* Smith (Rubiaceae) exerts antinociceptive effects in neuropathic pain induced by chronic constriction injury of the sciatic nerve



***Nauclea latifolia* Smith (Rubiaceae) exerts antinociceptive effects in neuropathic pain induced by chronic constriction injury of the sciatic nerve**

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## **Abstract**

Ethnopharmacological relevance: The roots of *Nauclea latifolia* Smith (Rubiaceae) popularly known as “koumkouma” is used in traditional Cameroonian medicine as neuropathic pain remedy and for the treatment of headache, inflammatory pain and convulsion.

Aim of the study: This study was conducted to evaluate the antinociceptive effects of the alkaloid fraction isolated from *Nauclea latifolia* in neuropathic pain induced by chronic constriction injury (CCI) of the sciatic nerve in rat.

Materials and methods: Bioactive-guided fractionation of the root extracts of *Nauclea latifolia* using the Von Frey in a rat model of neuropathic pain (Benett model), afforded a potent anti-hyperalgesic fraction IV. Further fractionation of this fraction was performed by high-performance liquid chromatography (HPLC), yielded eight sub-fractions (F<sub>1</sub>–F<sub>8</sub>) which were tested for antinociceptive effects. The alkaloid fraction (F<sub>3</sub>) collected by HPLC, exhibited potent antinociceptive effects, and the anti-allodynic and anti-hyperalgesic effects of this fraction (8, 16, 40 and 80 mg/kg) were determined using the von Frey and acetone tests respectively in a rat model of neuropathic pain. Rota-rod performance and catalepsy tests were used for the assessment of motor coordination.

Results: The alkaloid fraction (80 mg/kg) administered intraperitoneally induced a completely decreased hyperalgesia 90 min post-dosing. In the acetone test, the *Nauclea latifolia* fraction at 80 mg/kg showed its maximal anti-allodynic effects 120 min post-injection. The areas under the curve (AUC) of the anti-allodynic or anti-hyperalgesic effects produced by the alkaloid fraction at 80 mg/kg were significantly ( $p < 0.001$ ) greater than the AUC of effects

produced by vehicle in CCI rats. The alkaloid fraction did not exhibit any significant effects on the spontaneous locomotor activity of the mice in rota-rod performance and no sign of catalepsy was observed.

Conclusion: The analysis of the effects, expressed as the time course of AUC, supports the traditional use of *Nauclea latifolia* in neuropathic pain therapy. The pharmacological and chemical studies are continuing in order to characterize the mechanism(s) responsible for this anti-hyperalgesic and anti-allodynic action and also to identify the active substances present in the roots extracts of *Nauclea latifolia*.

Keywords: *Nauclea latifolia*, neuropathic pain, anti-hyperalgesic, anti-allodynic, traditional medicine.

## Abbreviations

ANOVA, analysis of variance; ATCC, American Type Culture collection; AUC, areas under the curve; CCI, chronic constriction injury; CHO, chinese hamster ovary; EC<sub>50</sub>, the 50% effective concentration; F<sub>1</sub>, fractions; HPLC, high-performance liquid chromatography; LD<sub>50</sub>, median lethal dose; L-NAME, N<sup>ω</sup>-L-nitro-arginine methyl ester; MTT; 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; NIH, National Institutes of Health.

## 1. Introduction

The pathophysiological mechanisms underlying neuropathic pain have been reviewed extensively in recent years, and the results reflect both peripheral and central sensitization mechanisms (Moalem and Tracey, 2006). The International Association for the Study of Pain defines neuropathic pain as pain initiated or caused by a primary lesion or dysfunction in the nervous system (Merskey, 1991). Clinically, the presence of neuropathic pain is often

characterized by stimulus-independent persistent pain or abnormal sensory perception of pain, such as allodynia (a painful response to a normally innocuous stimulus) and hyperalgesia (exaggerated pain sensations as a result of exposure to a mildly noxious stimulus) (Ueda and Rashid, 2003). Neuropathic pain is pain that arises from damage to the part of the nervous system that carries sensory information to the brain. It is difficult to treat because of its severity, duration and resistance to simple painkillers.

Examples of central neuropathic pain include post-stroke pain, affecting up to 8% of post-stroke patients, and neuropathic pain associated with multiple sclerosis in 50% of patients and with spinal cord injury in 40% of patients (Werhagen et al., 2007). Peripheral neuropathic pain states include painful diabetic neuropathy affecting approximately 25% of people with diabetes, sciatica, post-surgical and post-traumatic neuralgias. Post herpetic neuralgia is an example of a mixed neuropathic pain, with both peripheral and central mechanisms, affecting a significant proportion of patients following shingles (Andersen et al., 1995; Davies et al., 2006; Hewitt et al., 1997; Jung et al., 2004; Kehlet et al., 2006; Osterberg et al., 2005; Siddall et al., 2003). Most patients with neuropathic pain respond poorly to traditional analgesics and many require a multidisciplinary approach. With current available treatments, only 30-50% of patients with neuropathic pain experience meaningful improvement in pain and function, and a long-term commitment from the patient and physician are required to ensure compliance and appropriate outcomes (Bennett, 2007).

In the north of Cameroon, the leaves of *Nauclea latifolia* Smith (Rubiaceae) is used in traditional medicine for the treatment of cerebral malaria, behavioural disturbances in mentally-retarded children or central nervous system diseases, such as anxiety, depression and epilepsy (Dalziel, 1937; Adjanohoun et al., 1996; Arbonnier, 2000). The roots decoction of *Nauclea latifolia* is effective in cases of fever, headache, migraine, pain, inflammatory disorders and neuropathic pain (Biholong, 1986; Adjanohoun et al., 1996; Arbonnier, 2000;

Amos et al., 2005). Interested in investigating the pharmacological properties of *Nauclea latifolia* Abbah et al. (2009) and Taiwe et al. (2011) have demonstrated that oral administration of *Nauclea latifolia* extracts cause antipyretic, antinociceptive and anti-inflammatory effects. Earlier chemical studies of *Nauclea latifolia* have indicated the presence of Naucleamides A–F, a new monoterpene indole alkaloids, strictosamide (Shigemori et al., 2002, Ata et al., 2009), flavonoids, saponins, tannins, anthraquinones and phenols in the *Nauclea latifolia* extracts (Ngo Bum et al., 2009; Taiwe et al., 2011).

In the present study, in order to provide a pharmacological basis to the traditional use of *Nauclea latifolia* as antinociceptive therapy in neuropathic pain and to discover novel analgesic drugs from natural resources, we investigated the antiallodynic and anti-hyperalgesic effects of the alkaloid fraction from *Nauclea latifolia* roots on CCI of the sciatic nerve, a rat model of neuropathic pain, using the Von Frey and acetone tests.

## **2. Materials and methods**

### **2.1. Plant material**

The roots of *Nauclea latifolia* was harvested in March 2009 from the National Park of Benoué (North Cameroon) and identified in the National Herbarium, Yaoundé Cameroon by comparison to an existing voucher specimen No. 20144/SRF/Cam.

### **2.2. Solvent-guided fractionation of *Nauclea latifolia* and bioactivity guided studies**

The dried and powdered roots of *Nauclea latifolia* (1000 g) were extracted with acetone/H<sub>2</sub>O (7:3; 5 l) at room temperature. The combined extracts were evaporated *in vacuo* to afford a dark residue (381.61 g). The residue was suspended in warm water (1 l) and then extracted successively with ethyl acetate (0.5 l × 3) and n-butanol (0.5 l × 3), and concentrated to give residue A (133.25 g) and B (371.14 g), respectively. The latter was

resolved in warm water (1 l), acidified with HCl (1 N) to pH 4-5, and extracted with CHCl<sub>3</sub> (0.5 l × 3). The aqueous layer was neutralized with NaOH (1 N) to pH 9-10 and extracted with CHCl<sub>3</sub> (0.5 l × 3) once again and concentrated *in vacuo* to obtain the crude base (136.12 g; 36.67%). The crude base (136.12 g) was subjected to a chromatography column with silica gel and eluted with CHCl<sub>3</sub>/CH<sub>3</sub>OH/ 28% NH<sub>4</sub>OH (50:1:0.1 → 3:1:0.1) to afford thirteen fractions: Fr. I (12.51 g; 9.19%), Fr. II (6.34 g; 4.66%), Fr. III (17.68 g; 12.99%), Fr. IV (16.35 g; 12.01%), Fr. V (11.45 g; 8.41%), Fr. VI (92.42 mg; 0.07%), Fr. VII (75.43 mg; 0.05%), Fr. VIII (41.27 mg; 0.03%), Fr. IX (39.15 mg; 0.03%), Fr. X (4.21 g; 3.09%), Fr. XI (1.29 g; 0.95%), Fr. XII (56.94 mg; 0.04%), Fr. XII (513.54 mg; 0.38%). Bioactivity-guided studies on the extract and fractions using Von Frey test showed that the fraction IV from the roots of *Nauclea latifolia* significantly attenuated mechano-hyperalgesia of all CCI rats with  $85.26 \pm 9.17\%$ , compared to the control group.

### 2.3. Sample preparation, chromatographic analysis and bioactivity guided studies

10 mg sample of the fractions IV was dissolved in 10 ml methanol. The solution was filtered through membrane filter (pore size 0.45 μm) prior to high-performance liquid chromatography analysis. The sample was analysed by means of a HPLC system (AKTA™ Purifier, Amersham Biosciences). The alkaloid fraction derived from *Nauclea latifolia* was analysed by HPLC using a Vydac C18 column (218TP1010, 25 × 10 cm, 10 μm), and for elution of the constituents, a gradient of two solvents denoted A and B was employed. The mobile phases were (A) 90% acetonitrile with water and (B) 0.1% trifluoroacetic acid with water. The flow rate used was 1.0 ml/min and the injection volume was 10 ml. The retention time and UV spectrum of major peaks were analyzed. The eluant was monitored at 215 nm. The fractions eluted were combined, and then lyophilized [F<sub>1</sub>(0.03 mg; 0.30%), F<sub>2</sub>(0.1 mg; 10%), F<sub>3</sub>(0.13 mg; 1.30%); F<sub>4</sub>(0.07 mg; 0.70%), F<sub>5</sub>(0.11 mg; 1.10%), F<sub>6</sub>(0.04 mg; 0.40%),



F<sub>7</sub>(0.08 mg; 0.80%), F<sub>8</sub>(0.16 mg; 1.60%)] (Figure 1), to afford the products as powders. Further activity-guided studies on the sub-fractions (F<sub>1</sub>–F<sub>8</sub>) showed that the powders yielded by F<sub>3</sub> exhibited the most potent antinociceptive effects by causing a dose-dependent inhibition of mechanical hyperalgesia in CCI rats using Von Frey test. The collected fraction F<sub>3</sub> was dissolved in vehicle (saline containing dimethyl sulfoxide 2%) and screened for further activities.

## **2. 4. Animals**

Male Wistar rats (160-180 g), and male and female Swiss mice (25-30 g) were used. All animals were housed under standardized conditions in the animal facility on a 12 hrs light/dark cycle with food and water available *ad libitum*. The investigation conforms to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH; publication No. 85-23, revised 1996) and received approval of the local ethical committee for animal handling and experimental procedure (Ref n° FW-IRB00001954).

## **2.5. Production of neuropathy and behavioral testing**

### **2.5.1. Surgery**

The chronic constriction injury of the sciatic nerve model was employed according to methods described by Bennett and Xie (Bennett and Xie, 1988). Under thiopental sodium anesthesia (Sigma chemical, St Louis, USA, 35 mg/kg; i.p.), the right common sciatic nerve was exposed by blunt dissection through the biceps femoris, at the level of mid-thigh. Proximal to the sciatic trifurcation, the nerve was freed of adhering tissue, and four ligatures with 3-0 silk thread were tied loosely around the nerve with a spacing of about 1 mm, taking care not to interrupt the epineural circulation. After surgery, the muscle and skin were closed in two layers using absorbable chronic catgut 4-0 for the muscle and 3-0 silk thread for the

skin. In sham-operated controls, an identical surgical procedure was performed, except that the sciatic nerve was not ligatured. All surgical procedures were performed under normal sterile conditions by the same person. After operation, the rats were allowed to recover for 1 week.

### **2.5.2. Von Frey test**

To quantify mechanical sensitivity of the foot, a brisk foot withdrawal in response to normally innocuous mechanical stimuli was measured as described previously (Kim and Chung 1992). Six rats were used for each treatment (n = 6 per group). The rats were placed in acrylic cages on top of a wire mesh grid that allowed their paws access to the Von Frey filaments. The rats were adapted to the testing situation, and they were allowed to habituate until exploratory behaviour diminished for at least 10 min before stimulation was initiated. Bending forces of 1, 6, 10 and 15 g to the mid-plantar skin of each hind paw were then applied in increasing order from the weakest to the strongest. Beginning with the lowest force, the filament was placed on the skin until it bowed slightly, with each filament presented ten times at a rate of about 1/s. The paw-withdrawal threshold was defined as the percent force eliciting an active withdrawal on the affected ipsilateral paw. A response was recorded every 30 min for 180 min post-dosing if the rat withdrew its hind paw from the filament. The effects of acute administration of the alkaloid fraction from *Nauclea latifolia* (8, 16, 40 and 80 mg/kg; i.p.), morphine (Sigma chemical, St Louis, USA, 5.6 mg/kg; s.c.) or vehicle (10 ml/kg; saline containing dimethyl sulfoxide 2%, i.p.) on mechanical sensitivity were tested between at day 12 post-surgery.

### **2.5.3. Acetone test**

To quantify cold sensitivity of the foot, brisk foot withdrawal in response to acetone application was measured as described previously (Choi et al., 1994). Six rats were used for each treatment (n = 6 per group). With the animals inside acrylic cages on the elevated grid, a drop of acetone was delicately applied to the plantar surface of the hind paw without touching the skin using a blunt plastic needle connected to a syringe. A response was recorded if the rat withdrew its hind paw in response to acetone application. The time spent with the leg withdrawn from the floor during the 60 s following exposure to acetone was recorded. Both hind legs were tested in each animal, beginning with the unoperated left leg, and each stimulus was applied three times at intervals of approximately 5 min. The duration of lifting of the hind paw after acetone stimuli was recorded with a stopwatch every 30 min for 180 min post-dosing (Dowdall et al., 2005). The effects of acute administration of the alkaloid fraction from *Nauclea latifolia* (8, 16, 40 and 80 mg/kg; i.p.), morphine (5.6 mg/kg; s.c.) or vehicle (10 ml/kg; saline containing dimethyl sulfoxide 2%, i.p.; adequate controls were performed with the corresponding vehicles in CCI and sham rats) on cold sensitivity were tested between day 12 and day 14 post-surgery.

## **2.6. Motor assessment and signs of catalepsy**

The motor coordination test was performed to determine side effects of the *Nauclea latifolia* alkaloid fraction using the rotating rod method (Taiwe et al., 2011). The bar rotated at a constant speed of 16 revolutions per min. A preliminary selection of mice was made on the day of experiment prior to administration of extract or morphine ( $t_0b$ ), excluding those that did not remain on the rota-rod bar for two consecutive periods for 45 sec each. Six mice were used for each treatment (n = 6 per group). The integrity of motor coordination was assessed on the basis of the number of falls from the rota-rod in 180 sec. Selected animals were tested immediately at 0 ( $t_0a$ ), 30, 60, 90, and 120 min after administrations of the alkaloid fraction

from *Nauclea latifolia* (8, 16, 40 and 80 mg/kg, i.p.), morphine (5.6 mg/kg, s.c.), or vehicle (10 ml/kg; saline containing dimethyl sulfoxide 2%, i.p.).

Catalepsy was evaluated according to the standard bar hanging procedure by placing the naive mice with both forelegs over a horizontal bar, elevated 4.5 cm from the floor (Sanberg et al., 1988). Catalepsy was considered finished when the forepaw touched the floor or when the mouse climbed the bar. Eight mice were used for each treatment (n = 8 per group). Measurement was performed 30 and 60 min after administration of the alkaloid fraction from *Nauclea latifolia* (8, 16, 40 and 80 mg/kg; i.p.), morphine (5.6 mg/kg; s.c.) or vehicle (10 ml/kg; saline containing dimethyl sulfoxide 2%, i.p.; adequate controls were performed with the corresponding vehicles in CCI and sham rats). The time during which the mouse maintained the cataleptic position was recorded for up to 300 sec, with three attempts allowed to replace the animal over the glass bar.

## **2.7. Acute toxicity and cell viability assay**

The acute toxicity test for the alkaloid fraction from *Nauclea latifolia* was carried out to evaluate any possible toxicity. Mice of either sex (5 per sex = 10 per group) were divided into control and test groups. The first group served as a normal control treated with vehicle (10 ml/kg; saline containing dimethyl sulfoxide 2%, i.p.). The alkaloid fraction from *Nauclea latifolia* was administered intraperitoneally to different groups at the increasing doses of 160, 320, 640, 960, 1280, 1600 mg/kg. After injections of the alkaloid fraction from *Nauclea latifolia*, mice were allowed access to food and *water ad libitum* and all animals were observed for general behavioral and body weight changes, hazardous symptoms and mortality for a period of 14 days after treatment (Taïwe et al., 2011). The median lethal dose (LD<sub>50</sub>) was estimated according to the method described by Litchfield and Wilcoxon (1949).

Confirmatory tests were carried out and the LD<sub>50</sub> was calculated from the graph of probit mortality against dose of the extract.

Chinese hamster ovary (CHO) cell line from American Type Culture collection (ATCC) were maintained at 37°C in 5% CO<sub>2</sub> in F-12K nutrient medium (InVitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (InVitrogen) and 10,000 units/ml streptomycin and penicillin (InVitrogen). Chinese cell ovary cell were seeded into 96 well micro plates at density of approximately  $8 \times 10^8$  cells/well. After 2 days of culture, the cells were incubated for 24 hours at 37°C with various concentrations of the alkaloid fraction from *Nauclea latifolia* (0, 20, 40, 80, 160, 320, 640, 960, 1280 and 1600 µg/ml). Control wells containing cell culture medium alone or with cells, both without *Nauclea latifolia* fraction addition, were included in each experiment. The cells were then incubated 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT; Sigma, St Louis, MO, USA) for 30 min. Conversion of MTT into purple colored MTT formazan by the living cells indicated the extent of cell viability. The crystals were dissolved with dimethyl sulfoxide and the optical density was measured at 540 nm using microplate reader (Biotek ELx-800, Mandel Scientific Inc.) for quantification of cell viability. All assays were run in triplicates. Results were plotted as percent of cytotoxicity and concentration-response curves were fitted using Graph Pad Prism in order to determine the 50% effective concentration (EC<sub>50</sub>) (Poillot et al., 2010).

## **2.8. Data analyses and statistics**

Data are expressed as mean ± S.E.M. The cumulative anti-nociceptive effect during the entire observation period (180 min) was determined as the area under the curve (AUC) of the time course. The AUCs for each of the assayed drugs were calculated by the trapezoidal method (Rowland and Tozer, 1989). The data were compared using a two-way analysis of

variance (ANOVA) followed by Bonferroni's post-hoc tests. A significant statistical difference was determined by value of at least  $p < 0.05$ .  $ED_{50}$  values and 95% confidence intervals (95% CI) were determined at the time of the peak effect for each drug by semilogarithmic regression analysis or according to Litchfield and Wilcoxon (1949).

### 3. Results

#### 3.1. Effects of the alkaloid fraction from *Nauclea latifolia* on mechanical hyperalgesia

Ten days after surgery, CCI rats developed statistically significant increases in responses to 1, 6, 10 and 15 g von Frey filament stimulation. CCI rats showed  $7.25 \pm 1.14\%$  and  $17.29 \pm 3.18\%$  of response frequency to 1 g and 6 g von Frey filament stimuli, respectively, compared with the sham-operated group, which did not show any reaction. Thus, this effect was a mechano-allodynic response. However, the stimuli of 10 g or 15 g von Frey filaments produced mechano-hyperalgesic responses in rats on day ten post-surgery. For example, when a 10 g filament stimuli was used in the sham group,  $5.12 \pm 1.53\%$  was the observed response, while in CCI rats the percentage of response was  $65.81 \pm 2.14\%$ . Similarly, the stimuli of 15 g Von Frey filaments produced mechano-hyperalgesic responses in rats on day twelve post-surgery. When a 15 g filament stimuli was used in the sham group,  $86.87 \pm 2.59\%$  was the observed antihyperalgesia, while in CCI rats the percentage of response was  $8.54 \pm 1.83\%$  [ $F(6, 38) = 92.51$ ;  $p > 0.06$ ]. 90 min post-injection of 80 mg/kg of the alkaloid fraction from *Nauclea latifolia*, significantly attenuated mechano-hyperalgesia of all CCI rats with  $76.97 \pm 4.21\%$  [ $F(6, 38) = 92.51$ ;  $p < 0.001$ ], compared to the vehicle group (Figure 2A). The attenuation was most marked 60 min after injection for 40 mg/kg, and 180 min for 80 mg/kg. The maximum value of the AUC obtained in the Von test in sham rats (anti-hyperalgesic effects) under these experimental conditions was  $288.52 \pm 14.29$  area units (a.u.). At 180 min, the AUC was  $198.25 \pm 15.17$  a.u. in CCI animals administered with 80

mg/kg of the alkaloid fraction, indicating that mechano-hyperalgesia had been significantly reversed [ $F(6, 38) = 92.51$ ;  $p < 0.05$ ]. The alkaloids fraction showed potent and dose-dependent inhibition of mechanical hyperalgesia with an  $ED_{50}$  value (95% CI) of 35.94 (24.25 - 51.39) mg/kg (Figure 2B).

### **3.2. Effects of alkaloids fraction from *Nauclea latifolia* on cold allodynia**

The three hour time courses of the anti-allodynic effects of the alkaloid fraction from *Nauclea latifolia* and morphine after a single i.p. administration are shown as the anti-allodynic effects. Pronounced cold allodynia in response to acetone stimulation of the ipsilateral hind paw was observed in the CCI group in the acetone test 12 days after surgery. The alkaloid fraction from *Nauclea latifolia* at the dose of 80 mg/kg induced significant anti-allodynia effects of all CCI rats with  $89.38 \pm 3.59\%$  [ $F(6, 29) = 103.62$ ;  $p < 0.001$ ] using the acetone test (Figure 3A). The most pronounced effect was observed 2 hours after administration of the alkaloid fraction at the dose of 80 mg/kg. The percentage baseline allodynia in CCI rats, injected with vehicle, remained the same throughout the observation period, whereas the sham operated group did not present any response to this cold stimulus during the entire 3 hrs period of observation. The maximum value of the AUC obtained in the acetone test (anti-allodynic effect) under these experimental conditions was  $271.40 \pm 12.51$  a.u. An AUC of  $10.34 \pm 2.59$  a.u. was observed in the CCI rats which did not show anti-allodynic effects. The alkaloid fraction showed significant differences at 80 mg/kg ( $149.57 \pm 18.54$  a.u.) [ $F(6, 31) = 145.71$ ;  $p < 0.001$ ] compared with the CCI control group without treatment. The alkaloid fraction showed efficient anti-allodynic effects in the CCI model, with an  $ED_{50}$  value (95% CI) of 38.26 (22.31 - 49.67) mg/kg (Figure 3B).

### **3.3. Effects of the alkaloid fraction from *Nauclea latifolia* on motor coordination**

The effects of the alkaloid fraction from *Nauclea latifolia* on motor coordination were evaluated by the number of falls from the rotarod apparatus and compared to animals receiving the vehicle. This alkaloid fraction did not affect motor coordination at all doses tested [ $F(5, 72) = 103.52$ ;  $P > 0.07$ ]. However, a large effect on motor coordination ( $19.44 \pm 0.91$  falls) [ $F(5, 72) = 103.52$ ;  $p < 0.001$ ] was observed 30 min post administration with morphine at the dose of 5.6 mg/kg, compared with vehicle (Table 1).

The alkaloid fraction from *Nauclea latifolia* administered intraperitoneally did not induce any cataleptic effect either at low doses or at the higher dose. As can be seen in Figure 4, s.c. administration of morphine (5.6 mg/kg) induced catalepsy at 30 min [ $F(5, 32) = 124.39$ ;  $p < 0.01$ ] and 60 min [ $F(5, 32) = 124.39$ ;  $p < 0.001$ ] (Figure 4).

### **3.4. Acute toxicity and cell viability assay**

The results indicated that the acute treatment by intraperitoneal route at doses up to 1600 mg/kg of the alkaloid fraction from *Nauclea latifolia* did not produce any sign of toxicity or death in mice during 24 hours of observation. After 14 days, treated mice did not present any visible toxic effect. Furthermore, no lesions or bleedings were observed in internal organs such as lungs, kidneys, liver, heart and stomach. Neither absolute body weight nor body weight gain was affected by *Nauclea latifolia* fraction administration at all doses throughout the study. Since no death or damage was observed throughout the experiments, the  $LD_{50}$  could not be estimated.

The ability of the alkaloid fraction from *Nauclea latifolia* to induce cytotoxicity was further investigated by using CHO cells and a standard MTT bioassay (Figure 5). As shown, incubation for 24 hrs of these cell lines with the *Nauclea latifolia* fraction, up to a concentration of 160  $\mu\text{g/ml}$ , produces no cell toxicity. The toxicity values observed were not significantly different from baseline and lower than  $9.7 \pm 3.5\%$  [ $F(8, 32) = 147.25$ ;  $p > 0.07$ ].



However, incubation of CHO cells with a higher concentration (640 µg/ml) of the *Nauclea latifolia* fraction produced greater cell death ( $30.2 \pm 3.8\%$ ) [ $F(8, 32) = 147.25$ ;  $p < 0.001$ ]. The viability of CHO cells decreased in a concentration-dependent manner (Figure 5) with increasing concentrations of extract ( $EC_{50} = 772.35 \pm 13.41$  µg/kg) after 24h treatment.

#### 4. Discussion

The present study investigated the anti-allodynic and antihyperalgesic effects of the alkaloid fraction from *Nauclea latifolia* on chronic constriction injury of the sciatic nerve as a model of neuropathic pain. This model is based on a unilateral loose ligation of the sciatic nerve, which is one of the most frequently used models for the study of neuropathic pain and its treatment. Chronic constriction injury in rats simulates the clinical condition of chronic nerve compression such as that occurring in nerve entrapment neuropathy or spinal root irritation by lumbar disk herniation. This model also shows many of the pathophysiological properties of chronic neuropathic pain in human subjects, such as allodynia and hyperalgesia (Bennett and Xie, 1988; De Vry et al., 2004). In this study, 12 days after CCI, the rats showed a relatively high degree of similarity with other studies published on neuropathic pain in terms of the degrees of allodynia and hyperalgesia against cold and mechanical stimuli, demonstrated by the increased responsiveness to acetone stimulus and von Frey filaments (Bennett and Xie, 1988; De Vry et al., 2004; De la O-Arciniega et al., 2009).

The antinociceptive effects of the alkaloid fraction from *Nauclea latifolia* was well documented herein, as confirmed by the analysis of the AUC of anti-hyperalgesic effects over a time course of 3 hours post-dosing. Also, this alkaloid fraction induced a strong attenuation of cold allodynia, and the anti-allodynic effects persisted for 3 hours post-administration. Similarly, morphine demonstrated antinociceptive effects on mechanical hyperalgesia and cold allodynia at the dose of 5.6 mg/kg. It is the most widely used opioid and the standard

against which new agents are compared. This opioid drug has demonstrated antinociceptive efficacy in several models of nociception (López-Muñoz et al., 1993), including neuropathic pain (De Vry et al., 2004). Morphine mediates its actions by binding and activating receptors in the peripheral nervous system, as well as those found in inhibitory pain circuits that descend from the midbrain to the spinal cord dorsal horn via presynaptic and, to a lesser extent, postsynaptic  $\mu$ -opioid receptors modulating nociceptive transmission (Nicholson, 2003). Other studies suggest that *Nauclea latifolia* extracts remarkably decreased both the acute and delayed phases of formaline-induced pain in animals and also caused a significant reduction in both yeast-induced pyrexia and egg albumin-induced oedema in rats (Abbah et al., 2010; Taiwe et al., 2011). The antinociceptive effects exhibited by *Nauclea latifolia* extract in the formalin test was reversed by the systemic administration of naloxone (a non-selective opioid receptor antagonist),  $N_{\omega}$ -L-nitro-arginine methyl ester (L-NAME, a NO synthase inhibitor) or glibenclamide (an ATP-sensitive  $K^+$  channel inhibitor) (Taiwe et al., 2011). In the course of pharmacological studies, anticonvulsant, anxiolytic and sedative properties of *Nauclea latifolia* roots decoction (Ngo Bum et al., 2009; Taiwe et al., 2010) have already been reported from our laboratory. Neuropathic pain and epilepsy share neuronal hyperexcitability as a common underlying mechanism. There are established antiepileptic drugs that target the generation of neuronal hyperexcitability, and some of these have been proven to be effective in the treatment of various forms of neuropathic pain (Sindrup and Jensen, 2000).

The results obtained in our study suggest that inhibition of neuropathic pain by *Nauclea latifolia* is not related to the reduction of spontaneous locomotor activity of animals and it's not induced catalepsy. The catalepsy test has been used to predict tranquillizer activity as well as to evaluate motor effects of drugs, particularly those related to the extra-pyramidal system (Sanberg, et al., 1988).

In the present study, we did not observe any mortality case up to the doses of 1600 mg/kg of the alkaloid fraction from *Nauclea latifolia*. Therefore, we may suggest that the extract has no lethal toxicity in mice. As seen, using the more sensitive MTT assay, no cytotoxicity has been observed on CHO cells lines incubated with alkaloids fraction from *Nauclea latifolia* at 160 µg/ml. MTT assay measured the metabolism of 3- (4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide to form formazan precipitate by mitochondrial dehydrogenase which only present in viable cells. Formazan accumulation directly reflected mitochondrial activity, which was an indirect measure of cell viability (Mosmann, 1983).

In summary, a novel antinociceptive action of the alkaloid fraction from *Nauclea latifolia* has been confirmed against neuropathic pain induced by CCI of the sciatic nerve, a rat model of neuropathic pain, using the Von Frey and acetone tests, and showing no increase in side effects on motor coordination. Moreover, the anti-hyperalgesic and anti-allodynic action demonstrated in the present study supports, at least in part, the ethnomedical uses of this plant.

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## Figure legends

**Figure 1:** HPLC profile of the alkaloid fraction from the roots of *Nauclea latifolia* alone measured at the wavelength of 215 nm.

**Figure 2:** Effects of the alkaloid fraction from *Nauclea latifolia* on mechanical hyperalgesia. Time course of anti-hyperalgesic effects of *Nauclea latifolia* on mechanical hyperalgesia with a 15 g von Frey filament after CCI of the sciatic nerve (A). Control groups (sham and CCI) were treated with equivalent volumes of vehicle. Data are expressed as mean  $\pm$  SEM, n = 6, <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 and <sup>c</sup>P<0.001 *versus* vehicle (CCI). Dose-response curves expressed as the area under curve (AUC) for the antihyperalgesic effects of *Nauclea latifolia* with a von Frey filament (15 g) after CCI of the sciatic nerve in rats (B). Rats were treated with vehicle or morphine (Morp; 5.6 mg/kg, s.c.). Bars are means  $\pm$  SEM, n = 6. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 *versus* vehicle (CCI). Data were analyzed by a two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc tests.

**Figure 3:** Effects of the alkaloid fraction from *Nauclea latifolia* on cold allodynia. Time course of anti-allodynic effects of *Nauclea latifolia* on cold allodynia after CCI of the sciatic nerve (A). Control groups (sham and CCI) were treated with equivalent volumes of vehicle. Data are expressed as mean  $\pm$  SEM, n=6, <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 and <sup>c</sup>P<0.001 *versus* vehicle (CCI). Area under the curve (AUC) of anti-allodynic effects produced by *Nauclea latifolia* on cold allodynia after CCI of the sciatic nerve (B). Rats were treated with vehicle or morphine (Morp; 5.6 mg/kg, s.c.). Bars are means  $\pm$  SEM, n = 6. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 *versus* vehicle (CCI). Data were analyzed by a two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc tests.



**Figure 4:** Effects of the alkaloid fraction from *Nauclea latifolia* (8, 16, 40 and 80 mg/kg) on catalepsy in mice. Morphine (Morp; 5.6 mg/kg) was used as a positive control. Bars are means  $\pm$  SEM, n = 8. \*\*p < 0.01 and \*\*\*P<0.001 compared to vehicle by using a two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc tests.

**Figure 5:** Dose response curves for CHO cell line following 24 hours continuous exposure to the alkaloid fraction from *Nauclea latifolia*. Results are the mean of three determinations and are expressed as % cell survival.  $EC_{50} = 772.35 \pm 13.41 \mu\text{g/kg}$ .

## Tables

**Table 1:** Effects of the alkaloid fraction from *Nauclea latifolia* on motor coordination of mice on the rotarod apparatus.

Treatments	Dose (mg/kg)	Duration of study (min)					
		t <sub>0b</sub>	t <sub>0a</sub>	30	60	90	120
Vehicle	–	0.77 ± 0.11	1.11 ± 0.39	1.55 ± 0.83	1.88 ± 1.02	1.55 ± 0.48	1.44 ± 0.49
<i>N. latifolia</i>	8	0.88 ± 0.14	1.33 ± 0.44	1.22 ± 0.52	1.77 ± 1.31	1.88 ± 0.39	1.55 ± 0.61
<i>N. latifolia</i>	16	0.77 ± 0.44	1.22 ± 0.34	1.11 ± 0.39	2.16 ± 1.01	1.66 ± 0.58	1.66 ± 0.74
<i>N. latifolia</i>	40	0.55 ± 0.44	1.44 ± 0.59	1.33 ± 0.81	1.11 ± 0.61	1.88 ± 0.59	0.88 ± 0.19
<i>N. latifolia</i>	80	0.77 ± 0.44	1.33 ± 0.81	1.22 ± 0.69	1.66 ± 0.61	2.11 ± 0.39	1.11 ± 0.19
Morphine	5.6	0.66 ± 0.44	0.88 ± 0.39	16.55 ± 1.72***	19.22 ± 0.91***	19.44 ± 0.83***	18.77 ± 0.74 ***

Acquisition process of the rotarod performance as expressed by means ± S.E.M. of number of falls, n = 6 animals per group, \*\*\*p < 0.001, significantly different compared to the control. Data were analysed by two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc tests.

Figures

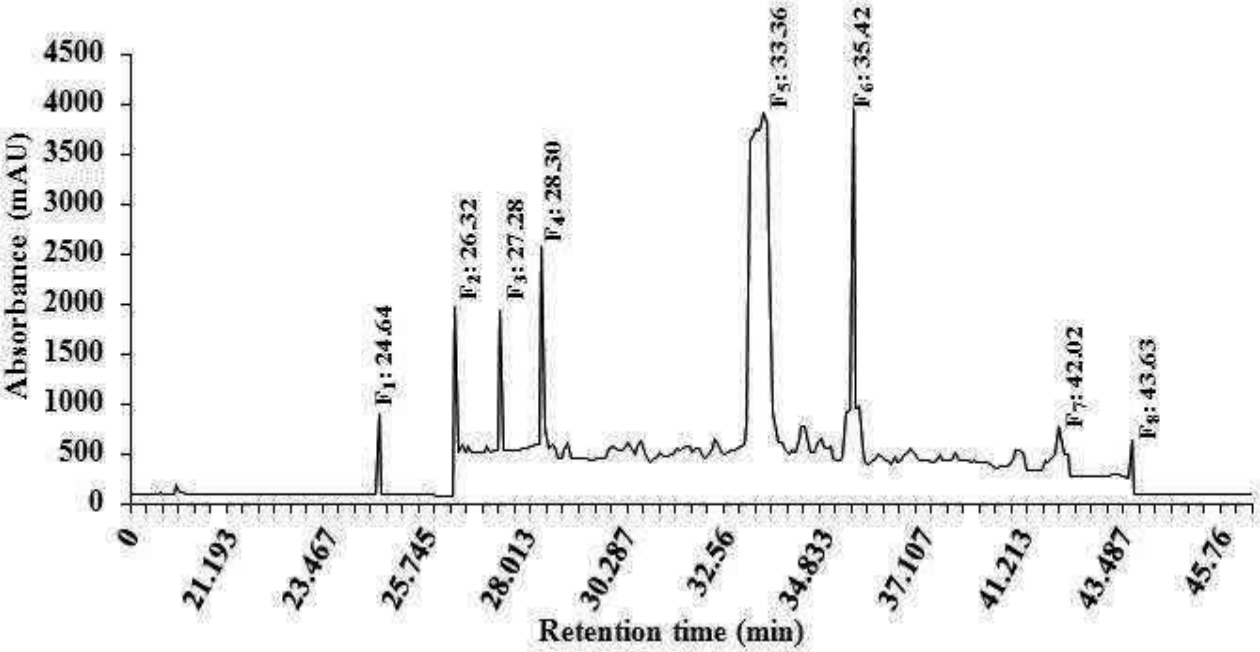


Figure 1: Germain Sotoing Taiwe et al.

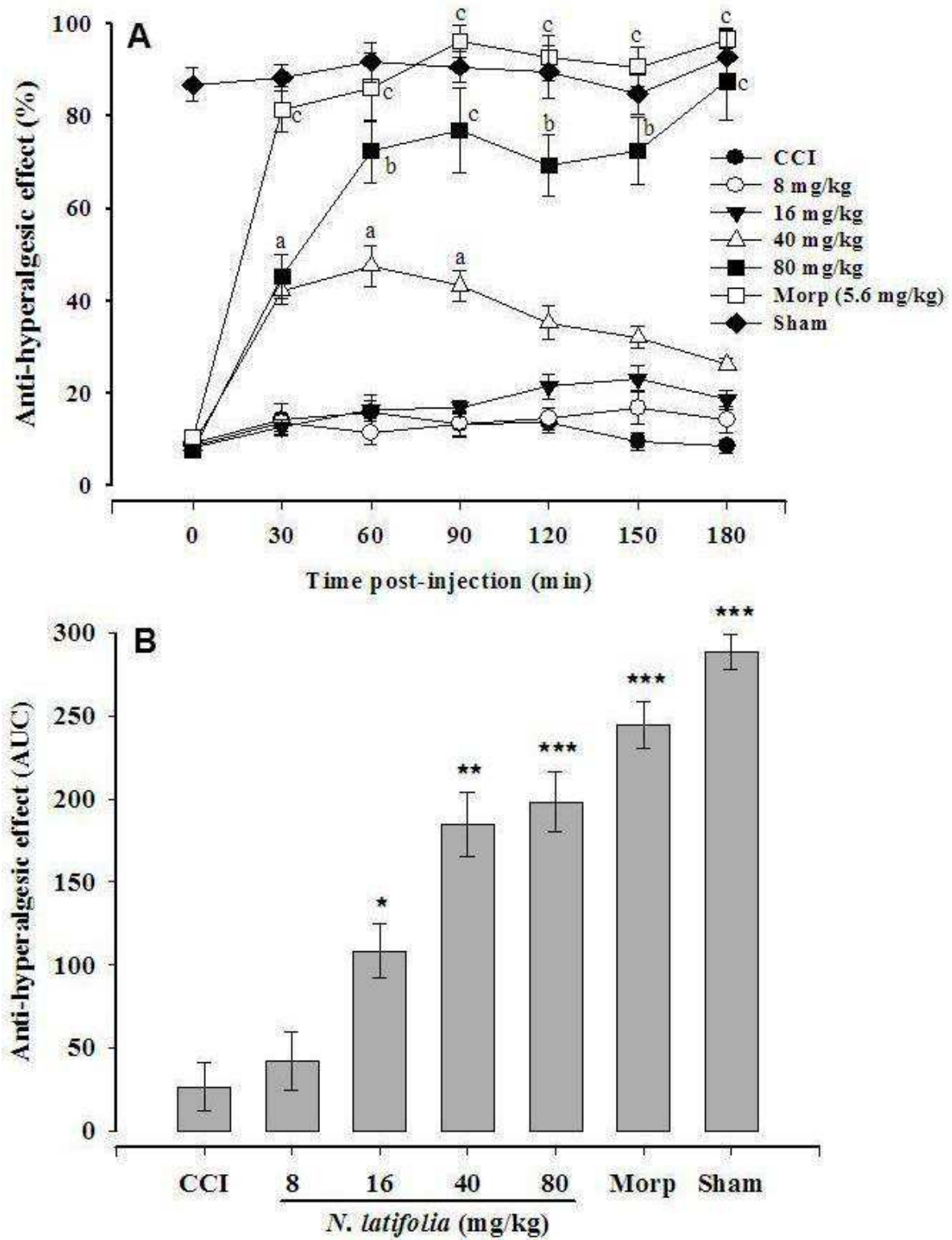


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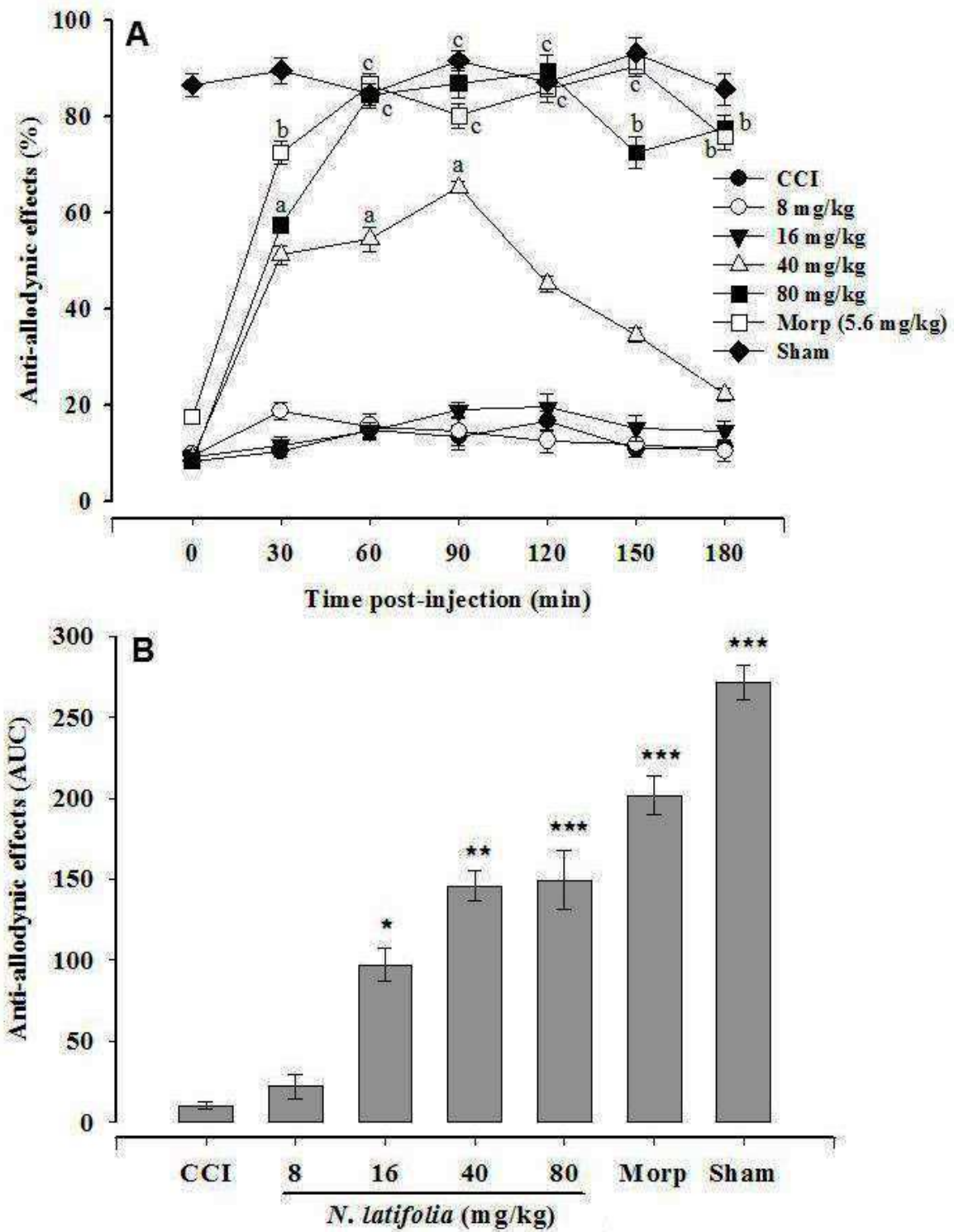


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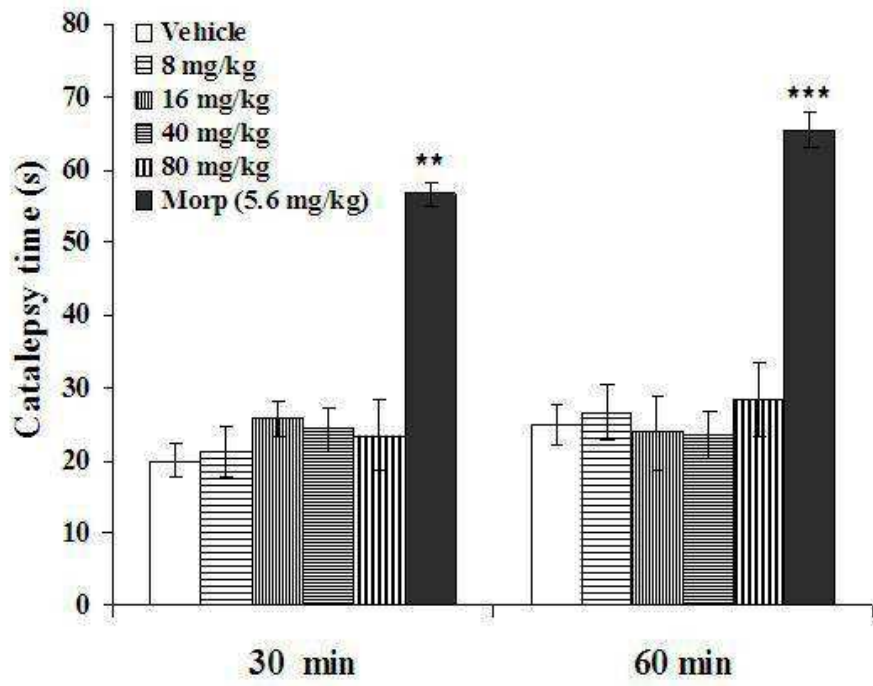


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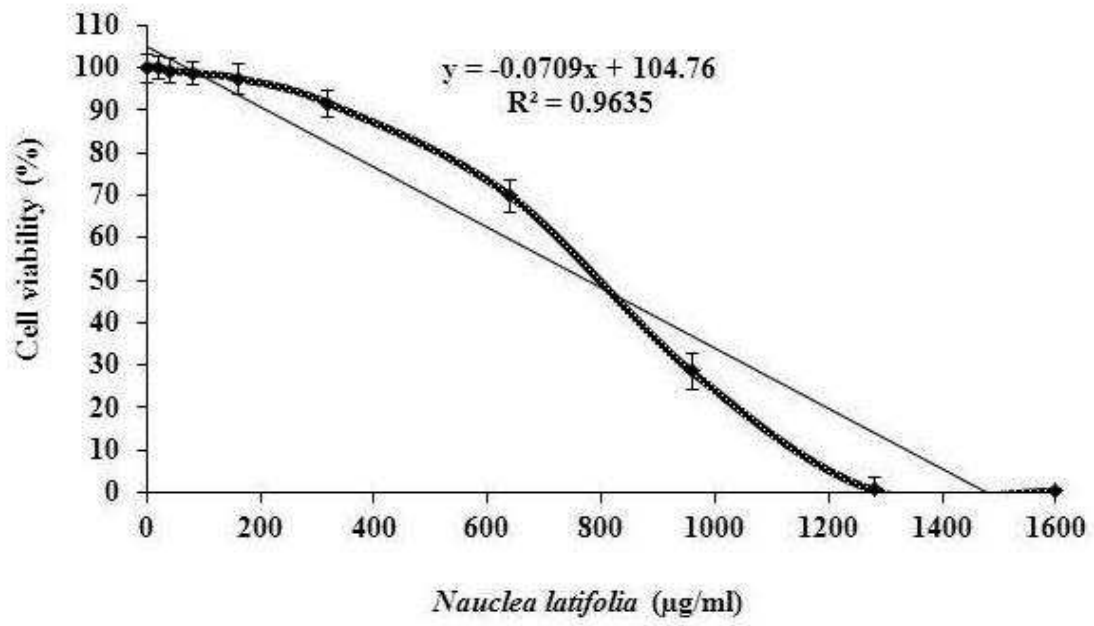


Figure 5: Germain Sotoing Taiwe et al.