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EFFECT OF *NEOCARYA MACROPHYLLA* LEAVES EXTRACTS ON MURINE MODEL OF PAIN

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ABSTRACT

Pain is the most common symptom of disease, which accompanies us from an early age. It is connected to the stimulus that it invokes and is also based on the observation of psychological interpretation of the phenomena taking place. The aim of this research is evaluation of the effect of *Neocarya macrophylla* leaves extracts on animal model of Pain. The pulverized leaves were extracted by maceration using 90 % methanol for 7 days and filtered to retrieve the residue known as the crude methanol leaf extract (MEL). Some part of MEL (60 g) was partitioned into fractions of different solvents with increasing polarity including chloroform (CF), ethylacetate (EF), n-butanol (BF) using liquid-liquid fractionation. Acute toxicity study was carried out to determine the LD_{50} of CF and it was estimated to be 565 mg/kg using Lorke's method. Analgesic studies was carried out on extract and partitioned fractions using acetic acid-induced writhing test in mice and the results revealed that the extract and fractions of the plant has significant ($p<0.05$) analgesic effect with EF being the most active fraction and CF being the least active; EF demonstrated significant ($p<0.05$) and dose-dependent analgesic activity with 83.9, 82.5 and 73.4 % inhibition of writhes at 20, 40 and 80 mg/kg, respectively; the standard drug, piroxicam (at 10 mg/kg) had 96.5 % inhibition of writhes. Qualitative phytochemical screening on the fractions revealed the presence of some phytochemicals.Conclusively, this study indicates that the leaves of *N. macrophylla* contains phytochemical constituents with analgesic activity and thus, validates the ethnomedicinal claim of the use of the plant in the management of pain.

Keywords: *Neocarya macrophylla*, Toxicity, Analgesic, Phytochemicals

INTRODUCTION

Pain can be experienced as an acute, chronic or intermittent condition or a combination of the three (Henschke *et al*., 2015). Specifically, chronic pain is a complex condition embracing physical, social, and psychological factors, consequently leading to disability, loss of independence and poor quality of life (Breivik *et al*., 2006).The feeling of pain can be caused by irritation of pain receptors, which can be found in the skin, joints and many internal organs. The cause of pain may also be damage to the nervous system, the peripheral nerves, brain and spinal cord. Experience of pain depends on the strength of the stimulus, individual susceptibility and individual resistance to pain. Pain receptors are sensitive to mechanical, thermal or chemical stimuli. The operation of noxious stimulus to these receptors results in the processing into an electrical signal. This impulse is conducted by nerve fibers into the spinal cord and then to the brain (Paulina *et al*., 2013).One of the most significant health problem all around the world are the acute chronic pain diseases. Although, numerous medications are known to treat and manage pain diseases, their prolonged use mostly leads to gastrointestinal intolerance, bone marrow depression, water and salt retention (Housseizadeh *et al*., 2000). Recent findings have introduced several different synthetic drugs of better efficacy, but most of them are associated with severe side effects. These drawbacks necessitate the need to search towards integrating complementary medicine with the mainstream medicine to increase efficacy and to decrease side effects and costs. Globally, opiods such as paracetamol, morphine, methadone and codeine and Non-steroidal anti-inflammatory drugs (NSAIDs) such as Aspirin, ibuprofen, Naproxen etc are the drugs of choice used in the management of mild and moderate pain due to their strong efficacy (Zarin *et al*.,2005).However , the drugs are associated with severe side effects such as tolerance, dependency, respiratory depression (Howland and Mycek,2006), gastric irritation leading to hyper acidity and aggravation of ulcer (Insel,2006). These drawbacks necessitate the need to search for effective alternatives with safe therapeutic profile.

The use of herbs as medicine is the oldest form of healthcare known to humanity and has been used in all cultures throughout history. Medicinal plants are the back bone of folk medicine. In fact, they are the oldest friend of humans (Fansforth, 1994) . According to the World Health Organization (WHO) more than 80% of the world population, mostly in third world rely on herbal/traditional plant-based medicines for their primary healthcare needs (WHO, IUCN 1993). Traditional medicine is derived from medicinal plants, minerals and organic matter and they are the sum total of knowledge, skill and practices based on theories, beliefs and experiences indigenous to different cultures that are used to maintain health as well as prevent, diagnose, improve and treat physical and mental illnesses (Acharya, 2008).

In this study, *Neocarya macrophylla* (Sabine) Prance (Chrysobalanacea) a native plant of Nigeria and Sudan with a long history of consumption, less side effects and cost-effectiveness has been used. *N. macrophylla* is a small, bushy, evergreen tree growing up to 10 m tall and it has a rounded, bushy crown with densely tomentose branchlets (Bayero *et al*., 2019). It is a genus in the Chrysobalanaceae family as described by Prance ex White in 1976 and it contains only one known species *Neocarya macrophylla* (Dressler *et al*., 2019). The plant family is composed of 17 genera and about 525 species that are mainly woody plants, shrubs and trees mostly found in tropical and subtropical regions (Yakandalawa *et al*., 2010). It is commonly known as Ginger bread plum in English or Neou oil tree, Gawasa or Farar rura in Hausa, Naawdi in fulfulde and Kobenci in Nupe. *N. macrophylla* is used

extensively in the Northern parts of Nigeria in ethnomedicine to treat numerous diseases such as asthma, skin infections, wounds, dysentery, inflammations, pulmonary troubles, ear, and eye infections (Warra , 2012).The ethanol leaf extract of *N.macrophylla* indicated the presence of flavonoids, steroids, palmitoleic acid, alpha tocopherol, beta tocopherol, tannins and glycosides (Datti *et al*., 2020). It reported that the decoctions of the leaves and bark are used as mouth wash, internal troubles and for inflamed eyes (Freidrick, 1961). The leaves are also chewed and applied topically for the relief of pain (Amza *et al*., 2010). Other pharmacological actions have been reported on the leaf of the plant, antimicrobial (Isaka *et al*., 2017, Olowo-okere, 2018), analgesic (Yusuf *et al*., 2019b) and anti-inflammatory (Yusuf *et al*., 2015c) studies have been reported on the stem bark extract of the plant. Antivenom (Yusuf *et al*., 2019a) and anthelmintic (Ajayi *et al*., 2018).

MATERIALS AND METHODS

Sample Collection and Identification

The Plant samples of *N. macrophylla* were collected at Jega Local Government area, in Kebbi State, Nigeria, in the Month of June 2019. It was identified by a taxonomist, Musa Magaji at the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto and a voucher specimen was obtained PCG/UDUS/CHRY/001.

Sample Preparation

The leaves of the plant were washed with water to remove external impurities and were shade dried and pulverized into powder. The sample was stored in plastic a container prior to use. The pulverized leaves were weighed at 1961g and extracted with 90% methanol using maceration method. It was extracted for 7 days with constant stirring and filtered using Whatman No.1 filter paper. The extracts were freed from solvent by evaporation with the aid of rotary evaporator at 40 ˚C to afford a residue referred to as methanol leaf extract (MEL). Some part of the crude methanol extract (60 g) was suspended in distilled water and it was filtered using filter paper and the filtrate was partitioned using chloroform, ethylacetate, n-butanol and aqueous to obtain the different fractions (Yusuf *et al*.,2019a).

Experimental animals

Locally bred Swiss albino mice weighing (15-22) g were used for the study. Standard commercial chow with water *ad libitum* and they were maintained under standard conditions (12 h light and 12 h dark cycle). The study was approved by the Research Ethical Committee, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto with approval number (PTAC/Nm/(Me)/OT/48-22). The care of the animals was conducted in accordance with established public health guidelines for handling experimental animals.

Qualitative Phytochemical Screening

The MEL and its fractions CF, EF and BF of *N.macrophylla* were subjected to preliminary phytochemical screening to check for the secondary metabolites such as saponins, alkaloids, reducing sugars, tannins, flavonoids, carbohydrates, steroids, oxalates and proteins according to standard procedures (Alebiosu and Yusuf, 2015).

Acute toxicity studies

The investigation of the acute toxicity was carried out according to the techniques outlined by Lorke, 1983. In the first phase, nine mice of either sex were grouped into 3 groups of 3 mice each. The first, second and third groups received 10,100 and 1000 mg/kg of the chloroform extract, respectively, and mice in all groups were observed for signs of toxicity and mortality for 24h. In the second phase, four groups of one mouse each were injected *i.p* with the chloroform extract at doses 200, 400, 800 and 1600mg/kg, respectively and observed for signs and symptoms of toxicity for 24.The route of administration was intraperitoneally. The median lethal dose was calculated using the following formula:

$$LD_{50} = \sqrt{Minimal\ lethal\ dose\ X\ Maximal\ survival\ dose}$$

The LD_{50} of the other extracts MEL, EF and BF was adopted from the previous work done by Yusuf *et al*. (2019a).

Analgesic studies

Acetic acid-induced writhing test in mice

The acetic acid-induced writhing test in mice as described by koster, 1959 was used: 25 albino mice were divided into 5 groups of 5 mice each. The first group received 10 mg/cm^3 distilled water (Normal saline) *i.p.* which served as the negative control, while the 2nd group received 10 mg/kg piroxicam intraperitoneally as the positive control. Groups 3, 4 and 5 received 20, 40 and 80 mg/kg of MEL of *N. macrophylla* respectively. The route of administration was intraperitoneal (*i.p.*).Thirty minutes later, mice in all groups were treated with acetic acid (1 % acetic acid of 1 cm^3 per kg body weight). Five minutes after acetic acid injection, mice were placed in an individual cage and the number of abdominal constrictions was counted for each mouse for a period of 10 minutes. Percentage inhibition of abdominal writhing was calculated using the expression;

$$\% \text{ Inhibition} = \text{hibition } \% = \frac{mean\ number\ of\ writhes\ (control) - mean\ number\ of\ writhes\ (test)}{mean\ number\ of\ writhes\ (control)}\ X\ 100$$

The above procedure was repeated for chloroform and ethylacetate fractions (at 20, 40 and 80 mg/kg) and n-butanol fraction (at 7.5, 15 and 30 mg/kg).

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA followed by Post Hoc for multiple comparisons. Values were considered significant at $p<0.05$ compared with control.

RESULTS

Phytochemical studies

The methanol extract of leaves of *N.macrophylla* and its fractions CF, EF and BF were subjected to phytochemical screening for the presence of saponins, alkaloids, reducing sugars, tannins, flavonoids, carbohydrates, steroids, oxalates and proteins (Table 1)

Table 1: Phytochemical screening of the ethyl acetate fraction

S/N	Phytochemicals	Observation	Abundance
1.	Saponins	Persistent froth	++
2.	Alkaloids	Yellow precipitate	+++
3.	Tannins	Greenish black precipitate	+++

4.	Flavonoids	Reddish colour	+++
5.	Anthraquinones	No color change	-
6.	Carbohydrates	Brick red	++
7.	Steroids	Reddish brown ring	++
8.	Oxalates	White precipitate	+
9.	Amino Acids	No color change	-
10.	Proteins	Yellow colour	+

Acute toxicity studies

The intraperitoneal LD_{50} of the chloroform fraction of the methanol leaf extract of *N. macrophylla* was estimated to be 565 mg/kg (Table 2).

Table 2: Determination of median lethal dose (LD_{50}) of chloroform fraction of *N. macrophylla*

First Phase		
Dose(mg/kg)	**Number of mice used**	**Mortality**
10	3	0/3
100	3	0/3
1000	3	2/3
Second phase		
Dose(mg/kg)	**Number of mice used**	**Mortality**
200	1	0/1
400	1	0/1
800	1	1/1
1600	1	1/1

Analgesic studies

Effects of methanol leaf extract on acetic acid induced writhing in mice

The investigation of the analgesic effect of methanol leaf extract of *N. macrophylla* using acetic acid induced writhing test in mice is indicated in Table 3; the extract significantly ($p<0.05$) reduced the number of abdominal constrictions by 32.8, 32.8 and 100 % at 20, 40 and 80 mg/kg while the standard drug, piroxicam had 96.5 % at 10 mg/kg.

Table 3: Effect of methanol leaf extract of *N. macrophylla* on acetic acid induced writhing in mice.

Treatment(mg/kg)	**Mean no of Writhing ± SD**	**% Inhibition (%)**
Normal saline	14.3±8.5	-
MEL 20	9.6±2.7*	32.8
MEL 40	9.6±3.7*	32.8
MEL 80	0.0±0.0*	100.0
Piroxicam 10	0.5±0.6*	96.5

Each value represents mean ± SEM * significantly better analgesic activity than normal saline (negative control) using One-Way ANOVA (Post Hoc Test) at $p < 0.05$;
Key: MEL: Methanol leaf extract

Effect of n-butanol fraction on acetic acid induced writhing in mice

The n-butanol fraction from the leaves of *N. macrophylla* exhibited highest percentage inhibition of writhes (86.6 %) at 30 mg/kg, though the effect was not dose-dependent while the standard drug, piroxicam had 96.5 % at 10 mg/kg as indicated in (Table 4).

Table 4: Effect of n-butanol fraction of *N. macrophylla* on acetic acid induced writhing in mice

Treatment(mg/kg)	**Mean no of Writhing ± SD**	**% Inhibition (%)**
Normal saline	14.3±8.5	-
BF 7.5	4.0±2.3*	72.0
BF 15	5.8±5.7*	59.4
BF 30	2.0±0.8*	86.6
Piroxicam 10	0.5±0.6*	96.5

Each value represents mean ± SEM * significantly better analgesic activity than normal saline (negative control) using One-Way ANOVA (Post Hoc Test) at $p < 0.05$;
Key: BF: n-Butanol fraction

Effect of ethylacetate fraction on acetic acid induced writhing in mice

The evaluation of the analgesic effect of ethylacetate fraction from the leaves of *N. macrophylla* using acetic acid induced writhing test in mice is seen in table 5; the fraction exhibited significant ($p<0.05$) and dose-dependent analgesic effect with 83.9, 82.5 and 73.4 % inhibition at 20, 40 and 80 mg/kg, respectively.

Table 5: Effect of Ethylacetate fraction of *N. macrophylla* on acetic acid-induced writhing in mice

Treatment(mg/kg)	Mean no of Writhing ± SD	% Inhibition (%)
Normal saline	14.3±8.5	-
EF 20	2.3±1.7*	83.9
EF 40	2.5±2.4*	82.5
EF 80	3.8±3.9*	73.4
Piroxicam 10	0.5±0.6*	96.5

Each value represents mean ± SEM *significantly better analgesic activity than normal saline (negative control) using One-Way ANOVA (Post Hoc Test) at $p < 0.05$; Key: EF: Ethylacetate fraction

Effect of chloroform fraction on acetic acid induced writhing in mice

Chloroform fraction of *N. macrophylla* was able to significantly and dose-dependently reduced the number of abdominal constrictions induced by acetic acid with 79.0, 69.9 and 41.9 % at 80, 40 and 20 mg/kg, respectively as indicated in table 6.

Table 6: Effect of chloroform fraction of *N. macrophylla* on acetic acid-induced writhing in mice

Treatment(mg/kg)	Mean no of Writhing ± SD	% Inhibition (%)
Normal saline	14.3±8.5	-
CF 20	8.3±5.1*	41.9
CF 40	4.3±4.0*	69.9
CF 80	3.0±1.4*	79.0
Piroxicam 10	0.5±0.6*	96.5

Each value represents mean ± SEM *significantly better analgesic activity than normal saline (negative control) using One-Way ANOVA (Post Hoc Test) at $p < 0.05$;
Key: CF: Chloroform fraction

DISCUSSION

Phytochemical screening of the methanol extract of leaves of *N.macropylla* revealed the presence of saponins, alkaloids, reducing sugars, tannins, flavonoids, carbohydrates, steroids, oxalates and proteins as presented in Table 1.Analgesic and anti-inflammatory effects of flavonoids,steroids and tannins have been reported by Das et al.,1989,hence the analgesic activity demonstrated by the fraction and its extracts may be attributed individually or collectively to the flavonoids.

Acute toxicity profile of the chloroform fraction of *N. macrophylla* was estimated to be 565 mg/kg suggesting the fraction to be toxic as presented in Table 2.

Acetic acid-induced writhing test is a very sensitive test and used to evaluate peripheral analgesic activity (Gene 1998). Local peripheral receptors are postulated to be partly involved in the abdominal constriction response (Bentley, 1983). MEL of *N. macrophylla* and its fractions has shown significant ($p<0.05$) analgesic activity in acetic-induced writhing test in mice suggesting that the plant possess peripherally-mediated analgesic activity. The mechanism may be mediated via inhibition of cyclooxygenases and/or lipoxygenases (Dhara *et al*., 2000).The abdominal constriction response is thought to involve in part local peritoneal receptors (Bentley *et al*., 1983) so the extract may have interfered with these peritoneal receptors to bring about analgesia. Higher inhibition was observed by the methanol leaf extract, however, fractionation of the extract lead to decrease analgesic activity. Ethylacetate fraction of the plant exhibited significant activity in a dose-dependent manner. NSAIDs relieve pain by suppressing the formation of pain inducing substances such as prostaglandins and bradykinn (Hirose *et al*., 1984, Prahbu *et al*., 2008) in the peripheral tissues (Jain *et al*., 2007).

The treatment at doses 20, 40 and 80mg/kg of the MEL significantly ($p<0.05$) reduced the number of acetic acid-induced writhes with inhibitions of 32.8%, 32.8% and 100.0% respectively. The activity of the extract against acute pain at the highest dose was higher when compared to the inhibitory activity of the standard drug, piroxicam 96.5% (Table 3).

The BF at doses 7.5, 15 and 30mg/kg signifacntly ($p<0.05$) reduced the number of acetic acid-induced writhes with inhibitions of 72.0%, 59.4% and 86.6% respectively. The activity of the extract against acute pain at the highest dose was lower when compared to the inhibitory activity of the standard drug, piroxicam 96.5% (Table 4).

The EF at doses 20, 40, 80 mg/kg signifacntly ($p<0.05$) reduced the number of acetic acid-induced writhes with inhibitions of 83.9%, 82.5% and 73.4% respectively. Piroxicam (10mg/kg) produced 96.5% reduction in abdominal constriction (Table 5).

The treatment at doses 20, 40 and 80 mg/kg of CF significantly ($p<0.05$) reduced the number of acetic acid-induced writhes with inhibitions of 41.9%, 69.9% and 79.0% respectively. . Piroxicam (10mg/kg) produced 96.5% reduction in abdominal constriction (Table 6).

The findings of this research differ from what was reported for the methanol stem extract of *N. macrophylla* where the extract exhibited a lower (63.9 %) percentage inhibition (Yusuf *et al*., 2015c).

CONCLUSION

In conclusion, the results of the phytochemical studies of *N. macrophylla* revealed the presence of some secondary metabolites such as saponins, reducing sugars , alkaloids, tannins, flavovoids, carbohydrates, steroids, oxalates and proteins. The intraperitoneal LD_{50} of chloroform fraction of *N.macrophylla* was determined to be toxic and *N. macrophylla* and its fractions have demonstrated significant analgesic activity by inhibiting the number writhes induced by acetic acid in animal model.

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AUTHOR'S CONTRIBUTION

HBB helped with the draft preparation; AYJ helped with data handling and design of experiment, data analysis,provision of study material and equipment, critically reveiwing and correcting the manuscript, SAZ helped with editing and organising the tables and figures , AIT helped with the supervisoin,writing and organizing . All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declared that they have no conflict of interests.

ETHICAL ISSUES

This reserch work was approved by the Department of Pharmacology and Toxicology research ethics committee of the Faculty of Pharmaceutical Sciences,Usmanu Danfodiyo University Sokoto with a ference number of (PTAC/Nm/(Me)OT/48-22).

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