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Phytochemical Analysis, Analgesic and Anti-inflammatory Activities of *Dalbergia saxatilis* Stem Bark in Mice

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Abstract
The decoction of the stem bark and leaves of *Dalbergia saxatilis* is used in traditional medicine for various ailments such as cough, small pox, skin lesions, bronchial ailments and toothache. The study aimed at establishing a safety profile, evaluating phytochemical constituents and some Pharmacological properties of methanol stem bark extract of *Dalbergia saxatilis*. Phytochemical constituents were screened using standard methods. Acetic acid induced writhing test in mice were used to evaluate the analgesic effect, while Carrageenan induced paw oedema model in rats was used to investigate the anti-inflammatory effect of the extract. The extract was administered at doses of 250 mg/kg, 500 mg/kg, and 1000 mg/kg. Acute toxicity study was carried out as described by Lorke Method in rats. The extract was found to contain alkaloids, flavonoids, tannins, saponins, cardiac glycosides, and triterpenes. The extract and standard significantly decreased the number of writhes caused by acetic acid. There was statistical significant increase in reaction time in standard group, extract 250 mg/kg, and 1000 mg/kg from 60 minutes.The median lethal dose in rats was found to be above 5000 mg/kg. The extract was found to possess constituents that may be associated with its analgesic and anti-inflammatory effects observed at doses tested. It has relative acute safety, but toxic on prolonged use.
Keywords: Analgesic effect, Anti-inflammatory, Carrageenan,*Dalbergia saxatilis*

INTRODUCTION
Pain is a disabling supplement of many medical conditions worldwide (Schim and Stang, 2004). It is associated with most pathological conditions in humans and affects thinking, sleeping, emotions, and performance of daily chores (Wilhelm *et al*., 2009; Dib-Hajj *et al*., 2010), thereby making its control an important therapeutic priority. Pain is the major cause of all first visits to hospitals for consultations and the most common symptom of disease or injury (Porth, 2011). Medical attention is often sought by patients to relieve pain which may range from mild discomfort to agonized distress. The management of pain is essential in most cases, and in some conditions like advanced cancer, the only viable therapeutic option is the use of analgesics; but potent and safe analgesics are limited (Schim and Stang, 2004). In many pathological conditions, particularly HIV/AIDS, diabetes, sickle cell disease and cancer, the management of pain remains a serious concern.

Inflammation is defined as the series of changes that occur in a living tissue when it is injured provided that the injury is not of such a degree that causes destruction. It is characterized by redness, swelling, heat and pain and a times loss of function (Punchard *et al*., 2004). Inflammation is a defensive mechanism of the body to remove injurious stimuli and initiate healing process for the tissue, but if it runs unchecked, it can lead to onset of certain diseases as vasomotor rhinnorrhoea, rheumatoid arthritis, and atherosclerosis (Sharma *et al*., 2010). Inflammation removes foreign matter from the body, disposes damaged cells, and initiates wound healing. It is controlled by mast cells that are in close proximity to autonomic nerves. Mast cells are constituents of connective tissues containing large granules that contain heparin, serotonin, bradykinin, and histamine (Kumar *et al*., 2012).

Plants used in traditional medicine are relatively safe, but some may have undesirable adverse effects which may be due to over dosage or certain factors and these may lead to toxicity and death (Okigbo*et al.,* 2009).*Dalbergia saxatilis* is a medicinal plant used for the treatment of pain, inflammation, and pyrexia, and has not been subjected to intensive toxicological evaluation. The use of analgesics also known as pain killers is the most common method in treatment of pain. These pain medications may cause serious side effects in some individuals. Orthodox drugs used in the treatment of pain include, non-steroidal anti-inflammatory drugs, opioid and non-opioid analgesics (Feinberg *et al.,* 2012). Non-steroidal anti-inflammatory drugs such as aspirin, ibuprofen, diclofenac and naproxen in the treatment of mild to moderate pain, inflammation are limited by their significant side effects such as gastrointestinal tract irritation, blood disorders, liver damage, renal damage, tinnitus, hypersensitivity reactions etc (Mishra *et al* ., 2011). Opioid analgesics, though very effective in chronic pain management, are associated with problems such as addiction and tolerance, and side effects such as constipation, weight gain and loss of libido. Opioids are also associated with dependence, and abrupt withdrawal can lead to withdrawal symptoms such as tiredness, diarrhoea, abdominal cramps and sweating (Stannard *et al*., 2007).

Majority of the world population depend on traditional medicine such as herbs for treatment of various ailments. Present day medicine was derived from herbal traditions (Ezeonwumelu *et al*., 2012). The use of traditional medicine is rapidly growing; most people are working in the field of ethnomedicine due to accessibility and affordability. Hence, there is need for the establishment of toxicological profiles of these medicines (Salawu *et al.,* 2009). Several species of *Dalbergia* genus are widely used in traditional medicine systems and relatively few of these have been investigated from an evidence-based pharmacological approach. The species are used in traditional system of medicines all over the world in the treatment of various ailments like diarrhoea, pain, inflammation, pyrexia, leucoderma, dyspepsia, dysentery, syphilis, gonorrhoea, stomach ache, leprosy, eye diseases, scabies, pain, and ringworm (Saha *et al.,* 2013). The anti-inflammatory and analgesic activities of *Dalbergia saxatilis* have not been established scientifically.

Problems associated with drugs used in fever, pain and inflammation are alarming, which necessitate the need for development of new drugs and variety of treatment option from bioactive constituents obtained from plants used in traditional medicine (Stark *et al.,* 2013).

MATERIALS AND METHODS

Collection and Identification of Plant Materials

The stem bark of *Dalbergia saxatilis*were collected from local farm in May, 2018 at Madobi Local Government Area, Kano state. The plant was identified and authenticated in the Herbarium of the Plant Biology Department of Bayero University, Kano and was compared with a voucher specimen number BUKHAN663.

Preparation of Plant extracts

Fifty grams (100g) each of the powdered stem bark was added to 1000 ml of methanol. Each was allowed to stand for 3days at room temperature (28 ±2^{o}C), with agitations at intervals. Each extract was sieved through a muslin cloth, filtered through a Whatman (no.1) filter paper, poured unto a clean evaporating dish and placed on a water bath at 50^{o}C until all the solvent evaporated.

Qualitative Phytochemical Screening of Methanol Extract of *Dalbergia saxatilis* StemBark

The plant extract was subjected to phytochemical screening in order to identify the phytochemical constituents of the plant using the method described below.

Tests for carbohydrates

Molish's (General) Test for Carbohydrates: To 1 ml of the filtrate, 1 ml of Molish's reagent was added in a test tube, followed by 1 ml of concentrated sulphuric acid down the test tube to form a lower layer. A reddish colour at the interfacial ring indicates the presence of carbohydrate (Evans, 2009).

Tests for Saponin

Frothing test

About 10ml of distilled water was added to a portion of the extract and was shaken vigorously for 30seconds. The tube was allowed to stand in a vertical position and was observed for 30mins. A honeycomb froth that persists for 10-15mins indicates presence of saponin.

Test for Flavonoids

Shinoda Test

A portion of the extract was dissolved in 1-2ml of 50% methanol in the heat metallic magnesium chips and few drops of concentrated hydrochloric acid were added. Appearance of red color indicates presence of flavonoids (Evan, 2009).

Test for Alkaloid
Wagner's Test
Few drops of Wagner's reagents were added to a portion of the extract, whitish precipitate indicates the presence of alkaloid (Evans, 2009).

Test for Steroid and Triterpenes
Liebermann-Burchard's test
To a portion of the extract, equal volume of acetic acid anhydride was added and mixed gently. 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer. A colour change observed immediately and later indicates the presence of steroid and triterpenes. Red, pink or purple colour indicates the presence of Triterpenes while blue or blue green indicates steroids (Evans, 2009).

Test for Cardiac Glycoside
Kella-killiani's test
A portion of the extract was dissolved in 1ml of glacial acetic acid containing traces of ferric chloride solution. This was then transferred into a dry test tube and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. Observed carefully at the interphase for purple-brown ring, this indicates the presence of deoxysugars and pale green colour in the upper acetic acid layer indicates the presence of cardiac glycosides (Evans, 2009).

Test for Tannins
Ferric chloride test
To a portion of the extract, 3-5 drops of ferric chloride was added. A greenish black precipitate indicates presence of condensed tannins while hydrolysable tannins give a blue or brownish blue precipitate (Evans, 2009).

Test for Anthraquinones
Bontrager's test
To a portion of the extract in a dry test tube, 5ml of chloroform was added and shaken for at least 5mins. This was filtered and the filtrate shaken with equal volume of 10% ammonium solution, bright pink colour in the aqueous upper layer indicates the presence of free anthraquinones (Evans, 2009).

Analgesic studies
Acetic acid induced writhing in mice
Acetic acid induced writhing method described by (Koster*et al.,* 1959) was adopted for evaluation of analgesic activity. Writhing is defined as a stretch, tension to one side, extension of hind legs, contraction of the abdomen so that the abdomen of mice touches the floor, turning of trunk (Mishra *et al.,* 2011). 30 Swiss albino mice of both sexes were divided into five groups, 1 and 5 served as negative control (distilled water 10 ml/kg) and positive control (Piroxicam 20 mg/kg) respectively, while groups 2, 3, and 4 received 100 mg/kg, 50 mg/kg, and 25 mg/kg of the extract respectively. Sixty minutes after treatment, the mice received 0.6% acetic acid

(10ml/kg) interperitoneally to induce pain. 5minutes after acetic acid injection, the animals were observed and number of writhes by each mouse was counted for 15minutes. Percentage inhibition was calculated using the following formular:
% inhibition = Average number of writhes (control) – Average number of writhes (test)/
Average number of writhes (control)

Anti-inflammatory Studies
Carrageenan induced rat paw ooedema model
The anti-inflammatory study was carried out using the method described by Winter *et al*.(1963). Wistar rats (30) were divided into five groups, 1 and 2 served as negative control (distilled water 10 ml/kg) and positive control (Aspirin 300 mg/kg) respectively, while groups 3, 4,and 5 received 250 mg/kg, 500 mg/kg, and 1000 mg/kg of the extract respectively. Treatments wereadministered 1hour before carrageenan injection. Carrageenan was prepared as 1% w/v solution in 0.9 % w/v NaCl and 0.1 ml was injected underneath the planter region. The paw size was then measured with digital Vernier calliper at 0, 1, 2, 3, 4, and 5 hours after Carrageenan injection (Sharma *et al.,* 2010).

Acute Toxicity Studies of Methanol Extract of *Dalbergia saxatilis*Stem Bark
Lethal Dose (LD_{50}) Determination
This is the determination of the lethal dose known as LD_{50}. The method of Lorke (1983) was employed. The phase I involved the oral administration of three different doses of 10, 100 and 1,000 mg/kg of the crude extract, to three different groups of three adult wistar albino rats. In a fourth group, three adult male wistar albino rats were administered with equivalent/volume of distilled water to serve as control. All the animals were orally administered the extract using a curved needle to which acatheter had been fixed. The animals were monitored closely every 30 minutes for the first 3 hours after administration of the crude extract and hourly for the next 6hours for any adverse effects. Then the animals were left for 72 hours for further observation.

When no death occurred, the phase II was employed, only one animal was required in each group. Groups 1–4, animals were orally given 1,500mg/kg, 2,200mg/kg, 3250mg/kg and 5,000mg/kg dose levels of the crude extract. All the animals were left for observation as in stage one.

RESULTS
Phytochemicals which include alkaloids, flavonoids, saponins, phenols, tannins, glycosides, carbohydrates, anthraquinones and triterpenes were detected in methanol extract of *Dalbergia saxatilis* stem bark.

Table 1. Qualitative Phytochemical Screening of Methanol Extract of *Dalbergia saxatilis* Stem Bark

Metabolites	Inference
Alkaloid	+
Flavonoid	+
Saponins	+
Cardiac glycoside	+
Tannins	+
Steroid	+
Triterpenes	+
Phenol	+
Anthraquinones	+
Carbohydrate	+

TLC Profile of *Dalbergia saxatilis* methanol extract using solvent system Butanol: Acetic acid: water (6:2:2). Four spots were detected with *p-Anisaldehyde* spray and the Rf values were shown alongside the spots.

Table 2. Thin Layer Chromatography results of Methanol extract of *Dalbergia saxatilis* stem bark

Extract	Solvent system	Number of Spots	Distance of spots	RF-Value
***Dalbergia saxatilis* (Methanol)**	BU:AA:H_2O (6:2:2)	4	5.7	0.35, 0.47, 0.61, 0.88

Key: BU (Butanol), AA(Acetic acid)

The extract significantly decreased the number of writhes caused by acetic acid in a dose independent manner as shown in Table 3.
The effects observed at 250mg/kg and 1000 mg/kg were more than that of 500 mg/kg of the extract. The effect observed in 1000 mg/kg group was comparable to that of the standard.

Table 3: Effect of Methanol Stem Bark Extract of *Dalbergia saxatilis*on Acetic Acid Induced Writhing in Mice

Treatment	Dose (mg/kg)	Mean No. of Writhes ± SEM	Inhibition (%)
Distilled water	10ml/kg	32.33±3.844^{a}	-
Piroxicam	10	4.47±1.451^{d}	86.2
Extract	250	10.52±3.124^{c}	67.5
Extract	500	12.52±3.451^{b}	61.2
Extract	1000	4.48±1.563^{d}	86.2
LSD	-	0.5	-

The methanol extract of *D. saxatilis* stem bark decreased the rat paw size at different doses though not statistically significant. Zero hour in each group was taken as control and compared to other times. There was statistical significant increase in paw size at the second hour compared to the first hour, a significant reduction was seen in the fourth and fifth hour compared to the second hour (peak increase) in the distilled water group. There was no statistical significant increase or decrease in paw size when time zero was compared to the other

times in the positive control group. In the 250 mg/kg group, the peak increase was observed in the third hour which was also statistically significant. In the 500 mg/kg group, the peak increase in paw size was observed in the first hour, while in the 1000 mg/kg group, there was statistical significant increase in paw size in all the hours when compared to the zero hour.

Table 4. Effect of Methanol Stem Bark Extract of *Dalbergia saxatilis* on Carrageenan Induced Rat Paw Ooedama

Treatment	Mean Paw Diameter (cm)						
	0hr	1hr	2hrs	3hrs	4hrs	5hrs	LSD
Distilled water	0.24±0.0^{d}	0.33±0.0^{c}	0.44±0.0^{a}	0.35±0.01^{b}	0.24±0.00^{d}	0.24±0.00^{d}	0.01
Aspirin	0.24±0.0	0.31±0.0	0.32±0.0	0.28±0.01	0.28±0.00	0.33±0.00	0.01
Extract 250mg/kg	0.24±0.0	0.28±0.0	0.30±0.0	0.37±0.01	0.31±0.00	0.33±0.00	0.01
Extract 500mg/kg	0.24±0.0	0.34±0.0	0.36±0.0	0.36±0.01	0.29±0.00	0.33±0.00	0.01
Extract 1000mg/kg	0.24±0.0	0.29±0.0	0.34±0.0	0.29±0.01	0.32±0.00	0.29±0.00	0.01

No death was recorded in the first phase of the study in rats. In the second phase, doses of 1500mg/kg, 2250mg/kg, 3250mg/kg and 5000mg/kg were used and no death was also recorded. The oral median lethal dose (LD_{50}) for the methanol stem bark-extract of *Dalbergia saxatilis* was therefore estimated to be greater than 5000mg/kg and no sign of behavioural changes were also observed.

Table 5: Acute Toxicity Study of Methanol Extract of *Dalbergia saxatilis* Stem Bark

Plant species	Group	Number of Animals	Dose (mg/kg)	Mortality recorded after 24hrs
Phase I	I	3	10	0/3
	II	3	100	0/3
	III	3	1000	0/3
Phase II	I	1	1500	0/1
	II	1	2250	0/1
	III	1	3250	0/1
	IV	1	5000	0/1

DISCUSSION

The preliminary phytochemical screening and thin layer chromatography of the methanol stem bark extract of *Dalbergia saxatilis* revealed the presence of several constituents. These constituents are known to be responsible for several pharmacological activities. Flavonoids were reported as prostaglandin synthetase inhibitors (Watanebe *et al.,* 2000). Prostaglandins are known to be involved in pain perception (Helms and Barone, 2008). This suggests that reduced availability of the prostaglandins by flavonoids might have been responsible for their analgesic activity. The presence of tannins and saponins possibly might have given rise to the observed anti-inflammatory property and contributed to the anti-pyretic activity of the plant extract. Saponins possess a wide range of therapeutic actions in the body including anti-inflammatory, expectorant, diuretic, anti-malarial and haemolytic effects on red blood cells, while tannins are

used in compress for cuts and wounds, haemorrhoids, varicose veins and in medicine for diarrhoea, catarrh, heavy menstrual flows and inflammatory conditions of the digestive tract (Evans, 1989). Cardiac glycosides increase the force of myocardial contraction and reduce conductivity within the atrioventricular (AV) node. They are used in the treatment of supraventricular tachycardias, especially for controlling ventricular response in persistent atrial fibrillation (Prassas and Diamandis, 2008).

Acetic acid-induced writhing is among the sensitive methods for evaluating potential analgesic drugs or compounds that act peripherally. Writhing is defined as a stretch, tension to one side, extension of hind legs, contraction of the abdomen so that the abdomen of mice touches the floor, or turning of trunk (Mishra *et al.,* 2011). This model of response is thought to be mediated by peritoneal mast cells, acid sensing ion channels and the prostaglandin pathway. The acetic acid induced writhing model has been associated with increased level of prostaglandins (PGE2 and PGF2α) in peritoneal fluids as well as lipoxygenase products; this enhances inflammatory pain by increasing capillary permeability (Lakshman *et al.,* 2006; Khan *et al.,* 2010). The mechanism, by which any substance that inhibits these writhings causes analgesia, will be preferably by inhibition of prostaglandin synthesis, which is a peripheral mechanism of pain inhibition (Somachit and Shahid, 2003).

The data presented suggests that the methanol extract of *D. saxatilis* stem bark possess peripheral analgesic property. The extract at all doses was shown to have analgesic property as evidenced in the model used. The reduction in the number of writhes caused by the extract, suggests that the analgesic effect may be peripherally mediated via the inhibition of synthesis and release of prostaglandins and other endogenous substances. Carrageenan induced rat paw oedema is a well-established animal model for evaluating the anti-oedematous effect of drugs or compounds (Sharma *et al.,* 2010). Formation of oedema caused by carrageenan is said to be in two phases; the first hour after carrageenan injection (first or early phase), involves the release of serotonin, histamine and bradykinin while the second or late phase (2 –5 hours) with increased oedema formation that remains up to the fifth hour involves the release of prostaglandins (Khan *et al.,* 2009). The second phase of swelling not only involves the elevated production of prostaglandins, but has been recently attributed to the induction of inducible cyclooxygenase (COX-2) in the hind paw (Nantel*et al.,* 1999). From the data presented, oedema was inhibited by both the standard (aspirin) and 250mg/kg group of the methanol leaf extract of *D. saxatilis* at the second hour. There was intense inhibition in the second, third, and fifth hour, this indicates that the extract may not have activity on the early phase of inflammation (1-1.5 hours), thus it may actby inhibiting the release of prostaglandins. Aspirin acts by inhibiting the activity of the enzyme cyclooxygenase (COX) which leads to the formation of prostaglandins (PGs) that cause inflammation, swelling, pain and fever (Vane and Botting, 2003).

The oral median lethal dose value for the methanol stem bark extract of *D. saxatilis* obtained in rats was found to be above 5000mg/kg. This suggests that the plant extract is non-toxic as no death was recorded. Acute toxicity studies are usually carried out to determine the dose that

will cause death or serious toxic manifestations when administered singly or severally at few doses in order to establish doses that should be used in subsequent studies (Wanda *et al.,* 2002). The Organization for Economic and Development (OECD), Paris, France, recommended chemical labelling and classification of acute systemic toxicity based on oral median lethal dose values as: very toxic if ≤ 5 mg/kg, toxic if > 5 mg/kg but ≤ 50 mg/kg, harmful if > 50 mg/kg but ≤500 mg/kg, and non-toxic or not harmful if > 500 mg/kg or ≤ 2000 mg/kg (Walum, 1998). Based on this classification, the oral median lethal dose obtained for rats found to be above 5000 mg/kg, is relatively safe orally. However, the situation may not be the same on repeat dose experiment in sub-chronic toxicity studies.

CONCLUSION

The methanol stem bark extract of *Dalbergia saxatilis* was found to possess several bioactive constituents including flavonoids, saponins, tannins, cardiac glycosides among others, associated with potent pharmacological activities. The extract was found to possess considerable analgesic and anti-inflammatory properties at doses tested. This partly justifies the claim for the traditional use of the plant in the treatment of toothache. The bioactive constituents responsible for the pharmacological activities should be isolated and characterized.

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