# Antinociceptive and Antioxidant Activities of Methanol Extract and Fractions of the Root Bark of Callichilia stenopetala Stapf. (Family Apocynaceae) In Mice

**Article** · September 2017

DOI: 10.26538/tjnpr/v1i3.7



**3 authors:**

Ifeoma Orabueze
University of Lagos
**15** PUBLICATIONS **71** CITATIONS
SEE PROFILE

Sunday ADELEKE Adesegun
University of Lagos
**39** PUBLICATIONS **657** CITATIONS
SEE PROFILE

Herbert A B Coker
University of Lagos
**89** PUBLICATIONS **1,619** CITATIONS
SEE PROFILE

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

# Antinociceptive and Antioxidant Activities of Methanol Extract and Fractions of the Root Bark of *Callichilia stenopetala* Stapf. (Family Apocynaceae) In Mice

Celestina I. Orabueze [1*], Sunday A. Adesegun[1], Herbert A. Coker[2]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, College of Medicine, University of Lagos, PMB 12003, Surulere, Lagos, Nigeria.*
[2]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, College of Medicine, University of Lagos, PMB 12003, Surulere, Lagos, Nigeria.*

ARTICLE INFO

*Article history:*
Received 01 August 2017
Revised 01 September 2017
Accepted 03 September 2017
Published online 09 September 2017

*Keywords:*
*Callichilia stenopetala,*
*Analgesic,*
*Pain reaction time,*
*Acetic acid,*
*Hot plate.*

ABSTRACT

The root of *Callichilia stenopetala* is used as chewing stick in the treatment of toothache and for oral hygiene in South-East Nigeria. The study assessed the antinociceptive and antioxidant activities of methanol root bark extract and fractions of *C. stenopetala*. Analgesic activity of methanol root bark extract of *C. stenopetala* and its fractions were evaluated using chemical (acetic acid and formalin) and thermal (hot plate and tail immersion) tests in mice. Morphine sulphate, acetyl salicylic acid and diclofenac sodium at 4, 100 and 10 mg $kg^{-1}$, respectively were used as standards. Antioxidant potential was evaluated using DPPH (1, 1-diphenyl-2-picrylhyrazyl) free radical scavenging activity and total phenolic content (TPC) assays. Oral acute toxicity was also evaluated. The extract (250 and 500 mg $kg^{-1}$), ethyl acetate and aqueous fractions (200 mg $kg^{-1}$) reduced acetic acid-induced writhing with percentage reduction of 65.14, 84.06, 93.27 and 50.63 %, respectively. The ethyl acetate fraction and methanol extract at 500 mg $kg^{-1}$ significantly increased reaction times in tail immersion and hot plate tests. The formalin test results for the ethyl acetate fraction suggested involvement of central mechanism of pain inhibition. In DPPH scavenging assay and TPC, the extract showed good antioxidant potential, with most of the activity prominent in the polar fractions. At 8000 mg $kg^{-1}$ no death of animal occurred. These findings suggested that *C. stenopetala* has pain alleviating effect which may be due to its antioxidant activity and this further substantiated the claimed traditional use of the plant in the management of acute toothache.

## Introduction

Plants are important part of the culture of any group of people. Natural medicinal products have been sourced from these plants over centuries to combat health challenges because of their effectiveness.[1] Today, these plants have greater value globally as they play important role at various level of healthcare system because of their therapeutic properties.[2] High percentage (25 %) of conventional drugs on the shelves in the pharmacies have their origin from medicinal plant.[3] The current re-insurgence of interest in ethnomedicinal use of medicinal plants particularly the ones locally available and with long history in the management of diverse ailments has been argued to be as result of treatment failures associated with some orthodox drugs.[4, 5] Some of the wide range of ideal properties promoted for medicinal plant products include high target effectiveness, low cellular toxicity, low cost, easy bio-degradability, abundant growth in endemic areas.[6, 7]

Pain is a major clinical symptom that serves as warning signal to patients of abnormality in the body system.[8] It serve as unpleasant body response to tissue injury that could affect both the physical and emotional well-being.[9, 10] The mechanism of pain initiation involves activating sensorial neurons that transmit nociceptive stimulus at spinal and supra-spinal levels.[11]

*Corresponding author. E mail: ifeoma_orabueze@yahoo.com
Tel: +234-803 343 5631

https://doi.org/10.26538/tjnpr/v1i3.7


Treatment of pain involves chemical agents or devices that have the ability to block these signal pathways, both at central and peripheral levels.[11] Managing pain has always been problematic and challenging in spite of progress made in medical research during the past decades. Drugs that are currently used with different levels of efficacy in management of moderate to severe pain are opioids or non-opioids analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) [12] but they come with limitations. Opioids, though useful in management of chronic and acute pain, exhibit a wide range of potential side effects, particularly addicting effect. [12] Studies have shown that NSAIDs are significantly associated with risk of gastrointestinal bleeding.[12] Other possible side effects include respiratory depression, decreased mental capacity, addiction, etc.[12] This stirs the need for continuous search for effective pain relieving agents with little or no side effects as therapeutic alternative to current medications. Medicinal plants already in use as folklore medicine need to be scientifically investigated to ascertain their level of effectiveness and safety.[13]

Pain-killing effects of antioxidants was investigated by Jensen and co-researchers and they reported an observed correlation between pain threshold and poor antioxidant status.[14] Robert Stephens, has shown that free radicals may contribute to severity of chronic pain.[15] The two researches directly agreed that the accumulation of free radicals in the body could further damage already-injured tissue and thus indirectly increase perceived pain. [15] The same observation was also reported by Rokytal *et al.*[16] of increased lipoperoxidation due to painful stimulation and ability of antioxidants in combination with analgesic being able to decrease levels of oxidative markers and pain. Thus, administration of antioxidants in pain treatment may decrease the doses of analgesics needed to control perceived pain and also prevent the negative impact of reactive oxygen species on nociception.[14, 16]

*Callichilia stenopetala* Stapf (family Apocynaceae), known locally as "Utu nkita" has been used by traditional medical practitioners in the South-Eastern part of Nigeria as a remedy for various ailments including different

types of "re-current" fever and problems associated with poor oral hygiene.[17] Root of *C. stenopetala* has been used as a chewing stick in the region for cleansing purpose and for its medicinal value in toothache therapy by traditional practitioners. Scientific data on the plant is highly limited.[17] The Present study was undertaken to evaluate the analgesic and antioxidant activities of methanol root bark extract of *C. stenopetala* in mice.

## Materials and Methods

*Experimental animals*

Healthy Swiss albino mice of both sexes (20 - 22 g) were used for the study. They were maintained at the animal house of the University of Lagos, Idi-Araba, Nigeria. The animals were housed in groups of 5 animals in clean polypropylene cage with wood shavings as bedding and fed on commercial animal feed and allowed free access to clean fresh water *ad libitum*. All experimental protocols were in compliance with internationally accepted principles for laboratory animal use and care. The Research Grants and Experimentation Ethics Committee of College of Medicine University of Lagos, Nigeria approved the protocol with ID number RGEEC/22/2015.

*Collection of plant material and identification*

The fresh root barks of *C. stenopetala* plants were procured locally at Uruagu village, Nnewi town, Anambra state, in July 2012 and authenticated by a taxonomist, Mr. Ozioko of Bioresources Development and Conservation Programme (B.D.C.P), Nsukka, Enugu State (retired staff, Department of Botany, University of Nigeria Nsukka, Enugu state) and further confirmed by Mr. Daramola, a curator, formerly of the University herbarium, Department of Botany, Faculty of Science, University of Lagos. The herbarium specimen was prepared and deposited with voucher number LUH 3622. After authentication, the root barks were cleaned and shade dried and milled into fine powder by a mechanical grinder.

*Preparation of methanol extract of C. stenopetala*

The dried powdered root bark of *C. stenopetala* (800 g) was extracted exhaustively using Soxhlet extractor and 1.8 L of 90 % methanol for 22 h. After extraction, the solvent was removed using a vacuum rotary evaporator (Buchi Rota vapor, Germany). The extract was then evaporated to dryness in water bath at 40ºC to obtain the crude extract.

The crude methanol extract (118.4 g) was suspended in distilled water and successively fractionated with organic solvents to obtain n-hexane, chloroform, ethyl acetate, and aqueous residual fractions. The extract and fractions were kept in airtight containers and stored in the refrigerator until needed.

*Phytochemical screening*

The crude extract was screened to identify phytoconstituents present in the extract using standard phytochemical screening methods.[18-20] Each test was qualitatively expressed as negative (-) or positive (+).

*Oral acute toxicity test*

The safety of the crude extract was evaluated by determining the oral acute toxicity using a modified Lorke's method.[21] The mice were starved for 24 h prior to drug administration. They were randomly divided into 5 groups of 6 mice per group (1 negative control group and 4 treated groups). The control group received 0.2 mL of 5 % Tween 80 and the treated groups were given 0.2 mL single oral dose of the extract prepared in 5 % Tween 80 (1000, 2000, 4000 and 8000 mg $kg^{-1}$ respectively). The mice were allowed to feed *ad libitum*, kept under continuous observation for first 4 h followed by regular intervals for 24 h up to 7 days for any mortality or behavioural changes. Behavoural changes watched out for include but not limited to, paw licking, salivation, loss of appetite, hair erection, convulsions, stretching of the entire body, weakness and respiratory distress.[22, 23]

*Analgesic Study*

*Acetic acid-induced writhing in mice*

Non-narcotic analgesic activity of the test extract and fractions were investigated as described by Dar *et al.* with some modifications.[24] Mice were randomly divided into 5 groups of 5 mice each. The negative and 2 positive control groups received 0.2 mL of 5 % Tween 80, acetyl salicylic acid (100 mg $kg^{-1}$) and diclofenac sodium (10 mg $kg^{-1}$) orally, respectively. The treatment groups received 250 and 500 mg $kg^{-1}$ orally of the plant extract respectively. Thirty minutes after test extract and standard drugs administration, 0.7 % (v/v) acetic acid (0.1 mL/10 g body weight) was administered intraperitoneal to all the mice to induce the abdominal writhing (abdominal contraction or elongation of the body with stretching of the hind limb). Antinociceptive activity was assessed by counting the number of abdominal constrictions or writhes induced by the acetic acid for 20 min and recorded. A significant reduction of writhes in test animals compared to those in the negative control group was considered as a positive antinociceptic response.[25]

The same procedure was repeated for the hexane, chloroform, ethyl acetate and aqueous fractions at single dose of 200 mg $kg^{-1}$ each and 5 animals per group.

*Hot plate test*

Hot plate test was used to estimate the latency of responses as described by Woolfe and MacDonald with some modifications.[26] Swiss albino mice of either sex were tested before administration of drugs to obtain the baseline and those that did not respond within 60 seconds were left out of the experiment. Twenty-five (25) mice were divided into 5 groups of five animals each. The animals were placed gently on the surface of hot plate heated to and maintained at 50 ± 0.5ºC. The pain reaction time (PRT) or response latency period was recorded which represented the time between placement and shaking or licking of the paws or jumping. A cut off time of 15 seconds was followed to avoid any tissue damage to the animal. Two doses of the extract (250 and 500 mg $kg^{-1}$) dissolved in 5 % Tween 80 were given orally to the mice 30 minutes before the test. Likewise, the control groups were pretreated with vehicle (0.2 mL of 5% Tween 80 solution), diclofenac sodium (10 mg $kg^{-1}$) and morphine sulphate (4 mg $kg^{-1}$) intraperitoneally, 30 min before the test.[7] Analgesic activity was expressed as the increase in response time with respect to negative control. The observations and record were made at 0, 30, 60, 90, 120 and 180 min following administration of drugs.

The same study was repeated for the n-hexane, chloroform, ethyl acetate and aqueous fractions at single dose of 200 mg $kg^{-1}$ each and 5 animals per group.

*Tail-immersion test*

The tail-immersion test was used to determine the central antinociceptive response of the test samples. The method is based on the observation that morphine-like drugs selectively prolong the reaction time of tail withdrawal reflex in animals. Sixty mice were assigned to 12 groups of 5 mice each and fasted for 12 h. The animals were given different doses (250 and 500 mg $kg^{-1}$ crude extract; 100 and 200 mg $kg^{-1}$ of the fractions) orally, 0.2 mL of 5 % Tween 80 solution (negative control) and morphine sulphate 4 mg $kg^{-1}$ (positive control), 30 min prior to the immersion of the distal end of the tail of each mice (2 - 3 cm) into hot water maintained at 50 ± 1°C. The reaction time (in seconds) was the time taken between tail immersion and deflection of the tail due to pain. The maximum reaction time was fixed at 20 sec to prevent tail tissue injury. Time taken for the animal to withdraw the tail was taken as the indicator of antinociception and was recorded at 60, 120, 150, and 180 min after the administration of morphine and test samples. Maximum possible effect was calculated.

*Formalin test*

Formalin test is a tonic pain model that is widely used in the assay of antinociceptive activity.[27] The antinociceptive activity of the ethyl acetate fraction of *C. stenopetala* was evaluated as described by Pandurangan *et al.*[27] Five mice per group housed in transparent cages (observation chambers), were administered with different doses of test fraction (100 and 200 mg $kg^{-1}$ ethyl acetate fraction), morphine (4 mg $kg^{-1}$) and 5 % Tween 80. After 15 min, 20 µL formalin (2.5 %) was injected to the dorsal surface of the left hind paw. The mice were observed for 30 min after the injection of formalin and duration of paw licking recorded as early phase (0 – 5 min) and late phase (15 – 30 min). The percentage inhibition of paw licking was calculated.

*Evaluation of Antioxidant Activity*

*DPPH free radical scavenging assay*

Scavenging activity against 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) radical was evaluated as described by Mensor *et al.* with slight modification.[28] Various concentrations (1, 0.8, 0.5, 0.2, 0.1 and 0.05 mg $mL^{-1}$) of sample extracts in methanol were prepared. To 1 mL of each solution, 3 mL of methanol was added and then 1 mL of a 1 mM DPPH in methanol was added to make up to 5 mL. After 30 min of protection from light at room temperature, the absorbance of the test and blank samples were recorded at 517 nm on a spectrophotometer. The experiment was

repeated with ascorbic acid (0.005, 0.01, 0.02, 0.05, 0.1, 0.2 mg $mL^{-1}$) which served as positive control. All determinations were carried out in triplicates. The same procedure was repeated using blank solution DPPH without the extracts. The procedure was repeated for 0.2 gm $mL^{-1}$ of the various fractions (hexane, chloroform, ethyl acetate and aqueous) in methanol and their absorbance compared with that of ascorbic acid. The decrease in absorbance was then converted to percentage scavenging activity (%SA) using the formula:

DPPH radical scavenging activity (%SA) = $[(A_b-A_s)/A_b] \times 100$
Where; $A_b$ = Absorbance of the blank solution
$A_s$ = Absorbance of the test (extract) solution or the standard (ascorbic acid)

*Determination of the Total Phenolic Content (TPC)*
The total phenolic content of methanol extract was determined with the modified Folin-Ciocalteu reagent.[29] For the preparation of the gallic acid calibration curve, 1 mL aliquots of 0.005, 0.01, 0.02, 0.05, 0.1 and 0.2 mg $mL^{-1}$ ethanolic gallic acid solutions were mixed with 5 mL Folin-Ciocalteu reagent (diluted tenfold) and 4 mL (75 g/L) sodium carbonate. The absorbance was read after 1 h at 610 nm and the calibration curve drawn. 1 mL ethanol extract of the extract was mixed with the same reagent as described above and after 1 h, the absorbance was measured for the determination of the total plant phenolic content. All determinations were performed in triplicates. The blank solution was made up of 5 mL Folin-Ciocalteu reagent, 4 mL sodium carbonate solution and 1 mL ethanol. The total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per gram of extract calculated thus;

T = CV/M
Where T = total phenolic content, mg $mL^{-1}$ of extract, in GAE
C = the concentration of gallic acid in the plant extract established from the calibration curve (mg/mL)
V = the volume of extract (mL)
M = the weight of ethanol extract (g)

*Statistical Analysis*
The data were expressed as mean ± S.E.M and were evaluated using one-way analysis of variance. $P < 0.05$ was considered significant.

## Results and Discussion

Pain is generally induced by tissue damage, infections, inflammation, malignancy and other disease-states or condition. In this study, chemical (acetic acid, formalin) and thermal (hot plate, hot water) methods were employed to induce pain. Acetic acid induced nociception was used to initiates formation of pro-inflammatory mediators that excite pain nerve endings while thermal-induced nociception (tissue damage) initiates non-inflammatory-mediated nociception.[30, 31] These actions in turn induce pathological pain.[29-31] Pain alleviating effect of the methanol root bark extract of *C. stenopetala* and its fractions on inflammation-mediated nociception (abdominal writhing test), non-inflammation-mediated nociception (hot plate test and tail flick test) and both types of nociception (the formalin-induced paw licking test) was evaluated on mice. These models were employed due to their sensitivity in the measurement of pathological pain.

Various laboratory models are employed in the evaluation of peripherally and central antinociceptive responses of test agents. These include acetic acid induced writhing assay which has been reported as a sensitive procedure to evaluate peripherally acting analgesics.[10] This model has been proposed to represents pain sensation by triggering localized inflammatory response.[32-34] Acetic acid induced writhing model cause the release of endogenous mediators which stimulate neurons that are sensitive to drug agents such as analgesics.[35] Drug agent that could reduce the frequency of twisting of dorsoabdominal muscles constrictions (writhing) initiated by released endogenous substances probably does so by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition. [32-34, 17] Hot plate method is considered to be selective for drugs acting centrally.[36] This *in vivo* model measures the complex response to a non-inflammatory, acute nociceptive input and is one of the models normally used for studying central nociceptive activity. [36] Narcotic analgesics exhibit both peripheral and central mechanisms of pain.[37, 38]

In the writhing test, data obtained showed that methanol extract of *C. stenopetala* root bark and its fractions (ethyl acetate and aqueous) caused significant reduction in the number of writhes in mice compared to control animals that received 5 % Tween 80 vehicle (Table 1). At both test doses (250 and 500 mg $kg^{-1}$), the root bark extract of *C. stenopetala* significantly ($P < 0.05$) reduced the frequency of the writhing thereby offering an appreciable level of protection (65.14 and 84.06 %, respectively) to the test animals while the standard drugs acetyl salicylic acid (aspirin) and diclofenac sodium exhibited 74 and 82.07 %, respectively. The highest percentage inhibition of abdominal constriction was observed at 200 mg $kg^{-1}$ of the ethyl acetate fraction. At tested dose of 200 mg $kg^{-1}$ ethyl acetate and aqueous fractions exhibited significant protection of the animals (93.27 and 50.63 % respectively). Ethyl acetate fraction at 200 mg $kg^{-1}$ exhibited a stronger analgesic effect (93.27 %) compared to the acetylsalicylic acid (74 %) and diclofenac sodium (82.07 %) (Table 1).

The significant reduction of writhes after administration of the root bark extract of *C. stenopetala* and its polar fractions can be attributed to the possibility of the plant possessing analgesic effect that may be in part or totally mediated peripherally. Non-steroidal anti-inflammatory and analgesic drugs mitigate inflammatory pain by interfering with the formation of pain mediators at the peripheral target sites where prostaglandins and bradykinin are proposed to play a significant role in the pain process.[17] Acetylsalicylic acid and diclofenac sodium are peripheral acting analgesic.[39] Active phytoconstituents of *C. stenopetala* root bark extract and its polar fractions may have exerted the observed peripheral antinociceptive action by inhibiting the synthesis, release and/or antagonizing the action of pain mediators at the target sites. The percentage in the number of reduction in the abdominal reflex is an indication of the degree of the analgesic effectiveness of the medicinal plant in the acetic acid writhing reflex model.[25] To assess the central mechanism of *C. stenopetala*, in producing analgesia, hot plate and tail-flick tests in mice were employed. Both methods can be differentiated, the hot plate reaction is based on supra-spinal reflex, involving higher brain functions while the tail immersion indicates the spinal reflex to nociceptive stimuli.[40]

The hot plate test has selectivity for opioid derived centrally mediated analgesia and an increase in the pain reaction time indicates the level of analgesia of the test substance.[22, 41] The longer the elevation in latency, the better the test agent as an analgesic agent[42, 41] Marked elevation in latency or mean post reaction time (PRT) was observed in the animals treated with test extract at 500 mg $kg^{-1}$, morphine and diclofenac sodium during the hot plate test compared to control group, 5 % Tween 80 (Figure 1). At 200 mg $kg^{-1}$ the polar fractions (ethyl acetate and aqueous) showed improved elevation in latency (Figure 2). Between 30 and 90 min the analgesic effects of these polar fractions were significant compared to the negative control (Figure 2). Thus, ethyl acetate and the aqueous fractions exhibited significantly superior pain reducing effect than the non-polar fractions (hexane and the chloroform fractions) but less compared to the standard drugs (Figure 2).

The result of the hot plate test also indicated that the root bark extract of *C. stenopetala*, has anti-nociceptive effect which was lower than the ethyl acetate and aqueous fractions (polar fractions).

The increased antinociceptive potential observed in the polar fractions may be due to increase in concentration of bioactive compound(s) in the fractions. The protection rank exhibited by the fractions is as follows ethyl acetate fraction > aqueous fraction> chloroform > hexane (Figure 2).

Tail immersion test further explored the possible central mechanism of analgesic action of *C. stenopetala*. The result of this test showed that the test samples (methanol extract at 500 mg $kg^{-1}$, polar fractions at 100 and 200 mg $kg^{-1}$) significantly ($P < 0.05$) increased the latency time when compared to the negative control (Table 2). Morphine, an opioid analgesic and centrally acting analgesic, showed 57.13 % ($P < 0.05$) activity.

**Table 1:** Effect of *C. stenopetala* methanol root bark extract on acetic acid-induced writhing in mice

| Groups | Dose (mg $kg^{-1}$) | No. of writhing | % inhibition |
|---|---|---|---|
| 5 % Tween 80 | 0.2 mL | 100.4 ± 1.03 | - |
| Acetyl salicylic acid | 100 | 26.1 ± 0.27* | 74.00 |
| Diclofenac sodium | 10 | 18.00 ± 1.2* | 82.07 |
| Extract 250 | 250 | 35 ± 0.43* | 65.14 |
| 500 | 500 | 16 ± 0.57* | 84.06 |
| Hexane Fraction | 200 | 82.61 ± 0.20 | 17.72 |
| Chloroform fraction | 200 | 97.86 ± 0.24 | 2.53 |
| Ethyl acetate | 200 | 6.76 ± 0.33* | 93.27 |
| Aqueous fraction | 200 | 49.57 ± 0.46* | 50.63 |

Each data represents mean ± SEM; n = 5
* $P < 0.05$ is significant when compared with the vehicle (5 % Tween 80)

**Table 2:** Effect of methanol extract of *C. stenopetala* on tail immersion test in mice

| Treatment/ Drug | Dose (mg/kg) | Pre-treatment RT (%) | Latency time in minutes | | | |
|---|---|---|---|---|---|---|
| | | | 60 | 120 | 150 | 180 |
| Control | 0.2 mL | 3.11 ± 0.01 | 3.15 ± 0.01 | 3.5 ± 0.01 | 3.5 ± 0.01 | 4.0 ± 0.01 |
| Crude extract | 250 | 3.5 ± 0.11 | 4.17 ± 1.27 (6.05) | 5.25 ± 0.68 (10.61) | 6.28 ± 1.31 (16.85) | 6.32 ± 1.06 (14.5) |
| | 500 | 3.00 ± 1.05 | 4.22 ± 1.53 (6.35) | 6.32 ± 1.36* (17.09) | 7.13 ± 0.67* (22.00) | 8. 21 ±1.19* (26.31) |
| Hexane fraction | 100 | 2.5 ± 0.63 | 3.7 ± 1.13 (3.26) | 4.13 ± 2.01 (3.82) | 4.56 ± 1.53 (6.42) | 5.06 ± 1.21 (6.63) |
| | 200 | 3.50 ± 0.74 | 3.8 ± 0.83 (3.86) | 4.86 ± 2.07 (8.24) | 4.93 ± 1.17 (8.61) | 6.03 ± 1.24 (12.69) |
| Chloroform fraction | 100 | 3.20 ± 1.36 | 3.27 ± 1.25 (0.71) | 3.84 ± 1.41 (2.06) | 4.37 ± 0.08 (5.27) | 4.56 ± 1.02 (6.42) |
| | 200 | 3.50 ± 1.21 | 4.27± 1.52 (4.67) | 4.43 ± 1.03 (5.63) | 4.72 ± 1.05 (7.39) | 5.22 ± 0.67 (7.63) |
| Ethyl acetate fraction | 100 | 4.00 ± 0.89 | 5.5 ± 1.20 (13.94) | 6.24 ± 2.13 (16.61) | 7.86 ± 1.44* (26.42) | 8.14 ± 1.64* (28.12) |
| | 200 | 4.50 ± 1.16 | 6.6 ± 1.07* (20.47) | 8.51 ± 0.52* (30.36) | 10.27 ± 0.56* (31.03) | 10.58 ± 0.83* (41.13) |
| Aqueous fraction | 100 | 4.00 ± 1.71 | 4.8 ± 1.50 (9.97) | 6.22 ± 1.05 (16.48) | 6.38 ± 1.33 (17.45) | 6.84 ± 1.32 (17.75) |
| | 200 | 4.50 ± 1.20 | 5.46 ± 0.47 (13.71) | 6.1 ± 1. 49 (15.76) | 7.34 ± 1.80* (23.27) | 7.79 ± 1.21* (23.69) |
| Morphine | 4 | 4.00 ± 1.05 | 7.00 ± 1.04* (22.85) | 9.39 ± 1.13* (35.70) | 12.58 ± 1.36* (55.03) | 13.41 ± 1.02* (57.13) |

Values are mean ± SEM, n = 5. * Significant at $p < 0.05$ compared to control.
Parenthesis represent percentage increase in pain reaction time (PRT).

**Table 3:** Effect of ethyl acetate fraction of *C. stenopetala* root bark on the licking time of mice in formalin test

| Drugs | Dose mg/kg | Licking time | | % Inhibition | |
|---|---|---|---|---|---|
| | | 0–5 (min) | 15–30 (Min) | 1st phase | 2nd phase |
| 5% Tween 80 | 0.2 ml | 57.06±1.50 | 69.62±1.51 | | |
| Ethyl acetate | 100 | 34.41±2.17* | 39.73±2.21* | 39.70 | 42.93 |
| Ethyl acetate | 200 | 25.11±1.15* | 27.40±0.87* | 60.64 | |
| Morphine | 4 | 21. 43 ± 3.50* | 25.13±1.24* | 63.90 | |

Values are mean ± SEM; (n=5). * Significant difference at $p < 0.05$ compared to control.

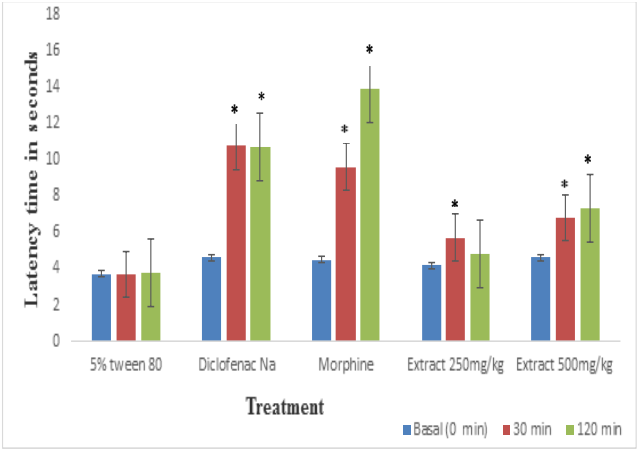


**Figure 1:** Effects of the methanol root bark extract of *C. stenopetala* on latency time in the hot plate test.
* Significant difference at $p < 0.05$ when compared to control.

The effect of ethyl acetate fraction at 200 mg $kg^{-1}$ at 180 min was comparable to the reference drug, morphin (4 mg $kg^{-1}$).
Increase in stress tolerance ability of animals in hot plate and tail immersion models indicates the possible involvement of higher center, possibly may have been mediated through central nervous system.[25] Morphine exhibited significant and good analgesic effects in both the hot plate (supra spinal) as well as the tail immersion (spinal) test, while *C. stenopetala* root bark extract (250 and 500 mg $kg^{-1}$) and its active fractions (ethyl acetate > aqueous) produced statistically significant but lesser degree of antinociceptive response to that of morphine in both test suggesting that the plant extract may act as mild narcotic analgesic.
Inhibition of acetic acid-induced writhing can detect non-analgesics such as antihistamines, muscle relaxants, monoamine oxidase inhibitors.[43] Therefore, formalin test was employed to confirm the antinociceptive effect of the sample extract and its active fractions. Formalin test encompasses neurogenic, inflammatory and central mechanisms of nociception.[43] The first phase of the formalin induced hind paw licking is selective for centrally acting analgesics such as morphine and represents direct chemical stimulation of the sensory nerve fibres (neurogenic). The first phase is characterized by an immediate acute pain occurring within the first ten (0 - 10) minutes after formalin injection. The second phase of formalin-induced hind paw licking is peripherally mediated and represents the events of inflammatory pain and mechanisms of central sensitization (15 - 30 min).[44] Thus, the test postulates the site and the mechanism of action of the analgesic. [45] Central analgesic drugs like narcotics (morphine) inhibits both phases, whereas peripheral acting analgesics affects mostly the second or late phase response. Animals treated with morphine (4 mg $kg^{-1}$) significantly reduced formalin-induced nociception in both phases with 62.44 % and 63.90 % ($P < 0.05$) for first phase and second phase respectively as shown in Table 3. The ethyl acetate fraction produced inhibition at both phases when compared to negative control group. At neurogenic phase (0 – 5 min), 39.70 and 55.99 % of licking time inhibition was recorded for 100 and 200 mg $kg^{-1}$ of ethyl acetate fraction respectively. While 42.93 and 60.64% inhibition was observed for 100 and 200 mg $kg^{-1}$ of test fraction respectively at inflammatory phase (15 –

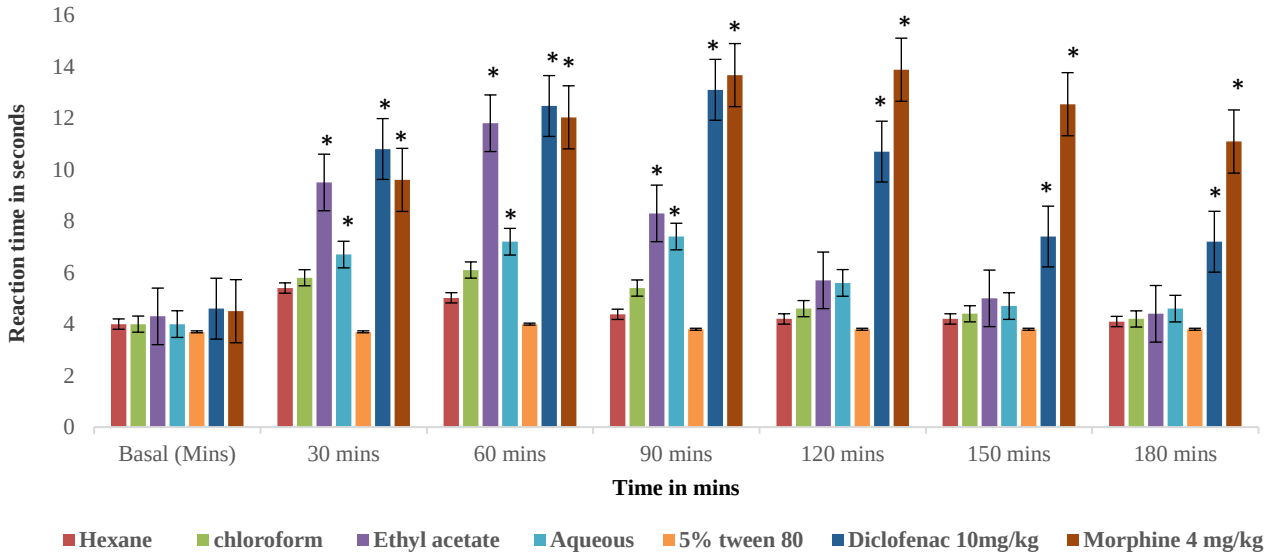


**Figure 2:** Effects of the fractions from methanol root bark extract of *C. stenopetala* on mouse reaction time in the hot plate model.
***** Significant difference at $p < 0.05$ when compared to control.

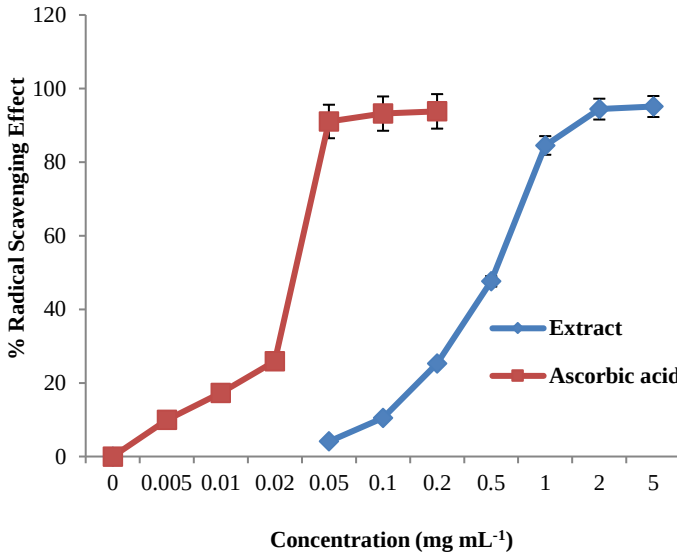


**Figure 3:** DPPH free radical scavenging effect of *C. stenopetala* root bark extract and ascorbic acid. Values are mean ± SEM, n = 3.

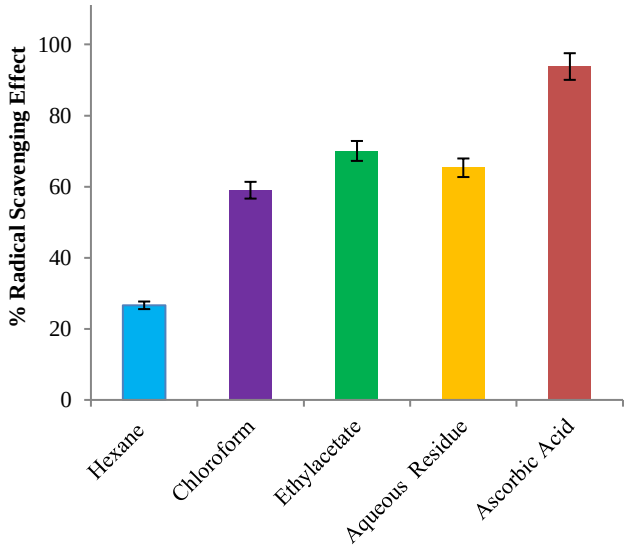


**Figure 4:** DPPH's radical scavenging activity of various fractions of *C. stenopetala* root bark and Vitamins C.
Values are mean ± SEM, n = 3.

30 min) of formalin-induced licking test when compared with the negative control (5 % Tween 80). The suppression of neurogenic and inflammatory pains by ethyl acetate fraction in this study might imply that it contains active analgesic principle that may be acting both centrally and peripherally similar to the reference drug morphine. However, the activity of ethyl acetate fraction was higher in the inflammatory phase than the first phase, confirming the high percentage of writhing inhibition observed in acetic acid induced writhing test (93.27 %) compared with its central acting potential (hot plate and tail immersion test). In the hot plate test, the effect was not long lasting compared to morphine and diclofenac sodium (Figure 2).

DPPH (1, 1-diphenyl-2-picrylhyrazyl) radical scavenging assay is an effective and widely used method for evaluation of the antioxidant activities of plants. [28] The percentage (%) scavenging of DPPH radical was found to be concentration-dependent both both extract and the standard (ascorbic acid). The $IC_{50}$ values of extract and ascorbic acid were calculated to be 7.54 µg $mL^{-1}$ and 4.87 µg $mL^{-1}$, respectively (Figure 3).

The result of this study reveals that DPPH radical scavenging activity resides more in the polar fractions than the non-polar fractions as compared with a known standard, ascorbic acid (Figure 4).

The free radical scavenging activities of the active polar fractions (ethyl acetate and aqueous) may be as a result of its phenolic status (Figure 5). Boye *et al.* had suggested that a relationship exist between antioxidant activity and antinociception.[46] Free radicals, ROS (reactive oxygen species) have been reported to trigger second messenger system involved in sensitization of neurons and spinal glial cells, which in turn stimulates pain.[47, 48] Thus an increase in the antioxidant in the body system to mop up the free radicals will likely reduce pain perception.[49] Antinociceptive agent having antioxidant effects may likely increase pain threshold and advantage in pain management.[46] In line with this observation, ethyl acetate and aqueous fractions have the highest DPPH free radical scavenging activity (Figure 4) and analgesic effect (Tables 1 and 2, Figure 2). The antioxidant effects of *C. stenopetala* may be responsible or has contributing effects to its antinociceptive activity. [49, 50] DPPH assay is a used for evaluating the antioxidant activity of plant extracts.[51]

Preliminary phytochemical analysis carried out on the crude methanol root bark extract of *C. stenopetala* indicated the presence of terpenoid, tannin, alkaloid which are generally known to be involved in the inhibition of synthesis of prostaglandins, superoxides and other endogenous substances that are responsible for triggering pain perception.[52, 53] Tannin and flavonoid, phenolic compounds are known to be powerful antioxidant and

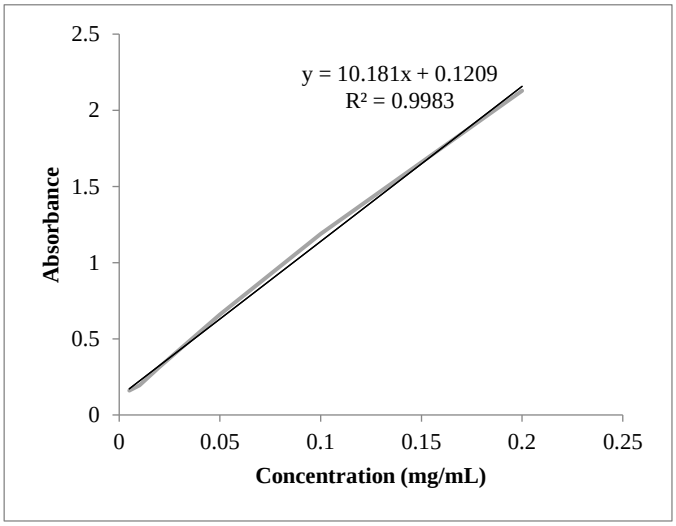


**Figure 5:** Gallic acid calibration curve.
TPC = 1 mL x 0.07/0.001 g = 70.00 mg $g^{-1}$

play role in analgesic activity primarily by their scavenging activities.[54, 55] The study showed that both the central and the peripheral analgesic effects of the test extract seem to be more pronounced or significant in the polar fractions (ethyl acetate > aqueous) which may be due to its polyphenolic content. The total phenolic content of the extract measured using Folin-Ciocalteu reagent was calculated as 70.00 mg $g^{-1}$in terms of gallic acid. No lethal effect was observed within 24 h after the administration of the extract at the doses used, even at the highest dose tested (8000 mg/kg). Therefore, the median lethal dose ($LD_{50}$) of the extract is greater than 8000 mg $kg^{-1}$.

Pain control assessing data from *C. stenopetala* crude root bark extract and its polar fractions indicate increase in pain threshold thereby justifying its use by herbalists for treatment of toothache.

## Conclusion

On the basis of these findings, it may be inferred that methanol root bark extract and polar fractions of *C. stenopetala* exhibit antinociceptive activities and may be acting through both central and peripheral nociceptive mechanisms. They also have DPPH free radical scavenging activities which may contribute to it effectiveness in the management of pain. The study also corroborates the traditional and folklore use of *C. stenopetala* in treatment of toothache.

## Conflict of interest

The authors declare no conflict of interests.

## Authors' declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

## Acknowledgements

The authors are grateful to Mr. Ozioko of Bioresources Development and Conservation Programme (B.D.C.P), Nsukka, Enugu State (retired staff Department of Botany, University of Nigeria Nsukka, Enugu state) and Mr. Daramola, a curator, former staff of University of Lagos, Department of Botany herbarium, Faculty of Science for identification and authentication of the plant. We also thank Mr. Ota and Department of Physiology, College of Medicine, University of Lagos, Lagos, for providing technical support through the course of the research.

## References

1. Bashir L, Oluwatosin KS, Adamu YK, Ali AJ, Maimuna BU, Eustace BB, Alozieuwa BU. Potential antimalarials from African natural products: A review. J Intercult Ethnopharmacol. 2015; 4 (4): 318–343.
2. Oladosu IA, Ogundajo AL, Alyalaagbe OO, Emenyonu N. Phytochemical and antituberculosis activity of *Coffea brivipes*, Hiern exracts. Res J Phytochem. 2011; 5: 130–135.
3. Cragg G, Newman DJ. Biodiversity: a continuing source of novel drug leads. Pure and Applied Chemistry. The Sci J IUPAC. 2005; 77 (1): 7–24.
4. Agrawal, S.S., Paridhavi, M., 2007. Introduction to medicinal plants. In Herbal Drug Technology. Universities Press Pvt. Ltd., Hyderabad, First Ed., 2007, India, 1–13p.
5. Saalu LC. Review article: Nigerian folklore medicinal plants with potential antifertility activity in males: a scientific appraisal. Res J Med Plant. 2016; 10: 201–227.
6. Marston A, Hostettmann K. Plant molluscicides. Phytochemistry. 1985; 24:639–652.
7. Muhammad N. *In-vivo* models for management of pain. Pharmacology & Pharmacy. 2014; 5: 92–96.
8. Gomase PPV, Shire PS, Nazim S, Choudhari AB. Development and evaluation of analgesic polyherbal formulation containing some indigenous medicinal plants. Int J Pharm Bio Sci. 2011; 2(3): 119–127.
9. Debono DJ, Hoeksema LJ, Hobbs RD. Caring for patients with chronic pain: Pearls and pitfalls. J Am Osteopath Assoc. 2013; 113(8): 620–627.
10. Deshmukk DB, Padwal KG, Gaikwad DD. Comparative study of analgesic activity of two Indian medicinal plants: *Jasminum sambac* and *Ficus recemosal*. World J Pharm and Pharm Sci. (WJPPS). 2014; 3(6): 1305–1311.
11. Santos CCP, Salvadori MS, Mota VG, Costa LM, Cardoso de Almeida AM, Lopes de Oliveira GU, Costa JP. Antinociceptive and antioxidant activities of Phytol *in vivo* and *in vitro* models. Neuroscience J. 2013; 2013: 1–9.
12. Kohn DF, Martin TE, Foley PL, Morris TH, Swindle MM, Vogler GA, Wixson SK. Public statement: guidelines for the assessment and management of pain in rodents and rabbits. J Am Assoc Lab Anim Sci. 2007; 46(2): 97–108.
13. Kumara N. Identification of strategies to improve research on medicinal plants used in Sri Lanka. In: WHO Symposium. University of Ruhuna, Galle, Sri Lanka. 2001
14. Jensen GS, Ager DM, Redman KM, Mitzner MA, Benson KF, Schauss GA. Pain Reduction and improvement in range of motion after daily consumption of an Açai (*Euterpe oleracea* Mart.) pulp–fortified polyphenolic-rich fruit and berry juice blend. J Med Food. 2011; 14(7-8): 702–711.
15. Stephens R. Antioxidants: new kid on the block for pain relief? Research communications, The Ohio State University. 2006
16. Rokyta R, Holecek V, Pekárková I, Krejcová J, Racek J, Trefi L, Yamamotová A. Free radicals after painful stimulation ares influenced by antioxidants and analgesics. Neuro Endocrinol Lett. 2003; 24: 304 –309.
17. Mensor L, Menezes F, Leitao G, Reis A, Santos T, Coube C, Leitao S. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res. 2001; 15: 127–130.
18. Duarte I, Nakamura M, Ferreira S. Participation of the Sympathetic System in Acetic Acid-Induced Writhing in Mice, Braz J Med Biol Res. 1998; 21(2): 341.
19. Ahmed F, Hossain MH, Rahman AA, Shahid IZ. Antinociceptive and sedative effects of the bark of *Cerbera odollam* Gaertn. Orient Pharm Exp Med. 2006; 6: 344–348.
20. Ferdous M, Rouf R, Shilpi JA, Uddin SJ. Antinociceptive activity of the ethanolic extract of *Ficus racemosa* Linn. (Moraceae). Oriet Pharm Exp Med. 2008; 8: 93–96.
21. Toma W, Graciosa JS, Hiruma-Lima CA, Andrade FDP, Vilegas W, Souza BARM. Evaluation of the analgesic and antiedematogenic activities of *Quassia amara* bark extract. J Ethnopharmacol. 2003; 85: 19–23.
22. Sabina EP, Chandel S, Rasool MK. Evaluation of analgesic, antipyretic and ulcerogenic effect of Withaferin A. Inter J Integrative Biol. 2009; 6(2): 52–56.
23. Elisabetsky E, Amador TA, Albuquerque RR, Nunes DS, Cavalho ACT. Analgesic activity of *Psychotria colorata* (Willd. Ex R. and S.) Muell. Arg. alkaloids. J Ethnopharmacol. 1995; 48: 77–83.

24. Pal S, Sen T, Chaudhuri AK. Neuro-psychopharmacological profile of the methanolic fraction of *Bryophyllum pinnatum* leaf extract. J Pharm Pharmacol. 1999; 51: 313–318.
25. Orabueze IC, Adesegun SA, Coker HA. Analgesic and antioxidant activities of stem bark extract and fractions of Peterianthus macrocarpus. Pharmacog Res. 2016; 8: 181–185.
26. Sofowora AE. Medicinal plants and traditional medicines in Africa. 2nd ed. Spectrum Books: Ibadan, Nigeria. 1993
27. Harborne JB. Textbook of phytochemical methods. 1st ed. London: Chapman and Hall Ltd; 1973.
28. Evans WC. Trease and Evans Pharmacognosy, Saunders an Imprint of Elsevier. 2005, 41 – 47p.
29. Lorke D. A new approach o practical acute toxicity test. Arch Toxicol. 1983; 54(4): 275-286.
30. Burger C, Fischer DR, Cordenunzzi DA, Batschauer de Borba AP, Filho VC, Soares AR. Acute and subacute toxicity of the hydroalcoholic extract from *Wedelia paludosa* (Acmela brasiliensis) (Asteraceae) in mice. J Pharm Pharm Sci. 2005; 8 (2): 370–373.
31. Sanmugapriya E, Venkataraman S. Toxicological investigations on *Strychnos potatorum* seeds in experimental models. J Health Sci. 2006; 52: 339–343.
32. Dar A, Faizi S, Naqvi S, Roome T, Zikr-Ur-Rehman S, Ali M, Firdous S, Moin ST. Analgesic and antioxidant activity of mangiferin and its derivatives: the structure activity relationship. Biol Pharm Bull. 2005; 28(4): 596–600.
33. Ezeja M, Omeh Y.S. Anti-nociceptive activities of the methanolic leaf extract of *Nicotiana tabacum* (Linn). Cont J Pharmacol Toxicol Res. 2010; 3: 5 – 10.
34. Woolfe G, MacDonald AD. The evaluation of analgesic action of pethidine hydrochloride. J Pharmacol Exp Ther. 1994; 80:300.
35. Pandurangan SB, Paul AS, Savarimuthu I, Ali AA. Antinociceptive, immunomodulatory and antipyretic activity of nymphayol isolated from Nymphaea stellata (Willd.) flowers. Biomol Ther. 2013; 21: 391–397.
36. Mcdonald S, Prenzler PD, Autolovich M, Robards K. Phenolic content and antioxidant activity of olive oil extract. Food Chem. 2001; 73: 73–84.
37. George KA, Eric W, David DO, George AK. Antinociceptive effects of *Newboulia laveis* (P. Beauv) stem bark extract in rat model. Pharmacog. Mag. 2009; 17: 49–54.
38. Rivest S, Lacroix S, Vallieres L. How the blood talks to the brain parenchyma and the paraventricular nucleus of the hypothalamus during systemic inflammatory and infectious stimuli. Proc Soc Exp Biol Med. 2000; 223: 22-38.
39. Onzago RO, Kiama SG, Mbaria JM, Gakuya DW, Githiji CG, Rukenya ZM. Analgesic activity of aqueous extract of *Vernonia hymenolepis* (A. Rich) a traditional medicine plant used in Kenya for toothache. The J Phytopharmacol. 2013; 2(6): 41–45.
40. Chapman CR, Casey KI, Dubner R, Foley KM, Gracely RH, Reading AE. Pain measurement: an overview. Pain. 1985; 22: 1–31.
41. Ibironke GF, Ajiboye KI. Studies on the antiinflammatory and analgesc properties of *Chenopodium ambrosioides* leaf extract in rats. Int J Pharmacol. 2007; 3: 111–115.
42. Ranadran K, Basinath L. A critical Analysis of the experimental evaluation of nociceptive reactions in animals. Pharm Res. 1986; 3: 253–270.
43. Boakye-Gyasi E, Tabiri Henneh I, Abotsi WKM, Ameyaw EO, Woode E. Possible mechanisms involved in the anti-nociceptive effects of hydro-ethanolic leaf extract of *Ziziphus abyssinica*. Pharm Biol. 2017; 55: 1962 – 1971.
44. Berken T, Ostunes L, Lermioglu F, Ozer A. Antiinflammatory analgesic and antipyretic effect of an aqueous extract of *Erythraea ceulaurum*. Planta Med. 1991; 57: 34 -37.
45. Chau TT. Analgesic Testing in Animal Models. Pharmacological Methods in the Control of Inflammation. New York, Liss. 1989, 342p.
46. Boye A, Amoateng P, Koffuor GA, Barku VY, Bawa EM, Anto OE. Antinociceptive and antioxidant activity of an aqueous root bark extract of *Daniellia oliveri* (Rolfe) Hutch. & Dalziel (Fam: Leguminosae (Fabaceae)) in ICR mice. J Appl Pharm Sci. 2013; 3 (12): 36–45.
47. Ali DW, Salter MW. NMDA receptor regulation by Src kinase signaling in excitatory synaptic transmission and plasticity. Curr Opin Neurobiol. 2001; 11: 336–342.
48. Zhang X, Wu J, Willis WD. The effects of protein phosphatase inhibitions on nociceptive behavioral responses of rats following intradermal injection of capsaicin. Pain 2003; 106: 443–451.
49. Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanism. J Pharmacol Sci. 2004; 96: 229–245.
50. Onoja SO, Ezeja MI, Omeh YN, Onwukwe BC. Antioxidant, anti-inflammatory and antinociceptive activities of methanolic extract of *Justicia secunda* Vahl leaf. Alexandria J Med. 2016;
51. Venkateshwarlu E, Reddy AR, Goverdhan P, Rani KS, Reddy GI. *In vitro* and *in vivo* antioxidant activity of methanolic extract of *Solena amplexicaulis* (whole plant). Int J Pharma Biol Sci. 2011; 14:522–533.
52. Yusriyya S, Harisha CR, Vinay J, Shukla A, Rabinarayan A. Pharmacognostical evaluation of *Launaea sarmentosa* (Willd.) schultz-bip.ex Kuntze root. J Res Ayurveda. 2013; 34(1): 90–94.
53. Nagalapur SK, Paramjyothi S. *In vitro* antioxidant activity of *Launaea Pinnatifida* cass leaves. Biogeosciences. 2010; 5(1): 105–108.
54. Rajnarayana K, Reddy MS, Chaluvadi MR., Krishna DR. Biflavonoids classification, pharmacological, biochemical effects and therapeutic potential. Indian J Pharmacol. 2002; 33: 2–16.
55. Moniruzzaman M, Khatun A, Imam MZ. Evaluation of antinociceptive activity of ethanol extract of leaves of *Adenanthera pavonina*. Evidence-Based Complementary and Alternative Medicine : eCAM. 2015; (2015): 1 – 8.