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Phytochemical Analysis, Analgesic and Anti-inflammatory Studies on the n-Hexane Soluble Fraction of *Vernonia glaberrima*

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Abstract

Vernonia species have been used in the management of pain and other ailments in traditional medicine. This study was aimed at investigating the phytochemical constituents, toxicity, analgesic and anti-inflammatory activities of the n-hexane soluble fraction of Vernonia glaberrima. Preliminary phytochemical screening of the n-hexane fraction revealed the presence of steroids and triterpenes while the intraperitoneal LD_{50} of the fraction using Lorke's method was 2154 mg/kg indicating the fraction to be slightly toxic. The analgesic property of the fraction was assessed using acetic acid-induced writhing test in mice and formalin-induced pain in rats while the anti-inflammatory effect was evaluated using formalin test in rats. The results of the study showed that the n-hexane fraction at the highest dose (500 mg/kg) decreased writhing response with 89.55 % inhibition. The fraction also exhibited significant ($P<0.05$) analgesic effect at both phases in the formalin test; the n-hexane fraction (250 and 500 mg/kg) and the standard drug, pentazocine (10 mg/kg) were able to significantly inhibit both phase while the extract 150 mg/kg diminished the pain induced by formalin in the first phase only. There was significant inhibition of oedema induced by carrageenan at the 1st hour (150 mg/kg) and the 4th hour at the graded doses of the fraction and the standard drug, piroxicam (10 mg/kg). The findings of this research suggests that the n-hexane soluble fraction of V. glaberrima contains bioactive constituents with analgesic and anti-inflammatory effect and thus, validates the use of the plant in the management of pain and inflammation in traditional medicine.

Keywords: *Vernonia glaberrima*; n-hexane fraction; toxicity; analgesic; anti-inflammatory.

1.0 INTRODUCTION

Pain is an enormous public health issue. Globally, it has been estimated that 1 in 5 adults suffer from pain and another 1 in 10 are diagnosed with chronic pain each year. About 10% of the world's population which is approximately 60 million people endure chronic pain (Goldberg and McGee, 2011). Pain is the main reason for emergency visits in more than 50% of clinical cases (Cordell *et al.*, 2002) and is present in 30% of family practice visits (Hasselström*et al.*, 2002). Epidemiological studies from

differentcountries have reported varying prevalence rates for chronic pain, ranging from 12 to 80% of the population (Abu-Saad, 2010). A studies conducted on4,703 patients revealed that 26% of the patients had pain in the last two years, increasing to 46% in the last month (Smith *et al.*, 2010).Major drugs used clinically for management of pain are the opioids and NSAIDs.The euphoria, tolerance, respiratory depression and dependence side effects associated with the opioid drugs (Bigal and Lipton, 2009; Emily and Gari, 2008) and the gastrointestinal irritation and renal damage caused by majority of the NSAIDs (Howland and Mycek, 2006) necessitate the search for readily available, safe and effective alternative analgesic agents.

2.0METHODOLOGY

2.1 Drugs and Chemicals

Piroxicam-Feldene manufactured by Pfixer Specialties LTD, Ikeja, Nigeria; Pentazocine injection Bp manufactured by Alpha Laboratories LTD, India, Acetic acid manufactured by BDH, England; Carrageenan 1%.

2.2 Experimental Animals

Locally bred adult Swiss albino mice and adult Wister rats of either sex weighing (15-31 g) and (121-184g) respectively were obtained from Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria, Nigeria. They were fed with laboratory diet and water *ad libitum* and maintained under standard conditions (12 hlight and 12 h dark cycle) in propylene cages at room temperature.

2.3 Collection, Identification and Preparation of the Plant Sample

The leavesof *Vernonia glaberrima* was collected in Nasarawa State, Northern-Nigeria in June 2012 during the rainy season. It was authenticated by U.S Gallah at herbarium section of Department of Biological Sciences, Ahmadu Bello University Zaria, where a voucher specimen (No.899) was deposited for future reference. The leaves were removed, air-dried, pulverized, labelled and stored in air-tight container prior to extraction.

For thousands of years, natural products from medicinal plants have been used extensively as remedies for human illnesses including pain(Thitiya, 2000). Their effect is attributed to the presence of active chemical compounds/constituents which produce definite physiological action on human body (Borris, 1996; Sasidharan*et al*., 2011).*Vernonia glaberrima* has been reportedly used in ethno-medicine for the treatment of pain and other ailments such as psoriasis, malaria etc. Earlier study on the crude methanol extract validated the pain inhibition and anti-inflammatory effect of the plant (Abdullahi *et al.*, 2015). This is a follow-up work to investigate the analgesic and anti-inflammatory effect of the n-hexane soluble fraction of *Vernonia glaberrima*.

2.4 Extraction and Fractionation

The powdered leaves of *Vernonia glaberrima* (2.5kg) was continuously extracted with methanol by maceration method. The extract was evaporated *in-vacuo*using rotary evaporator at 45 ℃ to obtain a dark green residue (418.7 g) referred to ascrude methanol leaf extract. A part of the methanol extract (220g) was suspended in distilled water, filtered and partitioned successively into n-hexane, chloroform, ethyl acetate and n-butanol fractions respectively.

2.5 Preliminary Phytochemical Screening

The n-hexane fraction was subjected to phytochemical screening to test for the presence of secondary metabolites including alkaloids, flavonoids, saponins, tannins and steroids/ triterpenes using standard procedures (Trease and Evans, 1996).

2.6 Thin Layer Chromatography

The TLC profile of the n-hexane fraction was conducted using hexane/ethyl acetate 4:1 as solvent system.A baseline (1 cm) was drawn at the bottomof silica gel coated TLC plate (0.25 mm layer). After spotting the n-hexane fraction using a capillary tube, the plates were developed in a TLC tank. The chromatograms were viewed

under UV at 254nm for number and color of spots. Each plate was then sprayed with 10% sulphuric acidand dried in an oven at the temperature of 105 ^{0}C for 10minutes. The color of the spots was recorded and their respective retention factors (R_f) values calculated.

$$R_f = \frac{Distance\ moved\ by\ the\ spot}{Distance\ moved\ by\ the\ solvent\ front}$$

2.7 PHARMACOLOGICAL STUDIES

2.7.1 Acute toxicity studies

The method described by Dietrich Lorke (1983) was employed. The route of administration was intraperitoneal (*i.p.*). In the first phase, nine mice of either sex were divided into three groups containing three mice each. The first, second and third groups received 10, 100 and 1000mg/kg of n-hexane fraction. In the second phase, three mice were used. Each of the three mice received different doses of the fraction; 1600, 2900 and 5000mg/kg. The median lethal dose (LD_{50}) was calculated using the following relation below**;**

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

2.7.2 Analgesic studies

I. *Acetic acid induced writhing response in mice*

The method described by Koster*et al.* (1959) was used; 25 albino mice of either sexes were divided into 5 groups of 5 mice each. Groups 1, 2 and 3 were injected with 125, 250 and 500mg/kg (*i.p.*) of the n-hexane fraction respectively. Group 4 was injected with 10mg/kg of piroxicam (positive control) and Group 5 was injected with 10 mL/kg (*i.p.*) of normal saline (negative control). 30 minutes later, each mouse was injected with 10mL/kg of aqueous solution of acetic acid (0.6% v/v). The number of writhes due to abdominal constrictions for each mouse was counted 5 minutes after injection of acetic acid for a periodof 10 minutes.

II. *Formalin test in rats*

The test was conducted in accordance with the method described by Dubuisson and Dennis (1977). Twenty five rats were used. The rats were divided into 5 groups each containing 5 rats. Groups 1, 2 and 3 were injected with 125, 250 and 500mg/kg (*i.p.*)of the n-hexane fraction respectively. Group 4 was injected with pentazocine 10mg/kg (*i.p.*)(positive control) and group 5 received 1 mL/kg of normal saline (*i.p.*)(negative control). Thirty minutes after this treatment, 50µl of freshly prepared 2.5% solution of formalin was injected subcutaneously under the planter surface of the left hind paw of each rat. The rats were placed individually in an observation chamber and monitored for about 1 h.

The severity of pain response was recorded for each rat based on the following scale:

(0) rat walked or stood firmly on the injected paw.

(1) The injected paw was partially elevated.

(2) The injected paw was clearly lifted off the floor.

(3) The rat licked, chewed or shook the injected paw.

Anti-nociceptive effect was determined in two phases. The early phase (phase 1) was recorded during the first 5 minutes, while the late phase (phase 2) was recorded during the last 45 minutes (Dubuisson and Dennis, 1977).

2.7.3 Anti-inflammatory studies

I. Formalin-induced paw edema

Formalin induced paw edema was used according to the method described by (Sayya*et al.*, 2003). A total of 25 rats were divided into 5 groups of 5 rats each. Groups1,2 and3 received hexane fraction *i.p.* at 125, 250 and 500mg/kg respectively.Group 4 received piroxicam at 10mg/kg which served as the positive control. Group 5 received normal saline 1 mL/kg. Thirty minutes later, the groups were treated with 2.5% v/v formalin (0.05 mL volumes in the subplantar region of the left hind paw of the rat). The paw diameter (cm) was measured using vernier caliper at interval of one hour for four hours.

3.0RESULTS

3.1Percentage Yield of the Plant Extract

The total weight of the extract obtained from 2500 g of the powdered leaves of *Vernonia glaberrima*after extraction was 418.7 g.The percentage yield was calculated to be 16.75 %.

The results of liquid-liquid fractionation of the crude methanol leaf extract *V. glaberrima* are presented in Table 1.

Table 1. Liquid-Liquid Fractionation.

S/No.	Fraction	Quantity (g)	Yield (%)
1.	Aqueous	28.24	12.84
2.	n-Butanol	23.20	10.55
3.	Ethyl acetate	17.70	8.05
4.	Chloroform	2.65	1.20
5.	n-Hexane	9.80	16.07

3.2 Phytochemical Screening

Preliminary phytochemical screening of the n-hexane soluble fraction revealed the presence of saponins, steroids and triterpenes (Table 2).

Table 2: Preliminary Phytochemical Screening of the n-hexane Soluble Fraction

Constituents	Test	Observation	Inferences
Glycosides	Fehling's	Red precipitate	-
Flavonoids	Ferric chloride	Green precipitate	-
	NaOH	Yellow coloration	-
Tannins	Lead acetate	Cream precipitate	-
Alkaloids	Wagner's reagent	Reddish-brown precipitate	-
Saponins	Frothing Test	Persistence of froth for 15mins	+
Steroids/terpenes	Liebermann- Buchard	Brown ring at interface	+

Key: + = positive, - = negative.

3.3 Thin-layer Chromatographic Analysis

TLC analysis of the n-hexane fraction using n-hexane/ethylacetate (4:1) revealed 4 major spots (Table 3).

Table 3: TLC Profile of n-Hexane Soluble Fraction

S/No. of spots	Colour under UV at 254nM	Type of spot	R_fValue
1	Brown	Minor	0.22
2	Brown	Minor	0.25
3	Brown	Minor	0.32
4	Brown	Major	0.42
5	Brown	Major	0.57
6	Brown	Minor	0.68
7	Brown	Major	0.77
8	Brown	Major	0.92

Solvent front: 6.5 cm.

3.4 Acute Toxicity Studies

The result of acute toxicity studies is presented in (Table 4).

Table 4: Determination of median lethal dose (LD_{50}) of n-hexane fraction

First phase		
Dose (mg/kg)	Number of mice used	Mortality
10	3	0/3
100	3	0/3
1000	3	0/3

Second phase		
Dose (mg/kg)	Number of mice used	Mortality
1600	1	0/1
2900	1	1/1
5000	1	1/1

The Intraperitoneal $LD_{50}$of the n-hexane fraction was found to be 2154mg/kg as calculated below;

$LD_{50}= \sqrt{2900 \times 1600}$= 2154 mg/kg.

3.5 Analgesic and Anti-inflammatory Studies

The results of analgesic and anti-inflammatory studies of the n-hexane soluble fraction are shown in Tables (5-7) respectively;

Table 5. Effect of n-Hexane fraction on acetic acid induced writhing in mice

Treatment (mg/kg)	Mean no. of writhes	% Inhibition
Normal Saline	44.00 ± 7.19	-
nHF (125)	7.00±6.75*	84.09
nHF (250)	11.40±4.88*	74.09
nHF (500)	4.60 ±4.12*	89.55
Piroxicam (10)	5.40 ±3.03*	87.73

*Values of the group with superscript * are statistically significant ($p<0.05$) compared to values of the group treated with normal saline. Values are expressed as mean ± SEM, n = 5*

Table 6: Effect of n-hexane fractionon formalin-induced pain in rats

	Mean Pain Scores	
Treatment (mg/kg)	First Phase	Second Phase
Normal Saline	2.12±0.10	2.36±0.14
nHF (125)	1.08±0.29*	2.09±0.17
nHF (250)	1.04±0.15*	1.73±0.21*
nHF (500)	0.28±0.19*	1.49±0.16*
Pentazocine (10)	1.32±0.23*	1.64±0.10*

*Values of the group with superscript * are statistically significant ($p<0.05$) compared to values of the group treated with normal saline. Values are expressed as mean ± SEM, n = 5.*

Table 7: Effect of n-hexane leaf extract on formalin-induced acute paw oedema in rats

		Mean paw diameter (cm)				
S/N	Drugs (mg/kg)	0hr	1hr	2hr	3hr	4hr
1	n-HF 125	5.19±0.28	0.52±0.22*	0.71±0.24	0.51±0.08	0.42±0.12*
2	n-HF 250	5.32±0.30	0.98±0.28	0.74±0.23	0.81±0.17	0.80±0.21*
3	n-HF 500	5.64±0.11	1.06±0.25	0.94±0.97	0.51±0.15	0.55±0.23*
4	Piroxicam 10	5.33±0.36	0.94±0.21	0.90±0.26	0.61±0.21	0.51±0.19*
5	N/S	5.03±0.07	1.48±0.31	1.30±0.25	1.22±0.46	1.27±0.30

*Values of the group with superscript * are statistically significant ($p<0.05$) compared to values of the group treated with normal saline (N/S).*

4.0 DISCUSSION

Preliminary phytochemical screening conducted on the n-hexane fraction revealed the presence of steroids and terpenes. The biological activity of plants are thought to be attributable to the presence of secondary metabolites (Cowan, 1999).

The thin-layer chromatographic analysis carried out on the fraction showed a very good resolution of constituents with different R_f values suggesting that the solvent systems employed could be adopted in the separation of constituents using column chromatography (Trease and Evans, 1996). The median lethal dose (LD_{50}) of the n-hexane fraction was 2154 mg/kg implying the fraction to be slightly toxic (Lorke, 1983).

Acetic acid-induced writhing test is a sensitive approach used to evaluate peripheral analgesic activity of a compound (Gene *et al.*, 1998). The n-hexane fraction of *V. glaberrima* showed a significant ($P<0.05$) peripheral analgesic activity on acetic acid-induced writhing in mice when compared to the negative control. The fraction at the highest dose (500mg/kg) decreased writhing response with 89.55 % inhibition, while the standard drug, piroxicam (10 mg/kg) had 87.73 %. An earlier work carried out on the methanol leaf extract of *Vernonia glaberrima*showed that the lower dose had the highest inhibition of writhes (Abdullahi *et al*., 2015). *Vernonia cinerea* showed a significant analgesic effect in acetic acid-induced writhing response and mechanical-induced pains(Mazumder*et al*., 2000). The analgesic effect of the fraction might be related to the presence of phytochemical constituents which could be acting in synergy to infer the analgesic effect of the fraction. Analgesic effect of steroids and terpenes have been reported (Sasidharan*et al*., 2011).The n-hexane fraction might possess peripherally-mediated analgesic activity and the mechanism of action may be mediated via inhibition ofcyclooxygenases and/or lipooxygenases (Deradt*et al*., 2000).

The first phase in the formalin test involves neurogenic pain while the second phase is due to inflammatory reactions. Peripherally acting drugs (e.g. NSAIDs) mainly inhibit the second phase while central acting drugs inhibit both phases. The n-hexane fraction (250 and 500 mg/kg) and the standard drug, pentazocine (10 mg/kg) were able to significantly inhibit both phases while the fraction at dose 125 mg/kg apparently diminished the pain induced by formalin in the first phase only. This is an indication that the extract may possess both central and peripheral analgesic activities.

NSAIDs are the drugs currently used in the management of inflammatory conditions (Insel, 1996). These drugs act by inhibiting both cyclooxygenase and lipoxygenase pathways of the arachidonic acid metabolism (Insel, 1996). The n-hexane fraction at the lowest dose (125 mg/kg) significantly inhibited the oedema induced by formalin at the 1st and 4th hours. However, the fraction (250 and 500 mg/kg) and the standard drug, piroxicam (10 mg/kg) were able to inhibit inflammation at the 4th hour implying that thefraction might have shorter rate of action and longer duration of action.

5.0 CONCLUSION

The n-hexane soluble fraction possessed significant analgesic and anti-inflammatory activities, and as such has lend credence to the use of the leaves of *Vernonia glaberrima* in ethno-medicine for treating some ailments like psoriasis, migraine, body pain, and dysmenorrhea.These activities may be attributed to the presence of bioactive constituents in the fraction.

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