STUDIES ON THE ANALGESIC EFFECT AND TOXICITY PROFILE

OF AN ETHANOLIC EXTRACTIVE OF DESMODIUM ADSCENDENS IN RODENTS

BY

AUDREY SERWAA BONSU

(10507323)

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In the

DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY,

SCHOOL OF PHARMACY

JULY 2017
DECLARATION

DECLARATION BY THE CANDIDATE

I hereby declare that this experimental work is a product of my own research undertaken under supervision and has neither been presented in whole nor in part for another degree elsewhere. I am solely responsible for any residual flaws in the work.

............................................ Date ...........................................

Audrey Serwaa Bonsu
(10507323)

DECLARATION BY SUPERVISORS

We hereby declare that the principal work and presentation of the thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

Principal Supervisor

............................................ Date ...........................................
(Prof. Kwasi Bugyei)

Co-Supervisor

............................................ Date ...........................................
(Dr. Patrick Amoateng)
ABSTRACT

Background
Pain is a major symptom usually associated with most disease states which alerts a patient to seek medical treatment. Pain management still remains unsatisfactory, despite countless efforts and therapies to solve it. This is a global burden which is gradually increasing and its prevalence, especially among Africans in recent findings, is on the rise. Medicinal plants have been used since medieval times for treating ailments, and are still being used today all over the world, both in its natural and isolated forms. Desmodium adscendens, popularly known as ‘sweetheart’, is used traditionally for the treatment of asthma, epilepsy, pain and inflammatory conditions. However, very little information exists in scientific literature on the effect of this plant on pain. This study sought to determine the analgesic effect and establish the safety profile of an ethanolic extract of the Desmodium adscendens plant in murine models.

Aim
The aim of this study was to investigate the analgesic effects and safety profile of an ethanolic extract of Desmodium adscendens in rodents.

Methodology
The pulverized plant material of Desmodium adscendens was extracted, using 70% (v/v) ethanol, and the extract screened for phytochemical constituents. A preliminary investigation of the general neuro-active behavior and acute toxicity of the ethanolic extract of Desmodium adscendens were assessed, using the Irwin’s test. Standard protocols, using various known noxious stimuli to induce acute, persistent, thermal nociception and neuropathic pain, were employed to determine the analgesic effect of the ethanolic extract of Desmodium adscendens. The possible mechanism(s) of analgesia of the extract were also investigated with pre-treatment of mice with various specific antagonists in the hot plate test. The toxicological evaluation of the extract was focused on haematological, serum biochemical parameters and histopathological changes of some isolated organs.

Results
The ethanolic extract of Desmodium adscendens contained several secondary metabolites. The extract produced sedation, analgesia, hypothermia and a characteristic upright elevation of the tail (Straub tail) in the Irwin test. DAE attenuated acetic acid-induced writhings (P=0.0012). The extract ameliorated formalin-induced nociceptive pain in both the first (P=0.0058) and second phase (P= 0.0116). There was pronounced latency in the reaction time in the hot plate test (P<0.0001). The extract, comparable to pregabalin, significantly reduced paclitaxel-induced neuropathic pain in both thermal hyperalgesia (P<0.0001) and cold allodynia (P=0.0024).

The analgesic effect exhibited by DAE was partly or wholly reversed after pre-treatment by the systemic administration of naloxone, glibenclamide, ondansetron, prazosin and yohimbine. However, the analgesic effects were not significantly affected in the presence of theophylline, atropine, L-NAME and nifedipine.

No deaths were recorded at a dose of 3,000 mg/kg. There were no significant effects on the body weight, organ weights, urinalysis and haematological parameters of the rats. However, in the biochemistry assay, significant changes were observed in the extract, especially at a dose of 100 mg/kg. Low levels of albumin, total protein, globulin, AST, ALT were observed at doses of 100
mg/kg and 300 mg/kg compared to the control; but ALP was elevated. HDL was elevated, but total cholesterol and LDL were reduced at a dose of 100 mg/kg. However, the triglycerides was unaffected in the lipid profile test. All other parameters were within the statistical range. The LD$_{50}$ was found to be above 3000 mg/kg.

**Conclusion**
The ethanolic extract of *Desmodium adscendens* has been shown to possess profound analgesic effect, especially against thermal nociception and paclitaxel-induced neuropathic pain. The extract may be acting via opioidergic, adrenergic, ATP-sensitive K+ channels and to a limited extent, the serotoninergic pathways. *Desmodium adscendens* has proven to be safe with an LD$_{50}$ and NOAEL (no-observed-adverse-effect-level) in murine species above 3,000 mg/kg with no deleterious effect on the major organs.
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<th>Description</th>
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<td>ABTS</td>
<td>2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid</td>
</tr>
<tr>
<td>ALB</td>
<td>Albumin</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphate</td>
</tr>
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<td>Alanine aminotransferase</td>
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<td>ASIC</td>
<td>Acid-Sensing Ion Channels</td>
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<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under Curve</td>
</tr>
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<td>CCI</td>
<td>Chronic Constriction Injury</td>
</tr>
<tr>
<td>CIPN</td>
<td>Chemotherapy-induced peripheral neuropathy</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<td>CYP</td>
<td>Cytochrome P</td>
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<td><em>D. adscendens</em></td>
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<td>Diclo</td>
<td>Diclofenac</td>
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<tr>
<td>DHS-I</td>
<td>Dehydrosoyasaponin I</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>ED</td>
<td>Effective dose</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>Ferric (III) Chloride</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma-glutamyl transferase</td>
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<tr>
<td>Abbreviation</td>
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<td>--------------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>GI</td>
<td>Gastro intestinal</td>
</tr>
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<td>Globulin</td>
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<tr>
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<td>Hydrochloric Acid</td>
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<td>Hematocrit</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<td>Hemoglobin</td>
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<tr>
<td>HST</td>
<td>Histamine</td>
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<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine</td>
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<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>ICR</td>
<td>Imprint control region</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
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<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>K$_3$Fe(CN)$_6$</td>
<td>Potassium ferricyanide</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LD</td>
<td>Lethal dose</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LYM</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean corpuscular hemoglobin</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean corpuscular hemoglobin concentration</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MPE</td>
<td>Maximal possible effect</td>
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<td>MPV</td>
<td>Mean platelet volume</td>
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<td>MOR</td>
<td>Morphine</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NOAEL</td>
<td>no-observed-adverse-effect-level</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NRS</td>
<td>numeric rating scale</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<td>PAG</td>
<td>Periaqueductal Grey</td>
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<tr>
<td>PAIN-AD</td>
<td>Pain Assessment in Advanced Dementia</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>p.o</td>
<td>Per os</td>
</tr>
<tr>
<td>PHN</td>
<td>Post Herpetic Neuropathy</td>
</tr>
<tr>
<td>PLT</td>
<td>Platelets</td>
</tr>
<tr>
<td>PK</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>PNL</td>
<td>Partial Sciatic Nerve Ligation</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RDW-SD</td>
<td>Red cell distribution with-standard deviation</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RVM</td>
<td>Rostral ventromedial medulla</td>
</tr>
<tr>
<td>SNL</td>
<td>Spinal nerve litigation</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>TBIL</td>
<td>Total bilirubin</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor –alpha</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential vanillloid</td>
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<td>Description</td>
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<td>------------------------------</td>
</tr>
<tr>
<td>TP</td>
<td>Total protein</td>
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<tr>
<td>WBC</td>
<td>White blood cell</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER ONE  
INTRODUCTION

1.1 BACKGROUND

The International Association for the Study of Pain (IASP) and the World Health Organization (WHO) define pain as “an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Taxonomy, 2014). Pain, according to McBeth and Jones (2007), is a defining trigger, symptom or feature of many diseases which alerts a patient to seek medical attention. It is a disabling and a major symptom associated with many medical conditions. It is purely subjective, making diagnosis solely reliant on the patient’s description of the intensity and quality of the pain (Clancy and McVicar, 1998). Poorly managed pain can interfere with many daily activities of a patient, including relationships, cognitive abilities and the capacity to work (Wilhelm et al., 2009). One of the main goals of pain therapy is to reduce its negative impact on the patient’s normal functionality and quality of life (Gordon et al., 2005). Pain management remains one of the most relevant therapeutic priorities (Schim and Stang, 2004) as it can serve as a measure of the severity and activity of an underlying condition, as well as a prognostic indicator often used by most clinicians (McBeth and Jones, 2007; Rang et al., 2007). It is, therefore, imperative to understand the anatomical and physiological pathways. The neuro-biochemical mediators involved in noxious transmission and pain perception is therefore vital to the optimization of pain management (Vadivelu et al., 2009).

Analgesics are drugs that are used in the management of pain. They act via several mechanisms to decrease pain peripherally by reducing the generators of the mediators of pain at the site of tissue damage, or centrally by inhibiting higher centres involved in the transmission and perception of pain (Craig and Stitzel, 2004).
However, current conventional analgesics are limited in potency and safety, and this has led to the upsurge in research to discover and develop newer alternatives so as to facilitate the provision of improved and effective alternatives in the management of pain. In this regard, many options are being investigated, and the use of traditional medicinal plants as potential sources of analgesics with high potency but with minimal adverse effects are being considered as alternatives (Woode and Abotsi, 2011).

Medicinal plants have been used since medieval times for treating ailments, and are still being used today all over the world, both in its natural and isolated forms. It is worth noting that the two main potent analgesics in use today are from plant sources; i.e. aspirin from *Salix alba* and morphine from *Papaver somniferum*, and it would be plausible to research into other plant-based sources for equally potent alternatives (Woode and Abotsi, 2011).

1.2 EPIDEMIOLOGY

Currently, data on the epidemiology of pain is scanty, mainly because of the subjective nature of the symptoms and a lack of agreement regarding specific diagnoses and definitions of the condition. Many pain conditions are sporadic, with the majority reporting recurred symptoms but not the incident for “first-time.” This makes the true incidence for most pain conditions remain unknown, though for the purpose of research, it is important to know the number of new cases within a given period of time (Henschke *et al.*, 2015). However, in spite of this, a world-scale epidemiology report of 2008 produced by Tsang *et al.* shows an age-related prevalence of chronic pain conditions in the previous 12 months of 37.3% in developed countries and 41.1% in developing countries, with an overall prevalence of 38.4%. Also, according to the
International Association for the Study of Pain (IASP), chronic pain affects about 20% of the adult population, particularly women and the elderly in developed countries with only 1-2% resulting from cancer. About 30-40% suffer musculoskeletal and joint pains, whereas neck and back pain accounts for another 30%. Headache and migraine account for less than 10% of the cases. A worldwide study indicates that pain in primary care is at least as prevalent in developing countries as in developed ones (Bond *et al.*, 2013).

In Ghana, these statistics are of no exception. In fact, over 60% of the adult population complains of lower back pain, popularly referred to as waist pains at one time or the other in lifetime, with a higher prevalence in females (56%) than males (44%) (Osei, 2000; Kyei *et al.*, 2015). The mean Lower Back Pain prevalence among the African adolescents was 12% and among the African adults 32% (range 10% to 59%) (Osei-Kyei and Chan, 2015).

**1.3 PROBLEM STATEMENT**

The general agreement among clinicians and researchers worldwide is that existing strategies for the management of pain are inadequate, in spite of the major advances in pain management strategies over the last twenty years (Stein and Baerwald, 2014). Current analgesics, especially for persistent pain, are relatively ineffective and do not reduce pain in all treated individuals (Woolf, 2010). Most are associated with major adverse effects or abuse tendencies. The opioids, e.g., morphine, pethidine, oxycodone, are currently one of the most potent groups of analgesics used clinically (Iwaszkiewicz *et al.*, 2013) with prescriptions increasing by 50% over the past decade for moderate-severe and chronic pain (Waterman *et al.*, 2013). However, there is a clear indication and consistent correlation between increase in opioid prescription rate and opioid overdose deaths,
abuse and addiction. These adverse effects are mainly due to their agonist effects on central opioid receptors causing dependence, tolerance, sedation, and respiratory depression (Hua and Cabot, 2010; Waterman et al., 2013). Non-steroidal and steroidal anti-inflammatory drugs have serious side effects such as gastric erosions, ulcer formation, bleeding, hypersensitivity reactions, cardiovascular toxicity, renal toxicity, and hepatotoxicity. In addition, they can cause a range of central adverse effects (Warner and Mitchell, 2008; Stein and Baerwald, 2014).

These findings reveal a gap in current treatment remedies and the need to discover and develop safer, less addictive, efficacious and readily available alternatives with relatively fewer and more tolerable side effects, so as to drastically reduce the global burden associated with pain management.

1.4 JUSTIFICATION

In many cultures, especially in Africa, the traditional uses of plants have been the mainstay of health maintenance since medieval times. The effect of these plants or extracts are known for a multitude of beneficial effects, including analgesic, anti-inflammatory, antibacterial, antiviral, anti-diabetic, anti-hypertensive or antioxidant effects. They have also been the subject of numerous studies (Nergard et al., 2005; Melendez and Capriles, 2006).

*Desmodium adscendens* is extensively used traditionally in Africa. In Ghana, *Desmodium adscendens* is traditionally used for the treatment of asthma and other diseases associated with smooth muscle contraction and its mechanism of action has been well established (Ampofo, 1977). Recently, publications from Africa, especially, from Ghana and Nigeria, on *Desmodium adscendens* have focused on the characterization and quantification of the chemical constituents
(Pothier *et al.*, 2006; Baiocchi *et al.*, 2013). Other publications are on the assessment of the general neuro-pharmacological effects, as well as the acute toxicity of the plant (N’gouemo *et al.*, 1996).

Anecdotal reports indicate its use traditionally in the management of pain, especially lower back pain. The analgesic effect of *D. adscendens*, using acetic-acid-induced writhing test has been ascertained (N’gouemo *et al.*, 1996). However, the acetic acid test is a general test for analgesia, of which weak analgesics and muscle relaxants all give positive results. Thus, the formalin test, which is considered to be the most predictive of acute pain and is believed to be a more valid model for studying clinical pain, should be employed to investigate the analgesic effect of the plant (Dubuisson and Dennis, 1977a; Tjolsen *et al.*, 1992). Additionally, the possible analgesic mechanism(s), as well as its potential as an anti-neuropathic agent, have not been established (Hunskaar and Hole, 1987; Tang *et al.*, 2007).

This research, therefore, evaluated the claimed analgesic effects, both anti-nociceptive and anti-neuropathic, the possible mechanism(s) of action, and the safety profile of the ethanolic extract from *Desmodium adscendens*, to scientifically validate its traditional uses.

1.5 AIM

The aim of this study was to determine the analgesic effect of the ethanolic extract of *Desmodium adscendens* in murine models.

1.6 SPECIFIC OBJECTIVES

1. To extract and perform phytochemical screening of *D. adscendens* to determine its phytochemical constituents.
2. To perform preliminary investigations, using the Irwin’s test to determine the general neuro-behavioral activity of the extract.

3. To assess the nociceptive analgesic effect of the extract of *D. adscendens* in thermal and chemical pain models, using standard protocols.

4. To determine the analgesic effects of the *Desmodium adscendens* extract against neuropathic pain, using chemotherapy-induced neuropathic pain models.

5. To investigate the possible mechanism(s) of the action of the extract.

6. To conduct safety assessment studies on the extract using both the acute and sub-acute toxicity tests.
CHAPTER TWO
LITERATURE REVIEW

2.1 THE DESMODIUM ADSCENDENS PLANT

Desmodium adscendens (DA) (Sw.) DC is a traditional perennial herb or shrub, indigenous to and widespread in many tropical countries, which commonly grows in open forests, pastures and along roadsides (Taylor, 2003). It belongs to the Plantae Kingdom; Class Magnoliopsida and of the family Fabaceae or Leguminosae-Papilioniodeae (Mshana et al., 2000; Nesom et al., 2006). It is commonly known as Sweetheart, Iron weed, hard man, hard stick, tick-trefoil and tick clover.

In Ghana it is known by the Akans as Akwamfanu or Nkatonkate; Nwomenle or ahule by the Nzemas and Azigbe, Anyigo or Anyigba-zi by the Ewes. In South America, it is called Amor seco or amor-do-campo by the Brazilians and Manayupa in Peru (Dokosi, 1998; Mshana et al., 2000; Taylor, 2003) Fig 2.1.

Fig 2.1: Desmodium adscendens plant (Taylor, 2003)
2.1.1 BOTANICAL DESCRIPTION OF D. ADSCENDENS

*Desmodium adscendens* is a herbaceous, multi-branched, weedy and perennial shrub that grows up to 50 cm tall. The leaves are trifoliate with leaflets that are elliptic to round or obovate (Fig 2.1). They are broadly rounded at the apex with sparsely distributed, very fine short hairs above, and thick, compact hair beneath (Hooker, 1879). Its stems are reddish-brown, willowy, erect and completely covered with fine, short grey hairs. The plant produces numerous flowers with standard whitish, greenish-blue, light-purple, pink petals. The fruits are small, green, semi-elliptic, bean-like pods joined by slender necks and covered with short hooked hairs (Hyde et al., 2009). The *Desmodium* genus is noted for its flattened loment of one-seeded, indehiscent sections, usually bearing uncinated trichomes. Because of the abundant small uncinated hairs on most species, the seed pods cling very tenaciously to clothing, human body and also to the feathers and hair of various animals, thereby ensuring a wide dispersal of the plants seed which is best sown ripe but develops a hard seed coat when stored (Croat, 1978).

2.1.1 GEOGRAPHICAL DISTRIBUTION

*Desmodium adscendens* is a vine, which grows wild and commonly found in the Amazon rainforest of Peru, other South American countries, India, Asia, Oceania and on the West Coast of Africa (Fig 2.2). It is also widespread in tropical areas of south and central America, the Caribbean and throughout tropical Africa (Ampofo, 1977; Croat, 1978).
2.2 ETHNOBOTANICAL AND MEDICINAL USES

*Desmodium adscendens* has been shown to have diverse affirmative effects on many diseases common in Africa. It is a highly valued folkloric medicinal plant and has been used for many years, because of its diverse pharmacological properties (Adjanohoun et al., 1988). Traditionally, it is prepared as a decoction and used by the native people in Amazonia, Peru, South America and West Africa, notably those along the coast. In Brazilian traditional medicine, the leaves of *Desmodium adscendens* are used to treat a wide range of conditions, including back pain, gonorrhea, diarrhea, body ache, excessive urination, and ovarian inflammation. In Guinea, Liberia, Ivory Coast and Trinidad, decoctions or bitters of the leaves are used in the treatment of pain, venereal diseases, psycho-neuro-pharmacological disorders and as aphrodisiac (Adjanohoun et al., 1988). In Ghana, it is extensively used to treat diseases connected to smooth muscle contraction, such as asthma and
bronchitis (Taylor, 2003). *D. adscendens* is considered as a food supplement and consumed traditionally in France for its protective effects on the liver, after scientific evidence of its positive curative effect on hepatic infection *in vivo* (Gyamfi *et al.*, 1999).

### 2.3 PHYTOCHEMICAL CONSTITUENTS

Some few key compounds have been isolated from the leaves of *Desmodium adscendens*. Phytochemistry reveals the presence of triterpenoid saponins, tetrahydroisoquinolones, phenylethylamines, polyphenols, flavonoids, anthocyanins, tannins and indole-3-alkyl amines from the leaf extract (Addy and Burke, 1987). Dehydrosoyasaponin I (DHS-I), soyasaponin I and soyasaponin III are the three active constituents isolated triterpenoid saponins that have been identified and characterized (McManus *et al.*, 1993). Dehydrosoyasaponin I, the major saponin of the plant, for instance, was shown to be a very potent potassium-channel (K+) opener. Tyramine and hordenine are also known to be constituents of the plant.(Addy, 1989; Muanda *et al.*, 2010)

### 2.4 ETHNO-PHARMACOLOGICAL STUDIES ON *DESMODIUM ADSCEDENS*

The pharmacological effects of the traditional medicinal herb, *Desmodium adscendens*, were extensively studied in the 1980s and 1990s. It is a plant which has been scientifically proven to possess diverse positive pharmacological activities. These activities include its relaxation effect on smooth muscle, hence its anti-asthmatic effect (Barreto, 2002, Addy and Dzandu., 1986). It is also reported to possess anti-inflammatory (Addy and Awumey, 1984; Addy and Dzandu, 1986; Addy and Burka, 1987; McManus *et al.*, 1993), CNS (N'gouemo *et al.*, 1996), anti-oxidant (Muanda *et al.*, 2011), immunological effects (Rammal and Soulimani, 2011) and is relatively not toxic (Magielse *et al.*, 2013).
2.4.1 Effects of *D. adscendens* On Selected Tissues and Systems

- Effects of *D. adscendens* on Smooth muscle

A research by Barreto revealed muscle relaxation of K+ induced contractions, but not phenylephrine- induced contractions, in an isolated anococcygeus muscle of a rat pretreated with the butanolic extract of *Desmodium adscendens*. It was also discovered that this fraction significantly reduced calcium levels in the muscle. It was, therefore, concluded that the butanolic extract of *Desmodium adscendens*, which inhibits the contraction of smooth muscle, may be acting partially through the blockade of voltage-sensitive Ca\(^+2\) channels (Barreto, 2002).

The relaxation effect of *Desmodium adscendens* on the smooth muscle, using varying concentrations of a hot water extract, reversed histamine-induced ilial contraction and dose dependently reduced lung histamine content, suggesting that the extract has the ability to cause a dose-dependent reduction in the amount of spasmogens released to relax the anaphylactic-induced ilial muscle contractions (Addy and Dzandu, 1986).

The anti-asthmatic (Addy and Awumey, 1984) and anti-inflammatory  (Barreto, 2002) effects of the *Desmodium adscendens* extract have all been demonstrated. Many studies have been carried out to ascertain these effects and to better understand and determine its mechanism of action (Addy and Awumey, 1984; Addy and Dzandu, 1986; Addy and Burka, 1987; McManus *et al.*, 1993). *In vivo* studies of its anti-anaphylactic property showed that an orally administered dose of both aqueous and ethanolic extracts of *Desmodium adscendens*, dose-dependently reduced anaphylactic smooth muscle contractions, inhibited histamine-induced contractions, and decreased the amount
of spasmogens released from lung tissue of guinea pigs which are implicated in asthma and inflammatory conditions Addy and Awumey, 1984; Addy and Dzandu, 1986; McManus et al., 1993). After a series of tests, this research eventually revealed that, *Desmodium adscendens* extract contained constituents that had the ability to activate calcium-dependent potassium channels and was the first example of a plant extract with such a high affinity and potency for opening the potassium channels.

- **Effects on Central Nervous System**

Its CNS effect was demonstrated in the area of pyrexia, algesia and epilepsy (N'gouemo et al., 1996). The ethanolic extract of *Desmodium adscendens* was proven to induce hypothermia, possessed analgesic effect and reduced the tonic phase of convulsion, and eventually mortality in PTZ-treated mice.

- **Anti-Oxidant Activity**

Its anti-oxidant activity was shown when the hydroethanolic extract of the leaves of *Desmodium adscendens* was subjected to the two most common radical scavenging assays, the 2, 2’-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays (Muanda et al., 2011). The ABTS, the DPPH tests and other cellular tests revealed that the extract of *Desmodium adscendens* leaves possess potent scavenging antioxidant properties. This is because pretreatment of granulocytes with the extract significantly inhibited the ROS generation induced by H$_2$O$_2$ (83.21 ± 6.21% reduction).
• Immunological effects

A research by Rammal and Soulimani demonstrated for the first time that the aqueous extract of *Desmodium adscendens* had immunological effects. Their findings revealed decreased counts of the total lymphocytes, particularly TCD4+, TCD8+ and NK cells; decreased humoral immunity but increased monocytes counts following a flow cytometry technique and the ELISA method. From this research, it can be said that *Desmodium adscendens* has possibly strong effects on both cellular and humoral immunity (Rammal and Soulimani, 2011).

With regards to the toxic properties of this plant, two major contrasting researches have been conducted, using the aqueous extract of *Desmodium adscendens*. An aqueous decoction of *D. adscendens* leaves and twigs exhibited a protective effect in rats against acute liver damage induced by D-galactosamine by decreasing aspartate transaminase (AST) and alanine transaminase (ALT) which are significant markers for liver damage. It also significantly decreased mortality in ethanol-induced liver damage but had no curative effects on chronic D-galactosamine-induced liver damage and acute paracetamol-induced liver damage (Magielse *et al.*, 2013).

In contrast, oral administration of the freeze-dried powder of aqueous crude extract of *D. adscendens* leaves to rats increased AST, ALT and direct bilirubin concentration dose-dependently, suggesting liver excretory dysfunction and hepatocellular damage. This resulted in 50% mortality (Quaye, 2001). This is contrary to the earlier findings indicated above. However, it was stated that doses that caused mortality are much higher (about 456 times higher) than the dose for effective therapy of the *D. adscendens* extract in humans (Quaye, 2001). The conclusion drawn by Quaye inferred that *Desmodium adscendens* is safe with a wide therapeutic window.
2.5 DEFINITION OF PAIN AND PAIN ASSESSMENT TECHNIQUES

2.5.1 OVERVIEW OF PAIN

Algesia (pain) is an ill-defined, unpleasant sensation, usually evoked by an external or internal noxious stimulus and has the propensity of affecting the quality of life of the sufferer. The term *pain* is derived from the Latin word *poena* meaning punishment which reveals the harmful effects that can be inflicted upon the body (Craig and Stitzel, 2004; Tripathi, 2013). The International Association for the Study of Pain and the World Health Organization define pain as “an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Taxonomy, 2014). Although pain can be unpleasant, incapacitating and a major symptom associated with many medical conditions, it serves as an important adaptive, protective and warning sign, alerting the individual, stimulating immune function, promoting healing and preventing further damage (Craig and Stitzel, 2004). It is typically a direct response to an outward event often linked with tissue damage such as injury, infection, inflammation or cancer. It can also arise as a result of no known predisposing cause (e.g. trigeminal neuralgia), or nerve injury, e.g. following a stroke or herpes infection (Rang *et al.*, 2007; Banning *et al.*, 1991). In fact, pain is now considered the fifth vital sign alongside, temperature, pulse, blood pressure, and respiratory rate thus making pain assessment an important tool in clinical practice (Bradshaw *et al.*, 2005).

A definition by McCaffrey and Beebe (1989) describes pain as “a highly subjective experience with no objective tests to appropriately measure it” (McCaffery *et al.*, 1989). It is also quite unfortunate that some patients cannot provide a self-report of pain verbally, in writing, or by other
means, such as finger span or blinking of the eyes to answer yes or no questions (Merkel et al., 2002; McCaffery et al., 2011).

Pain assessment is an extremely important step to providing good pain management and it is difficult to achieve good pain control without any assessment (Anderson et al., 2000). There are many recommendations and guidelines that stipulate what adequate pain assessment should consist of, although quite a number of them seem impractical in acute care practice. It is therefore, important that health care professionals working with patients with pain select the appropriate elements of pain assessment, with respect to the prevailing clinical situation. Pain assessment has to be done on a regular basis (e.g., every 2 hours, once a shift) using a standard format. Assessment parameters need to be specifically directed by health policies and procedures so as to meet patients’ needs. Pain reassessment, after each intervention, is important to evaluate the effect and determine whether there is the need for any modification. Again, this should be directed by health policies and procedures (Max et al., 1995; Carr and Jacox, 1997; Popp, 2003). The choice for pain intervention, including the type of analgesic and dosing, is based upon intensity and therefore every pain assessment must be brief and simple to complete. Numerous pain intensity measures have been developed and validated, and several tools provide a numeric rating of pain intensity e.g., visual analogue scale, numeric rating scale (NRS) (Carr and Jacox, 1997). Simpler tools such as the verbal rating scale, which classifies pain as mild, moderate or severe, are also commonly used. Furthermore, patients with limited cognitive ability use scales with drawings or pictures available (e.g., the Wong-Baker FACES scale Fig 2.3). Also, patients with advanced dementia require behavioral observation to determine the presence of pain and tools such as the PAIN-AD (Pain Assessment IN Advanced Dementia) are available for this patient population (Carr and
The selection of any pain assessment tool should be a joint decision by the patient and the health care provider, and the patient must be familiar with the scale. This should be based on the age, physical, emotional, cognitive status and preference of the patient (Carr and Jacox, 1997). The pain tool selected should be used on a regular basis to assess pain and the effect of interventions, but must not be used as the sole measure of pain perception (Gordon et al., 2005).

2.6 CLASSIFICATION OF PAIN

There are two main and well-recognized broad categories of pain: the nociceptive and the neuropathic pain. However, there are other types that are not specified and categorized, e.g. pain resulting from fibromyalgia and also classification due to duration; acute and chronic pain (Rang et al., 2003 Fig 2.4).
2.6.1 NOCICEPTIVE PAIN

Nociceptive pain occurs as a result of activities in the neuronal pathways secondary to actual tissue damage or potentially tissue-damaging stimuli. This occurs as a result of the activation of the nociceptive system by noxious stimuli, inflammation or disease by a process known as nociception (Rang et al., 2003). Examples of nociceptive pain include surgical pain, arthritis pain, low back pain and pain from sharp or hot objects.

Nociceptors are the specialized sensory receptors responsible for the detection of noxious (unpleasant) stimuli, transforming the stimuli into electrical signals, which are then conducted to the central nervous system. These are free nerve endings of primary sensory afferent neurons with a single cell body located in the dorsal root ganglion. There are different types of afferent neurons and are classified based on their fiber size, conduction velocity and myelination. There are three major groups: A which is further sub-divided into four groups (α, β, γ, δ), B, and C. Primary sensory afferent fibres that respond to various noxious stimuli are the thinly-myelinated, small
diametric (Aδ) fibers or tiny diametric, unmyelinated C-polymodal fibers. These fibers attached to the nociceptors are widely distributed throughout the body (under skin in the epidermis and dermis, muscles, joints and viscera) and are activated by different stimuli such as heat, pressure and vexatious chemicals (Monitto LC et al., 2015). Inflammatory mediators such as bradykinin, serotonin, prostaglandins, cytokines, and H+ that are released from damaged tissues can stimulate nociceptors directly or sensitize the nociceptors by reducing the activation threshold. (Reddi et al., 2013).

Pain is expressed in two main ways classified as First pain and second pain. First pain, defined as a fast, sharp, localized pain, is short-lived and is conducted and transmitted by the Aδ nociceptors, whilst the C-fiber, polymodal nociceptors conduct and transmit the second pain which is usually a slow, diffuse, persistent, burning and lasts for several minutes, even after termination of the stimulus (Reddi et al., 2013).

2.6.2 NEUROPATHIC PAIN

Neuropathic pain is caused by damage to nerves in the central or peripheral nervous system. Damage can be due to a number of mechanisms including trauma or surgery, diabetes mellitus, chemotherapy, radiotherapy, ischaemia, infection or malignancy. Neuropathic pain is more likely to be spontaneous and is described as burning or ‘like an electric shock’. This pain may be experienced in response to a stimulus that does not normally cause pain (allodynia), or may be an exaggerated response to a stimulus that is usually painful (hyperalgesia) (Millan, 1999).
Pain elicited by peripheral nerve injury is sometimes used synonymously with `neuropathic' pain although it also includes `central' pain associated with damage to the CNS (Millan, 1999). Majority of patients with neuropathic pain frequently report sensory abnormalities, including burning sensations, exaggerated responses to noxious stimuli, pain sensations resulting from innocuous stimuli (allodynia) and spontaneous pain episodes (dysesthesia) (Benbouzid et al., 2008). Neuropathic pain can also alter the patient’s quality of life by interfering with emotional well-being (Benbouzid et al., 2008). Because of its severity, chronic nature and resistance to some classical analgesics, neuropathic pain is a challenge in clinical practice (Backonja and Stancey, 2004). Of the symptoms of this pain type, mechanical allodynia comprises the most striking perturbation of sensation. Also allodynia to cold may be pronounced, in particular in sympathetically maintained painful states. (Millan, 1999).

2.7 NEUROPHYSIOLOGY OF PAIN

Pain transmission goes beyond a mere a series of nociceptive neuronal transmissions from a site of injury to the brain where it is generated and perceived. Instead, it involves multifarious processes of in which the somatosensory and limbic system are involved. This accounts for its subjective nature, and no two persons experience pain in the same way (Lowry et al., 2007).

Many painful states are associated with abnormalities of the normal physiological pathway. There are two main components involved in this pathway; the peripheral and central component. The peripheral component, which is due to the action of mediators acting on the nerve terminals, and the central component which reflects the facilitation of synaptic transmission in the dorsal horn through to the brain (Fig. 2.5) (Rang et al., 2003).
2.7.1 PERIPHERAL PROCESSING OF PAIN

When a hot object or noxious substance touches the skin, the stimulus is detected and transduced into electrical stimuli by nociceptors. These nociceptors (A\(\delta\) and C-fibres) attached to the primary afferent neuron conduct the action potential along the neuron to the cell body in the dorsal root ganglion. In cases of tissue or nerve injury, activation of the nociceptors lead to the production and release of a number of chemical and inflammatory mediators including hydrogen ions, potassium ions, histamine, leukotrienes, prostaglandins, cytokines, serotonin (5-HT), bradykinins, and nerve-growth factors, which can originate locally or from cells that infiltrate the site of injury (Millan, 1999). A mixture of these agents form what is known as inflammatory milieu or ‘soup’ and this largely contributes to changes in vascular permeability, resulting in erythema and edema. The
inflammatory ‘soup’ also sensitizes peripheral nociceptors primarily C-fibres by initiating a cascade of events that change and sensitize the nociceptors by lowering the pain threshold at the site of injury. This process is known as peripheral sensitization and have become novel targets for pharmacological intervention (Lowry et al., 2007). Hyperalgesia (the increased response to a noxious stimulus) and allodynia (non-nociceptive fibers transmit noxious stimuli resulting in the sensation of pain from non-noxious stimuli) are resultant effects observed in peripheral sensitization. The nociceptive input is further processed spinally (Lowry et al., 2007; Millan, 1999).

2.7.2 PAIN TRANSMISSION IN THE DORSAL HORN OF THE SPINAL CORD

In the spinal cord, Aδ and C fibers synapse with secondary afferent neurons in the dorsal horn of the spinal cord. The dorsal horn can be divided histologically into ten layers called Rexed laminae. Aδ and C fibers transmit information to nociceptive-specific neurons in Rexed lamina I and II, in addition to projections to other laminae (Reddi et al., 2013). Primary afferent terminals release a number of excitatory neurotransmitters including amino acids, such as glutamate and aspartate, or they may release excitatory peptides, such as substance P, neurokinin A, calcitonin gene-related peptide (CGRP), cholecystokinin and somatostatin. Complex interactions occur in the dorsal horn between afferent neurons, inter-neurons and descending modulatory pathways (Reddi et al., 2013). These interactions determine activity and eventual relay process of the secondary afferent neurons. It is the dorsal horn, more specifically the laminae II, that is the gateway for inhibition or facilitation of the pain transmission (Millan, 1999).
2.7.3 PAIN TRANSMISSION IN THE ASCENDING TRACTS IN THE SPINAL CORD

The second-order neurons then ascend contra-laterally in the spinothalamic, spinoreticular and spinomesencephalic tracts and transcend messages to the supraspinal centers; hypothalamus, thalamus, periaqueductal grey, locus coeruleus and cerebral cortex. Here, nociceptive signals are localized and generated together with sympathetic, thermoregulatory and arousal responses. They then synapse with third-order neurons in the somatosensory cortex (Reddi et al., 2013)

2.7.4 CENTRAL PROCESSING OF PAIN

The central analgesia system is mediated by three major components: The periaqueductal grey matter, the nucleus raphe magnus and the nociception inhibitory neurons within the dorsal horns of the spinal cord, which act to inhibit nociception-transmitting neurons also located in the spinal dorsal horn. Pain transmission through the spinal cord involves the lateral spinothalamic tract pathway. The lateral spinothalamic tract has two pathways for nociceptive information to reach the brain i.e. the neospinothalamic tract for "fast spontaneous pain" and the paleospinothalamic tract for "slow increasing pain". The neospinothalamic tract is responsible for fast pain which travels via type Aδ fibers to terminate on the dorsal horn of the spinal cord, where they synapse with the dendrites of the neospinothalamic tract (Millan, 1999; Kivell and Prisinzano, 2010). The axons of these neurons travel up the spine to the brain and cross the midline through the anterior white commissure, passing upwards in the contralateral anterolateral columns and then terminate on the ventrobasal complex of the thalamus and synapse with the dendrites of the somatosensory cortex. The paleospinothalamic tract is involved in slow pain transmission via slower type C fibers to laminae II and III of the dorsal horns, together known as the substantia gelatinosa. Impulses are then transmitted to nerve fibers that terminate in lamina V, also in the dorsal horn, synapsing with neurons that join fibers from the fast pathway, crossing to the opposite side via the anterior white
commissure, and travelling upwards through the anterolateral pathway. These neurons terminate throughout the brain stem, with one-tenth of fibers stopping in the thalamus and the rest stopping in the medulla, pons and periaqueductal grey of the midbrain tectum (Millan, 1999; Kivell and Prisinzano, 2010).

2.8 INHIBITION OF PAIN TRANSMISSION

There are mechanisms that act to inhibit pain transmission at the spinal cord level and via descending inhibition from higher centers and are as follows:

- **Gate control theory of pain**

The gate control theory of pain was proposed by Melzack and Wall in 1965 to describe a process of inhibitory pain modulation at the spinal cord level (Reddi *et al.*, 2013). It helps to explain why when we bang our head, it feels better when we rub it against an obstacle. By activating Aβ fibers with tactile, non-noxious stimuli, inhibitory interneurons in the dorsal horn are activated leading to inhibition of pain signals transmitted via C fibers (Reddi *et al.*, 2013 Fig 2.6).
Descending inhibition

The periaqueductal grey (PAG) in the midbrain and the rostral ventromedial medulla (RVM) are two important areas of the brain involved in descending inhibitory modulation. Both these centers contain high concentrations of opioid receptors and endogenous opioids, which helps explain why opioids are analgesics. Descending pathways project to the dorsal horn where there are adrenergic, serotoninergic, as well as opioid receptors, and inhibit pain transmission. These pathways are monoaminergic, utilizing noradrenaline and serotonin as neurotransmitters (Reddi et al., 2013).

2.9 PAIN MANAGEMENT

Consequences of Poor Management of Pain

Aggressive and multimodal pain management is highly recommended because pain, when inadequately managed, can culminate into adverse physical and psychological patient outcomes for individual patients and their families (McGowan et al., 2002).
There are also legal implications of poorly managed pain in some developed countries (Bair et al., 2007). Hospitals stand to lose reputation as well as profit if pain is poorly managed (Bair et al., 2007). Continuous, unrelieved pain stimulates the pituitary-adrenal axis, which can suppress the immune system, activate the sympathetic system and result in further complications (Wells et al., 2008). Sympathetic activation can also have negative effects on the cardiovascular, gastrointestinal and renal systems, predisposing patients to adverse events, such as cardiac and renal ischemia, as well as paralytic ileus (Wells et al., 2008). Unrelieved pain reduces patient mobility, leading to complications, such as deep vein thrombosis, pulmonary embolus, and pneumonia. Furthermore, complications related to poor pain management affects the patient’s general well-being, because of extended lengths of stay and re-admissions, both of which increase the cost of care (McGowan et al., 2002; Wells et al., 2008).

Common psychological responses to pain include anxiety and depression. The inability to escape from pain may create a sense of helplessness and even hopelessness, which may predispose the patient to a more chronic depression and, therefore, patients who have ever experienced inadequate pain management may be reluctant to seek medical care for other health problems (Wells et al., 2008).

Poor management of pain may put clinicians at risk for legal action as there are instances of lawsuits filed for poor pain management by physicians (Furrow, 2001; D'arcy, 2005). Current standards for pain management, such as the national standards outlined by the Healthcare Joint Commission (formerly known as the Joint Commission on Accreditation of Healthcare Organizations, JCAHO), require that pain is promptly addressed and managed (Hick et al., 2004).
Analgesics are the drugs pharmacologically used in the management of pain. They include opioids, NSAIDs, paracetamol, local anesthetics, α₂-adrenergic agonists, NMDA antagonists, some anti-epileptics and anti-depressants.

Over the past few years, the concept of multimodal analgesia has been introduced. This has greatly helped to decrease the dose of single dosed medication, exert maximum pain control and drastically reduce drug-induced adverse effects (DeLeo, 2006). Its usage since its inception has expanded and advanced, and it includes targeting various sites along the pain pathway concurrently (Ballantyne, 2001; Katz and McCartney, 2002). This multimodal approach also includes non-pharmacological therapies, such as distraction, massage, exercise and acupuncture (Rusy and Weisman, 2000).

2.10 ANALGESICS

These are drugs that inhibit and prevent pain perceptions. They can also be defined as any drug that has the potential to interfere with the various neurochemical systems that are involved in pain transmission. These drugs more often act as pain receptor antagonists which interfere and inhibit the descending transmission of pain. They act mainly by:

- Decreasing the activation and excitability of nociceptors and/or
- Decreasing or inhibiting the transmission of pain in the peripheral nervous system and/or
- Blocking or inhibiting the transmission of pain signals in the CNS and/or
- Upregulating or activating the descending pain modulatory pathways in the body (Rang et al., 2010)
Analgesics are classified into non-opioid analgesics (nonsteroidal anti-inflammatory drugs (NSAIDs) and Acetaminophen, opioid analgesics and adjuvant analgesics (WHO, 1996).

**FIG 2.7**: A flow diagram depicting peripheral, spinal and supra-spinal targets of analgesics along the pain pathway: ASA = aspirin; LAs = local anesthetics; NSAIDs = nonsteroidal anti-inflammatory drugs; SNRIs = serotonin-norepinephrine reuptake inhibitors; SSRIs = selective serotonin reuptake inhibitors; TCAs = tricyclic antidepressants (www.medscape.com).
2.10.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are the first line of drugs used in the treatment of pain. They are indicated for mild to moderate pain relief. These drugs are useful for treatment of pain, fever, and inflammation and for reduction of platelet aggregation. Although the NSAIDs are not as effective as the opioids in providing pain relief; unlike the opioids, they do not produce tolerance and physical dependence. Generally, the mechanism of action of traditional NSAIDs involves the inhibition of the enzyme cyclooxygenase (COX), a critical enzyme responsible for the production of prostaglandins. This leads the inhibition of synthesis and a decrease in the amount of prostaglandins at the site of injury in the periphery (Chacko et al., 2002; Craig and Stitzel, 2004; Fig 2.7).

Owing to the suppression of prostanoid synthesis through inhibition of the cyclo-oxygenase (COX) of the arachidonic acid pathway, NSAIDs have three major pharmacologically desirable actions, these are:

1. **An anti-inflammatory action:** the decrease in prostaglandin E$_2$ and prostacyclin reduces vasodilatation and, indirectly, oedema.

2. **An analgesic effect:** decreased prostaglandin generation results in less sensitization of nociceptive nerve endings to inflammatory mediators, such as bradykinin and 5-hydroxytryptamine.

3. **An antipyretic effect:** The antagonizing effect of NSAIDs prevents interleukin-1 mediated release of prostaglandins in the central nervous system, where they elevate the hypothalamic set point for temperature and fever control. (Rang et al., 2007).

Examples of NSAIDs include Aspirin, Diclofenac, Ibuprofen, Naproxen, Celecoxib and Ketorolac (Harvey et al., 2012).
2.11.1 OPIOID ANALGESICS

Opioids are usually the drug of choice for the management of severe or chronic malignant pain and for moderate pain where NSAIDs have failed. They are natural or synthetic compounds that produce morphine-like effects. All drugs in this category produce their effects by binding to specific opioid receptors in the CNS that mimic the action of endogenous peptide neurotransmitters (Rang et al., 2007). Even though the opioids have a broad range of effects, their primary use is to relieve intense pain and the anxiety that accompanies it irrespective of the aetiology of the pain (Harvey et al., 2012).

Opium, an extract of the juice of the poppy plant *Papaver somniferum*, contains many alkaloids of which morphine is an example. The basic structure of morphine was determined in 1902, and since then many semisynthetic compounds and fully synthetic opiates have been studied and derived from this structure (Rang et al., 2007). The basic structure of morphine can be altered in minor ways that drastically change the effects of the drug. For example, acetylation of the hydroxyl groups leads to the synthesis of heroin (diacetyl morphine) which has a much greater ability to pass the blood-brain barrier than morphine. The methylation of the phenolic hydroxyl group of morphine results in codeine and its derivatives which have longer duration of action than morphine. This is because the codeine derivatives are less susceptible to glucuronidation and suffer reduced first pass effect (Craig and Stitzel, 2004; Fig 2.8).
Figure 2.8: A figure showing morphine and its derivatives (Opioid agonists, mixed agonist–antagonists, and antagonists (Craig and Stitzel, 2004)

### 2.11.2 Opioid Receptors

Opioid receptors are located on the membranes of certain cells in the CNS, nerve terminals in the periphery, on cells of the gastrointestinal tract and other anatomic regions. The major effects of opioids are mediated by three major receptor families, μ, κ and δ (Harvey et al., 2012). Each receptor family exhibits different specificity for the drug(s) it binds. The analgesic properties of
the opioids are primarily mediated through the µ receptors; however, the κ receptors in the dorsal horn also contribute. The enkephalins interact more selectively with the δ receptors in the periphery. All three opioid receptors are members of the G-protein coupled receptor (GPCR) family and inhibit adenylyl cyclase. They are also associated with ion channels, increasing postsynaptic K\(^+\) efflux (hyperpolarization) or reducing presynaptic Ca\(^{2+}\) influx, thus impeding neuronal firing and transmitter release (Harvey et al., 2012; Fig 2.9).

Figure 2.9: Schematic diagram depicting the mechanism of action of µ-opioid receptor agonists in the spinal cord (Harvey et al., 2012)
2.11.2.1 Distribution of opioid receptors

Opioid receptors involved in pain perception are present in five general areas of the CNS. These are in the thalamus, hypothalamus, Periaqueductal Grey (PAG) matter, cerebral cortex (limbic system) and the substantia gelatinosa of the spinal cord. They have also been identified on the peripheral sensory nerve fibers and their terminals and on immune cells.

2.11.3 ANTI-CONVULSANTS USED AS ANALGESIC ADJUVANTS

The two commonly used anti-convulsants in analgesia, specifically neuropathic pain, are Gabapentin and Pregabalin. Studies have revealed their effectiveness in treating different forms of neuropathies i.e. diabetic peripheral neuropathy, mixed neuropathic pain, phantom limb pain, Guillain–Barre´ syndrome and acute and chronic pain from spinal cord injury (Pan et.al.; 2009). They act on calcium channel found on the pre-synaptic spinal terminals of the primary afferent nociceptors. They bind to α2δ1 subunit of voltage-sensitive calcium channels and inhibit spontaneous neuronal firing. Generally, they are safe, well tolerated, and have very few drug interactions. Other examples less commonly used are carbamazepine, tomiperate and lamotrigine (Harvey et al., 2012).

2.11.4 ALPHA2 (α2) ADRENERGIC AGONSITS

Alpha-2 adrenoceptors belong to the family of G-proteins and transmembrane superfamily of receptors. They are stimulated by norepinephrine and epinephrine (Bylund DB, 2007). Three major receptor subtypes have been identified and isolated. These are α2A, α2B, and α2C of which α2A-receptors seem to be the major receptor subtype responsible for the analgesic effects associated with the use of α2-agonists (Elliott JA, 2009).
Most of the analgesic effect of $\alpha_2$-agonists likely occurs via stimulation of $\alpha_2$-receptors in the dorsal horn of the spinal cord. Stimulation of Gi, which leads to the inhibition of adenylate cyclase and the eventual reduction in the formation of cAMP, is the definitive, but not the only, mechanism of alpha-2 adrenoceptor action. Activation of the receptor also results in opening of potassium channels, causing K+ efflux and hyperpolarization of the membrane and decreased calcium conductance. The resultant effect of these is to decreased neurotransmitter release and neuronal impulse transmission. However, there may also be diminished neurotransmitter release from peripheral sympathetic nerve terminals, and an overall reduction in sympathetic outflow both centrally and peripherally may contribute to analgesia (Finnerup et al.; 2005).

The prototypical alpha-2 agonist and antagonist are clonidine and yohimbine, respectively. Alpha-2 adrenoceptors are implicated in diverse physiological functions in the heart, and presynaptic alpha-2 receptors inhibit the release of norepinephrine and other neurotransmitters in both the central and peripheral nervous systems (Rang et al., 2007; Fig 2.7).

**2.11.5 TRICYCLIC ANTI-DEPRESSANTS USED AS ANALGESICS**

The effectiveness of Tricyclic Anti-depressants (TCAs) in treating neuropathic pain conditions may account for their broad range of pharmacological actions. These compounds inhibit the reuptake of monoaminergic transmitters. They are believed to potentiate the effects of biogenic amines in central descending pain modulating pathways. In addition, they block voltage-dependent sodium channels and $\alpha$-adrenergic receptors. Venlafaxine and duloxetine, which block both serotonin and noradrenaline reuptake are efficacious in Diabetic Peripheral Neuropathy (DPN). Comparing the analgesic effect of venlafaxine and imipramine in patients with painful
polyneuropathy, both antidepressants demonstrated superior pain-relief activity compared with a placebo, but did not differ from each other. Selective serotonin reuptake inhibitors (SSRIs) have fewer adverse effects and are generally better tolerated than TCAs; however, they have not shown convincing efficacy in neuropathic pain states (Siddall and Middleton, 2006; Fig 2.7).

2.11.6 NMDA ANTAGONISTS AS ANALGESICS
These drugs block excitatory glutamate receptors in the CNS that are thought to be responsible for the increased central excitability (central sensitization) following noxious stimuli. Clinically available substances with NMDA-receptor blocking properties include ketamine, dextromethorphan, memantine, and amantadine. Studies of small cohorts have generally confirmed the analgesic effects of ketamine in patients suffering from PHN. However, studies with oral NMDA-antagonist formulations (e.g., dextromethorphan) showed positive results in DPN without beneficial effect in PHN (Dworkin and Schmader, 2003; Fig 2.7).

2.12 ANIMAL MODELS FOR THE STUDY OF ANALGESIC EFFECTS OF DRUGS
In drug discovery, animal models are conventional pharmacological models used as obligatory tools at the preclinical (toxicological and pharmacological) stages of the drug study (Parvova et al., 2011).

A battery of pain models has been developed to simulate the clinical pain conditions with diverse etiology. These models have been classified into nociceptive (thermal, chemical, inflammatory and mechanical) and neuropathic pain models. Some certified well-known pain models used in animal studies discussed below are the acetic acid-induced writhing test, formalin-induced
nociception, hot plate test, spinal nerve injury, ligation of sciatic nerve and drug-induced peripheral neuropathy.

2.12.1 Nociceptive Pain Models

The hot plate test is one of the oldest (Eddy and Leimbach, 1953; Knoll et al., 1956) and most widely used nociceptive animal model. The hot plate test is a source of thermal stimulation of pain induction which evaluates supraspinal-mediated responses to a noxious stimulus and is usually carried out in rats and mice. It involves the placing of the test naïve animal on a heated hot plate surface (50–56 °C), and the latency time taken for the animal to lick or withdraw the hind-paw or jump is noted and recorded (Kamal et al., 2002). This test can be said to be quantitative, with low tissue damage after repetitive experimental tests. It appears there is a good correlation between drugs that produce positive responses in the hot plate test and drugs clinically used in pain management (Taber, 1973).

Although highly advantageous, there are a few challenges associated with this model. The comparison of anti-nociceptive effect at different temperatures is extremely challenging, because baseline latencies tend to vary significantly at the varied temperatures (Morgan et al., 2006). Also, numerous studies have shown that hot plate latency reduces with repetitive testing (Bardo and Hughes, 1979; Lai and Chan, 1982). This decrease could be due to learning, a reduction in stress, habituation to the stimuli associated with the test, or other unknown factors (Knoll et al., 1956; Hunskaar and Hole, 1986; Plone et al., 1996). This demerit is reduced by the reduction in repetitive experiments and/or the propagation in of fallow periods before repeating the experiment.
The acetic acid–induced abdominal writhing test is also extensively used as a selection tool for evaluating analgesic or anti-inflammatory effects of drugs (Koster, 1959). This is considered as a visceral pain model, as pain is induced by the injection of 0.6% - 0.9% acetic acid into the peritoneal cavity of rodents (Koster, 1959; Singh and Majumdar, 1995; Ito et al., 2002). The animals exhibit a characteristic stretching action which is called writhing (Burke et al., 2006). Writhing is defined as an explicit response to the intense pain. It is characterized by episodes of stretching and extension of hind legs or contraction of the abdomen so that the abdomen of the rodent touches the floor, or turning and twisting of the trunk. This is mainly due to the activation of C fiber poly-modal nociceptors via the release of inflammatory mediators, such as prostaglandins and the sensitization of these nociceptors (Collier et al., 1968; Mishra et al., 2011; Fig 2.10). Analgesia is inferred from a reduction in the frequency of writhing after 30 minutes.

Due to the irritant nature of acetic acid, the rodents are prone to lesions. Also usually the pain is progressive and somewhat irreversible and, in most cases, rodents need to be discarded. This demerit is managed by the use of the lower dose (0.6%) of acetic acid to attenuate the overt pain and inflammatory response.

The Formalin Test is also considered as a persistent, chronic, inflammatory model of animal nociception. It is both sensitive to centrally-acting and peripheral analgesics. Intraplantar injection of 1-5% formalin into the right hind limb reveals two distinctive phases of painful behavior. Many studies have unveiled the first-phase also known as neurogenic pain. This usually occurs within 0 - 10 minutes after formalin injection and it is as a result of direct chemical activation of C fiber poly-modal nociceptive primary afferent fibers. The second, later phase, also known as
inflammatory pain, has been said to represent ongoing activity in the primary afferents, increased sensitivity of dorsal horn neurons in the spinal cord following inflammation-induced injury and inflammatory-stimulated sensory activity (Hunskaar et al., 1987b; Tjølsen et al., 1992; Munro, 2009). Albeit, just like the acetic acid-induced writhing test, the formalin test is associated with severe pain, and rodents are prone to lesions, there is no doubt that the formalin test is a predictive test for assessing potential analgesic compounds of both central and peripheral activity and remains a golden model in the screening of potential analgesic compounds (Dubuisson and Dennis, 1977b; Saddi and Abbot, 2000; Vissers et al., 2003; Fig 2.10).

2.12.3 Neuropathic Pain Models

There are several animal models to simulate neuropathic pain. Although all of these models feature some degree of direct neuronal damage, they differ in the time course and patho-mechanisms that are associated with the development of hyperalgesia and allodynia (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992; Woolf and Salter, 2000; Fig 2.10). These include:

2.12.3.1 Nerve-injury neuropathy

The three major models under this type of neuropathy are the Seltzer model, Bennett model and Chung model. In the Seltzer model, also known as the fractional ligation model, a portion of sciatic nerve is tightly ligated, while in the Bennett model, the entire sciatic nerve is loosely and constrictively ligated. It is also called the chronic constriction injury model. The Chung model is distinctive in that the segmental spinal nerve (especially L5 or L6) is ligated in this case (Mizoguchi et al., 2009).
2.12.3.2 Drug-induced neuropathy

The use of this model is limited to the technicality associated with this model. This includes chemotherapy-induced neuropathy which involves the use of chemotherapeutic agents, such as vincristine, cisplatin and paclitaxel administered cumulatively to induce neuropathy which is known to last several weeks after administration. The anti-retroviral agents, dideoxycytidine and didanosin, and anti-tuberculosis drugs, such as isoniazid and ethambuthol, are known to cause neuropathy after continuous administration in rodents (Contreras et al., 1997; Authier et al., 2003; Siau and Bennett, 2006; Ledeboer et al., 2007). Diabetes-induced neuropathy is employed with use of streptozotocin-induced diabetic rats, causing persistent hyperglycemia-induced changes in the nerves, whilst trigeminal neuralgia involves the constriction or compression of the trigeminal ganglion (Courteix et al., 1993; Zhang et al., 2002). This model is popularly used due to its ease of operation with little technical know-how.

2.12.3.3 Spinal cord injury

There are various experimental models under this model. The excitotoxic spinal cord injury involves the intraspinal injections of excitatory amino acids leading to continuous firing and eventually nerve damage injury. Also, dropping of heavy weights over the exposed spinal cord and spinal hemi-section are common examples under this model (Yezierski and Park, 1993; Siddall et al., 1995; Genovese et al., 2008; Fig 2.10). Although numerous examples exist under this model of pain, the drug-induced model is usually deployed due to its ease of operation with little or no technicalities required. It is also less cumbersome and reversible after a couple of weeks.
Fig: 2.10 Summary of pain states and animal models (Millan, 1999).
CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals and reagents

The chemicals and reagents used in this study include: acetic acid, formalin, theophylline (Theophylline anhydrous API Ernest Chemists Ltd, Ghana), diclofenac (Troge medical GMBH, Hamburg, Germany), morphine (Morphine sulphate, Sterop Belgium), naloxone (naloxone HCl from Hamelin pharma plus, GMBH), yohimbine pentylenetetrazole, glibenclamide (Glibenclamide anhydrous API from Ernest Chemists Ltd, Ghana), L-Nitro arginine methyl ester (L-NAME), ondansetron, nifedipine (Adalat® LA, Bayer pharmaceutical, Germany), prazosin (Hypovase™ from Pfizer pharmaceutical, Germany), atropine (Atropine sulphate from Hamelin pharma plus, GMBH) and paclitaxel (Intaxel® from Fresinus Kabi, oncology Baddi India) and pregabalin (Lyrica® from Pfizer pharmaceutical, Germany).

3.2 Experimental animals and housing

Imprint Control Region (ICR) mice (20-30g) and Sprague-Dawley rats (150-200g), of either sex were obtained from and maintained at the Centre for Research into Plant Medicine, Mampong. The animals were housed in groups of five in stainless steel cages (34 cm x 47 cm x 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet and were given water ad libitum. They were maintained under laboratory conditions at temperature 25±2 °C, relative humidity 60-70%, and a 12-hour light-dark cycle. Approval for the study protocols were sought from the Scientific and Technical Committee (STC) of the University of Ghana. All animal procedures and techniques to be used in these studies were in accordance with the Noguchi Institute of Animal Care and Use Committee (NIACUC), College of Health Sciences, University
of Ghana, with protocol number NIACUC-2017-06-2R. It was ensured that all experiments carried out on animals conformed to the OECD guidelines.

3.3 TIME OF EXPERIMENTATION

All behavioral studies were performed in the light cycle between 7:30 am and 2:30 pm with experimentally naïve mice.

3.4 PLANT COLLECTION AND EXTRACTION

Samples of the whole plant were collected from the Aburi Botanical Gardens, and were identified and authenticated at the Ghana Herbarium, Department of Botany, University of Ghana, Legon, Accra where a voucher specimen was lodged.

The samples of the collected plant were air-dried in a well-ventilated room at Mampong for seven days and powdered. Two kilograms of the powder was cold-macerated for 14 days with 70 % v/v of ethanol in water. The ethanolic extract was then evaporated, using a rotary evaporator (Buchi Rotavapor® R-300, Flawil, Switzerland) under reduced pressure below 20ºC to remove the ethanol. The dried extract obtained was labeled as DAE and kept in a desiccator.

3.5 QUALITATIVE PHYTOCHEMICAL SCREENING OF DESMODIUM ADSCENDENS EXTRACT (DAE)

The extract was screened for the presence of phytochemical constituents such as alkaloids, glycosides, tannins, sterols, flavonoids etc. The following chemicals and reagents were used: flavonoids (NaOH and HCl), alkaloids with Mayer’s and Dragendoff’s reagents, saponins
(frothing test), tannins (FeCl$_3$), glycosides (Glacial acetic acid and Fehling’s solutions A and B) cardiac glycosides (Salkowski test), anthraquinones (Borntrager’s reaction) phenols (FeCl$_3$ and K$_3$Fe(CN)$_6$), steroids (Chloroform, H$_2$SO$_4$) and terpenoids (Chloroform and H$_2$SO$_4$) (Trease and Evans, 2002).

### 3.6 PRIMARY OBSERVATION TEST

**Irwin Test**

The behavioural and neuroactive effects of the extract was first evaluated according to standardized observation grid, as described by (Irwin, 1968). ICR mice were randomly divided into various groups (n=5) and kept in the experimental environment for 7 days to acclimatize. Animals were fasted overnight with access to water *ad libitum*, and then treated orally with the extract at various doses or with the vehicle. The mice were observed and assessed at 0, 15, 30, 60, 120 and 180 min, up to 48 hours after treatment. Observations for general changes in behavior and physiological function, neurotoxicity as well as mortality were carried out. Effects on autonomic functions were also noted. This test determined the selection of the pharmacological doses required for the experimental study.

### 3.7 ANALGESIC MODELS

#### 3.7.1 Acetic acid-induced Writhing

The test was carried out as described by (Koster *et al.*, 1959; Tang *et al.*, 2007). Briefly, male ICR mice were randomly divided into seven groups (n=5) and kept in the experimental environment to acclimatize. Each group received either the vehicle (10 ml/kg, p.o), DAE (100-1000 mg/kg, p.o) or diclofenac (10-100 mg/kg, i.p.), 60 minutes prior to the intraperitoneal administration of acetic
acid (0.6%, 10 ml/k.g.) (Tab 3.1). Mice were then placed individually in a testing chamber (a Perspex chamber, 15 × 15 × 15 cm).

Injection of acetic acid-induced a nociceptive behavior, writhing and an exaggerated extension of the abdomen combined with the outstretching of the hind limbs. Responses were captured on a camcorder for 30 minutes for analysis. A public domain software Behavior Tracker was used in tracking the behavior to obtain the frequency of writhes per 5 min, starting immediately after acetic acid administration. A nociceptive score for every 5 mins was determined. These data were expressed and analyzed in a time course, which enabled the observation of changes in the writhing induced to be obtained.

Table 3.1

<table>
<thead>
<tr>
<th>No. of Groups</th>
<th>Animals per group</th>
<th>Drugs</th>
<th>Dose</th>
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3.7.2 Formalin- Induced Nociception

The formalin test was carried out as described by (Dubuisson and Dennis, 1977). Briefly, male ICR mice were randomly divided into seven groups (n=5), and kept in the experimental environment to acclimatize. Each group received either the vehicle (10 ml/kg, p.o), DAE (100-1000 mg/kg, p.o), morphine (1-10 mg/kg, i.p) or diclofenac (10-100 mg/kg ,i.p.) (Tab 3.2), 60
minutes prior to intraplantar injection of 10 μl of 5% formalin into the right hind paw. The animals were immediately placed into the testing chamber, and their nociceptive behaviors scored.

Formalin-induced nociception is characterized by biting, licking and favoring of treated paw with the inability to place both paws on the ground. The pain response was scored for 1 h, starting immediately after the formalin injection. A nociceptive score was determined for each 5min time block by measuring the amount of time spent biting/licking of the injected paw. Tracking of the behavior was done using public domain software Behavior Tracker. The data was expressed and analyzed as the mean ± SEM of scores between 0-10min (first phase) and 10–60 min (second phase) after the formalin injection.

**Table 3.2**

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**3.7.3 Hot Plate Test**

The method described by (Eddy and Leimbach, 1953) was used with little modifications. Briefly, male ICR mice (n = 5) were placed on a hot plate (Model 7280, Ugo Basile Inc., Milan, Italy) heated to 52 ± 0.5 °C, and the baseline reaction time of the animals to nociceptive responses (licking/shaking of the paws, jumping) recorded as baseline reaction latency. They were treated
with the test preparations (similar to that described for the writhing test and formalin test above),
and the reaction times taken again at 0.5, 1 and 2 hours intervals, after a latency period of 30
minutes following the administration of the vehicle (10 ml/kg, p.o), DAE (100, 300, 1000 mg/kg, 
p.o), morphine (1-10 mg/kg, i.p) (Tab 3.3). A cutoff reaction time was set at 20 seconds to prevent
damage to tissues of the foot. The maximal possible effect (MPE) in % of individual mice was
measured by following the formula:

\[
\text{% MPE} = \left[ \frac{\text{post-treatment latency} - \text{pre-treatment latency}}{\text{cut-off time} - \text{pre-treatment latency}} \right] \times 100
\]

The AUC calculated graphically.

**Table 3.3**

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**3.8 INVESTIGATION OF POSSIBLE MECHANISMS OF ACTION OF EXTRACT**

Various mechanisms of action are involved in the analgesic effects of many analgesics. Several
neurotransmitters, mediators and via various pathways are implicated to exhibit analgesia
peripherally or centrally. Different agonists and antagonists are therefore used to explore the
various possible mechanism(s) by which these analgesics act. This principle was replicated for
ethanolic extract of *Desmodium adscendens* (DAE), containing several and different secondary
metabolites, to investigate the possible mechanism(s) of analgesic action. In this experiment, mice
were pre-treated with specific antagonists with selected doses based on data from literature prior to administration of test drug (DAE or standard reference drug) and subjected to the hot plate test to determine some possible mechanism(s) by which the extract exerts its analgesic effect. The standard reference drug used for this test was morphine. The respective median doses of DAE (300mg/kg, p.o) and morphine (3mg/kg,i.p) were determined.

3.8.1 Possible involvement of the opioid system
Male ICR mice were pretreated with naloxone (a non-selective opioid receptor antagonist; 2 mg/kg, i.p.), followed by 15mins later by DAE (300mg/kg, p.o), morphine (3 mg/kg, i.p.), or vehicle (10 ml/kg, p.o.). The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or the vehicle, and 30 mins after the administration of morphine (Woode and Abotsi, 2011).

3.8.2 Possible involvement of the nitric oxide pathway
Male ICR mice were pretreated with L-nitro-arginine methyl ester (L-NAME - a NO synthase inhibitor, 10 mg/kg, i.p.), followed 15 mins later by administration of DAE (300mg/kg, p.o) , morphine (3 mg/kg, i.p.), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle, and 30 mins after morphine administration(Woode et al., 2011).

3.8.3 Possible involvement of ATP-sensitive K⁺ channels
Briefly, male ICR mice were pretreated with glibenclamide (an ATP-sensitive K⁺ channel inhibitor, 8 mg/kg, p.o.) and after 15 minutes received DAE(300mg/kg, p.o) , morphine (3 mg/kg,
i.p.), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 mins after morphine administration (Woode et al., 2011).

### 3.8.4 Possible involvement of the adenosinergic system

Male ICR mice were pretreated with theophylline (3 mg/kg, i.p., a nonselective adenosine receptor antagonist), followed 15 minutes later by administration of DAE (300mg/kg, p.o), morphine (3 mg/kg, i.p.), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle, and 30 mins after morphine administration (Woode and Abotsi, 2011).

### 3.8.5 Possible involvement of 5-HT3-serotonergic receptors

Male ICR mice were pretreated with ondansetron (0.5 mg/kg, i.p., a 5-HT3 receptor antagonist), followed 15 minutes later by the administration of DAE (300 mg/kg, p.o), morphine (3 mg/kg, i.p.), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 mins after morphine administration (Woode and Abotsi, 2011).

### 3.8.6 Possible involvement of Adrenergic (α1 and α2-adrenoceptors)

Male ICR mice were pretreated with prazosin (1 mg/kg, p.o., a selective α1-adrenoceptor antagonist) and yohimbine (3 mg/kg, p.o., a selective α2-adrenoceptor antagonist). After 15 minutes, DAE (300mg/kg, p.o), morphine (3 mg/kg, i.p.), or vehicle was administered. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle, and 30 mins after morphine administration (Woode and Abotsi, 2011).
3.8.7 Possible involvement of voltage-gated calcium channels
Male ICR mice were pretreated with nifedipine (10 mg/kg, p.o., L-type voltage-gated calcium channel (VGCC) blocker) followed 15 minutes later by the administration of DAE (300mg/kg, p.o), morphine (3 mg/kg, i.p.), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle, and 30 mins after morphine administration (Woode and Abotsi, 2011).

3.8.8 Possible involvement of the muscarinic cholinergic system
Male ICR mice were pretreated with atropine (5 mg/kg, i.p., a nonselective muscarinic receptor antagonist) followed 15 minutes later by the administration of DAE (300mg/kg, p.o), morphine (3 mg/kg, i.p.), or vehicle. The reaction latencies in the hot plate test were recorded 1 h after the administration of DAE or vehicle and 30 mins after morphine administration. (Woode and Abotsi., 2011)

3.9 Investigation of Paclitaxel- Induced Neuropathic Pain
The experimental study was carried out as described by Ameyaw et al., (2013) with modifications. Male Sprague Dawley (SD) rats were allowed to acclimatize to the behavioral testing environment. Baseline measurements of hot and cold stimuli were performed. Neuropathic pain was induced in the rats by intraperitoneal (i.p.) injection of paclitaxel (2 mg kg-1) dissolved in distilled water on four alternate days (days 0, 2, 4 and 6) as described by Ameyaw et al., (2013); Flatters and Bennett, (2004). On day 16 of post-paclitaxel treatments, vehicle (10ml/kg; p.o; group 1), DAE (100, 300 and 1000 mg /kg p.o; groups 2-4) and pregabalin (10, 30 and 100 mg/kg; groups 5-7) were
administered to the rats after confirmation of neuropathic pain in the various tests. The effect of the vehicle, DAE and pregabalin treatments on paclitaxel-induced neuropathic pain were evaluated in the thermal hyperalgesia and cold allodynia tests.

3.9.1 Thermal Hyperalgesia
The hot plate test was used to determine the effect of the vehicle, DAE (100-1000 mg/kg) and pregabalin (10-100 mg/kg) on thermal hyperalgesia (Thirumal et al., 2013). The rats were placed individually on the hot plate maintained at 52°C temperature and nociceptive responses (licking/shaking of the paws, jumping) recorded.

3.9.2 Cold allodynia
The tail-immersion/ flick test was used to determine the analgesic effect of the vehicle (10ml/kg), DAE (100-1000 mg/kg-1) and pregabalin (10-100 mg/kg on cold allodynia (Kim et al., 2005). The distal portion of the rat’s tail (3 - 4 cm) was immersed in cold water maintained at < 4°C until the tail was withdrawn. The duration of immersion was recorded and a cut-off time of 20 s was used.

Latencies to reaction times after drug treatment were measured in the hot plate and tail flick tests on days 16, 18 and 20 (labelled as Day 0,3 & 5) post-paclitaxel administration. A cutoff reaction time was set at 20 seconds to prevent damage to tissues of the foot. The maximal possible effect (MPE) in % of individual mice was measured by the following formula:
\% MPE = \left[\frac{\text{post-treatment latency} - \text{pre-treatment latency}}{\text{cut-off time} - \text{pre-treatment latency}}\right] \times 100. The AUC was determined graphically and plotted as the total nociceptive score against the dose in mg/kg.

3.10 TOXICITY STUDIES OF THE EXTRACT

3.10.1 ACUTE TOXICITY

Signs and symptoms of acute toxicity were observed during a 48-hour period. Six groups of rats were used, with five animals in each group. Five groups of animals were administered with the following doses of the extract (30 mg/kg, 100 mg/kg, 300 mg/kg, 1g/kg and 3g/kg p.o) and the sixth group served as the control and received the vehicle (10 ml/kg). Six hours after administration of the extract, the animals in each group were observed for 24 hours for changes in movement, salivation, respiratory pattern, and frequency and consistency of stool and mortality within twenty-four hours. Mortality before and during and at 24 and 48-hour post treatment, if any, were recorded and the LD\textsubscript{50} (the lethal dose-50) was calculated.

3.10.2 SUB-ACUTE TOXICITY

Four groups of rats were used with 5 animals in each group. Three groups received DAE in doses 100 mg/kg, 300 mg/kg and 1000 mg/kg and the fourth group served as the control. The rats were then monitored daily for a period of 14 days and euthanized on the 15\textsuperscript{th} day for postmortem examinations to be conducted. On day 15 of the study period, blood samples were collected from each animal by cardiac puncture into a BD microtainer brand tube with EDTA (1 ml) and BD vacutainer SST – II Advance (5 ml) for haematological and biochemical analysis, respectively. An automated haematology analyzer (KX-21N, Sysmex Corporation, Japan) was used for the
haematological analysis, and a Selectra Junior version 04 autoanalyzer (Vital Scientific Bv, Netherlands) was used for the biochemical assays indicative of renal function, lipid profile and liver function. The rats were euthanized in a chloroform chamber and immediately autopsied. All highly perfused organs and tissues harvested were macroscopically examined and stored in formalin.

3.10.3 Parameters investigated

3.10.3.1 Clinical observations and body weights

All animals were observed twice daily for morbidity and mortality. Clinical examinations included any abnormal physical and behavioral changes. The observations included changes in skin, fur, eyes, mucus membrane, and autonomic activity like lacrimation, piloerection, pupil size and unusual breathing pattern. Changes in gaits, posture, response to handling, presence of clonic or tonic movements, stereotypic activities like excessive grooming, repetitive circling, etc, were observed. The time of onset, intensity and duration of such symptoms, if any, were recorded. Ocular examinations were conducted on all animals prior to the initiation of experiments and during the day prior to euthanasia.

Individual animal body weights for treatment and control groups were recorded at intervals during the investigation period, beginning on the day before the initiation of treatment.

Mean body weights and mean body weight changes were calculated for the corresponding intervals (Day 0, 5, 9, 14). Final body weights were recorded one day prior to the scheduled necropsy.
3.10.3.2 Clinical pathology (urinalysis, hematology and serum chemistry)

Urine and blood samples for clinical evaluations were collected from all animals on the 15th day of the study. Urine samples were collected overnight from all animals a day before termination, using metabolic cages (Nalgene, USA).

Urine analysis parameters included: appearance, specific gravity, pH, total volume, protein (albumin), glucose, and microscopy of sediment.

For hematology and clinical chemistry analysis, blood samples were collected from all animals through cardiac puncture. Hematology parameters included: hemoglobin concentration, red blood cell count, white blood cell count, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, total and differential leukocyte count, platelet count, blood clotting time, activated partial prothrombin time, and reticulocyte time.

For the chemistry analysis, blood was allowed to coagulate and serum was separated after centrifugation.

Serum chemistry parameters included: glucose, triglycerides, cholesterol, urea, creatinine, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), total bilirubin, blood urea nitrogen (BUN), albumin, globulin, total protein, chloride, potassium, and sodium.
3.10.3.3 Macroscopic and microscopic examinations

A complete necropsy was performed on all animals. Animals were euthanized under chloroform inhalation anesthesia. The necropsies included, but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. Organs collected were placed in 10% neutral-buffered formalin. At the scheduled necropsies, the following organs were weighed from all animals: kidneys, liver, spleen, lungs and heart.

3.10.3.4 Histological Examination of Isolated Organs

The isolated hearts, livers and kidneys were preserved in 10% neutral buffered formalin solution for 7 days and washed with water. Tissues were cut with a disposable microtome blade, into approximately 3 mm thick slices. Three slices each were obtained from each of the organs under investigation. Dehydration of the organs was done using ethanol. This was followed by immersion in xylene to remove the alcohol and allow infiltration with paraffin wax. The tissues were then embedded in paraffin. Paraffin wax blocks of the tissues were prepared and tissue blocks were cast using a molten wax dispenser, plastic cassettes and mould boxes. The tissue blocks were sectioned at 4µm, using a rotary microtome. They were then mounted onto microscopic slides and then dried overnight. The slides were later observed under a light microscope after being staining with hematoxylin and eosin (H&E) dyes. The slides were identified with codes written on the frosted sides of the slides.
CHAPTER FOUR

RESULTS

4.1 PHYTOCHEMISTRY OF DAE

Preliminary qualitative phytochemical screening of DAE revealed the presence of alkaloids, saponins, flavonoids, tannins, glycosides, triterpenoids and sterols (Table 4.1).

Table 4.1: Preliminary qualitative phytochemical screening of DAE from the *Desmodium adscendens* plant

<table>
<thead>
<tr>
<th>Test</th>
<th>DAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>-</td>
</tr>
<tr>
<td>Sterols</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: Present (+) Absent (-)
4.2 IRWIN TEST

No observable signs of toxicity were exhibited by the mice during the 48-hour observation period in all the groups of mice, except for an initial excitation (characterized by jumping) at doses 100 and 300mg/kg and sedation at doses from 100 – 3000 mg/kg. There were signs of Straub tailing, analgesia and defecation in all treated groups. Altered reactivity to touch and hypothermia were also observed in all treated groups except at the dose of 30 mg/kg. Mortality was not recorded after 48 hours and the mice resumed normal physical activity by the 24th hour (Table 4.2).
Table 4.2: Physiological & Pharmacological Effects observed in the Irwin’s test of the ethanolic extract of *Desmodium adscendens* at various doses

<table>
<thead>
<tr>
<th>Dose mg/kg</th>
<th>Mortality (d/t)</th>
<th>Latency (min)</th>
<th>Observable physiological/pharmacology effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>0/7</td>
<td>60</td>
<td>straub tail, ↑analgesia, defecation, jumping, straub tail</td>
</tr>
<tr>
<td>100</td>
<td>0/7</td>
<td>30</td>
<td>↑ sedation, ↑↑analgesia, ↓altered reactivity to touch, hypothermia, jumping</td>
</tr>
<tr>
<td>300</td>
<td>0/7</td>
<td>30</td>
<td>straub tail, ↑↑sedation, ↑↑analgesia, ↓↓altered reactivity to touch, hypothermia</td>
</tr>
<tr>
<td>1000</td>
<td>0/7</td>
<td>30</td>
<td>straub tail, ↑↑↑sedation, ↑↑↑analgesia, ↓↓↓altered reactivity to touch, hypothermia</td>
</tr>
<tr>
<td>Dose mg/kg</td>
<td>Mortality (d/t)</td>
<td>latency (min)</td>
<td>observable physiological/ pharmacology effects</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>3000</td>
<td>0/7</td>
<td>15</td>
<td>↑↑ ↑sedation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>straub tail</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑↑ analgesia</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓↓↓ altered reactivity to touch</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hypothermia</td>
</tr>
</tbody>
</table>

D/T – DEAD/ TREATED

↑ - mild; ↑↑- moderate; ↑↑↑- intense

### 4.3 ANALGESIC EFFECT OF ETHANOLIC EXTRACT OF DESMODIUM ADSCENDENS

#### 4.3.1 Acetic-acid induced Writhing

In this assay, pre-treatment of mice with DAE significantly and dose-dependently reduced the frequency of writhes caused by intraperitoneal injection of acetic acid [P=0.0012, F₃,₂₄= 7.294; Fig 4.1A]. Assessment using total writhes, calculated as AUC, revealed that DAE significantly decreased the writhing effects of acetic acid and this was also dose-dependent when compared to the vehicle-treated group [P< 0.0001; F₃,₁₆= 17.80; Fig 4.1B]. Correspondingly, the non-steroidal anti-inflammatory drug diclofenac (10-100mg/kg) also significantly and dose-dependently attenuated the frequency of writhes induced by acetic acid [P<0.0001, F₃,₂₄ =26.88; Fig 4.2A]. Calculated AUCs also revealed a significant inhibition of writhing in comparison to the vehicle-treated groups, and this inhibition of writhes were also dose-dependent [P< 0.0001; F₃,₁₆ =42.83;Fig 4.1B]. From the ED₅₀ values obtained by log dose graph, [Fig 4.3], diclofenac was about 59 times (389.2/6.647 mg/kg) more potent than DAE.
Fig 4.1: Effect of DAE (100, 300, 1000 mg/kg) on the time course curves (A) and the total nociceptive score (calc. as AUCs) (B) of acetic acid-induced writhing in mice. Nociceptive scores are shown in 5 min time blocks up to 30 min for the time course curves.

Data presented as Mean ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control group (ctrl) (Two-way repeated measures ANOVA followed by Bonferroni’s post hoc). †P < 0.05, ††P < 0.01, †††P < 0.001 compared to control group (ctrl) (one-way ANOVA followed by Dunnet’s post hoc test)
Fig 4.2: Effect of diclofenac (1, 3, 10 mg/kg) on the time course curves (A) and the total nociceptive score (calc. as AUCs) (B) of acetic acid-induced writhing in mice. Nociceptive scores are shown in 5 min time blocks up to 30 min for the time course curves.

Data presented as Mean ±SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control group (ctrl) (Two-way repeated measures ANOVA followed by Bonferroni’s post hoc). †P < 0.05, ††P < 0.01, †††P < 0.001 compared to control group (ctrl) (one-way ANOVA followed by Dunnet’s post hoc test)
4.3.2 Formalin-Induced Nociception

In the DAE treated group, there was a general reduction in pain as described by nociceptive scores, and this reduction was dose-dependently significant for all the doses of DAE (100-1000 mg/kg) used in comparison to the vehicle \( P=0.0111; F_{3,48}=4.122 \), Fig 4.4A.

Oral administration of DAE (100, 300, 1000 mg/kg) 30 min before formalin injection dose-dependently inhibited both the first and second phases of formalin-induced paw biting/licking, but this inhibition was only significant for DAE (1000 mg/kg) during the first phase \( P=0.0058; F_{3,16}=6.092 \); Fig 4.4B and DAE (300-1000 mg/kg) during the second phase \( P=0.0116; F_{3,16}=5.085 \); Fig 4.4 C].
Fig 4.4: Effect of DAE (100, 300, 1000 mg/kg) (A) and the total nociceptive score (calc. as AUCs) (B,C) in the 1st and 2nd phases of formalin-induced nociception in mice. Nociceptive scores are shown in 5 min time blocks up to 60 min for the time course curves. Data are presented as mean ± SEM (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 compared to control group (ctrl) (Two-way repeated measures ANOVA followed by Bonferroni’s post hoc). †P < 0.05, ††P < 0.01, †††P < 0.001 compared to control group (ctrl) (one-way ANOVA followed by Dunnet post hoc).
Similarly, morphine (1, 3, 10 mg/kg) which is an opioid agonist significantly and dose-dependently reduced nociceptive scores in comparison to the vehicle [P=0.0024; F₃,₄₈=4.122, Fig 4.5A]. Also, when nociceptive scores were calculated as AUCs, MOR (1, 3, 10 mg/kg), significantly and dose-dependently inhibited both the first and second phases of formalin-induced nociceptive behaviors [P=0.0001; F₃,₁₆=13.290 and P=0.0123; F₃,₁₆=5.011, Fig 4.5B and 4.5C].

Figure 4.5: Effect of morphine (1, 3, 10 mg/kg) (A) and the total nociceptive score (calc. as AUCs) (B,C) in the 1st and 2nd phases of formalin-induced nociception in mice. Nociceptive scores are shown in 5 min time blocks up to 60 min for the time course curves. Data are presented as mean ± SEM (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 compared to control group (ctrl) (Two-way repeated measures ANOVA followed by Bonferroni’s post hoc). †P < 0.05, ††P < 0.01, †††P < 0.001 compared to control group (ctrl) (one-way ANOVA followed by Dunnet post hoc).
Furthermore, Diclofenac (10, 300, 100 mg/kg) which is an NSAID, also significantly and dose-dependently reduced total nociceptive scores induced by formalin injection, when compared to the vehicle. \(P=0.00363; F_{3,48}=3.076,\) Fig 4.6A. Diclofenac reduced nociceptive response during the first phase, but this reduction was only significant at the highest dose of diclofenac (100 mg/kg) used. \(P=0.0051; F_{3,16}=6.280,\) Fig 4.6B. However, during the second phase diclofenac (10, 30, 100 mg/kg) significantly and dose-dependently reduced nociceptive responses induced by formalin \(P=0.0116; F_{3,16}=6.406,\) Fig 4.6C.

Figure 4.6: Effect of Diclofenac (10-100 mg/kg) \((A)\) and the total nociceptive score (calc. as AUCs) \((B, C)\) in the 1st and 2nd phases of formalin-induced nociception in mice. Nociceptive scores are shown in 5 min time blocks up to 60 min for the time course curves.

Data are presented as mean ± SEM \((n = 5)\). *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) compared to control group (ctrl) (Two-way repeated measures ANOVA followed by Bonferroni's post hoc). †\(P < 0.05\), ††\(P < 0.01\), †††\(P < 0.001\) compared to control group (ctrl) (one-way ANOVA followed by Dunnet post hoc)
Comparison of ED$_{50}$ obtained from a log-dose response curve revealed a sigmoid displayed dose–response relationship as shown in Fig 4.7.

The ED$_{50}$ values were approximately 244.0, 0.2815, 20.83 mg/kg for DAE, morphine and diclofenac respectively in the first phase and 75.85, 0.099, 4.091 mg/kg for DAE, morphine and diclofenac respectively in the second phase. The comparison of ED$_{50}$ obtained revealed that the extract was more potent in the second phase than in the first. The graph also revealed that morphine was the most potent, followed by diclofenac, and then DAE in both first and second phases of formalin-induced nociceptive behaviours. Morphine and diclofenac were approximately 867 (244/0.2815) and 11.7(244/20.83) times more potent respectively than DAE during the first phase (fig 4.6A), and 766(75.85/0.09903) and 19.3(75.85/4.091) times more potent respectively than DAE during the second phase (Fig 4.6B).

Figure 4.7: ED$_{50}$ of DAE (100, 300, 1000 mg/kg), Morphine (1, 3, 10 mg/kg) and Diclofenac (10, 30, 100 mg/kg) in the 1st and 2nd phases of formalin-induced nociception test.
4.3.3 Assessment of analgesia using the Hot Plate Test

There were differences in the maximum possible effect (% MPE) between the vehicle and the treatment groups (DAE). However, this difference was not statistically significant, except at a dose of 1000 mg/kg (P=0.0005, F_{3,8}= 19.32; Figure 4.8A). When MPE was calculated as Area Under the Curve (AUC), significant differences were noted between the vehicle and the treatment groups (DAE) at doses of 100 mg/kg and 1000 mg/kg (P<0.0001, F_{3,10}= 24.95;Figure 4.8B), as revealed by the post hoc test.

Figure 4.8: Effect of DAE (100-1000 mg/kg, p.o) and vehicle (Veh) on the assessment of analgesia using %MPE (A) and AUC (B) in the hot plate test.

Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA, followed by a Dunnett’s multiple comparison post hoc test).
There was also no statistically significant difference between the vehicle and the treatment groups (MOR) on %MPE except at dose 10mg/kg (P<0.0001, F \( (3,8) = 32.08 \); Figure 4.9D). However when MPE was calculated as AUC there was a significant difference noted between the vehicle and the treatment groups (MOR) at all doses used (1-10)mg/kg and more pronounced at 10mg/kg as revealed by the post hoc test (P<0.0001, F \( (3,10) = 22.16 \); Figure 4.9E).

Figure 4.9: Effect of Morphine (1, 3, 10 mg/kg, i.p) and vehicle (Veh) on the assessment of analgesia using %MPE (D) and AUC (E) in the hot plate test.

Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA) followed by a Dunnett’s multiple comparison post hoc test)
4.4 INVESTIGATION OF POSSIBLE MECHANISMS OF ACTION OF THE EXTRACT

The results presented in Fig 4.10(A) show that the pre-treatment of mice with naloxone (2 mg/kg, i.p.) significantly inhibited ($P<0.0001$) antinociception by DAE (300 mg/kg, p.o.) in the hot plate test. Naloxone also significantly reversed the antinociception caused by morphine (3 mg/kg, i.p.) ($P<0.0001$; Fig 4.11B).

Systemic pre-treatment of the mice with glibenclamide (8 mg/kg, i.p.) significantly reversed the antinociception caused by DAE (300 mg/kg, p.o.) ($P<0.0001$; Fig 4.10A), but had no significant effect on morphine.

Systemic oral pre-treatment of mice with prazosin (1mg/kg p.o) significantly blocked antinociceptive action of DAE (300 mg/kg p.o) in the test ($P<0.0001$; Fig 4.10A). It also reversed the antinociception of morphine (3 mg/kg i.p) ($P<0.005$; Fig 4.11B)

Yohimbine (3 mg/kg i.p), as well as morphine (3mg/kg i.p) ($P<0.01$; Fig 4.11B) also significantly reversed antinociception of DAE (300 mg/kg p.o) ($P<0.01$; Fig 4.8A)

Theophylline and ondansetron also significantly reversed anti-nociceptive activity caused by morphine (3 mg/kg, i.p.) ($P<0.0001$ and $P<0.05$ respectively; Fig 4.11B), but did not affect antinociception produced by DAE (300 mg/kg p.o).

Ondansetron (0.5 mg/kg, i.p.) did not significantly block antinociception caused by DAE (300 mg/kg, p.o.). However, in contrast, ondansetron significantly reversed the antinociception caused by morphine (3 mg/kg, i.p) ($P<0.05$, Fig 4.11B).
L-NAME (10 mg/kg, i.p.), atropine (5 mg/kg, i.p) and nifedipine (10 mg/kg, p.o.) did not significantly inhibit the antinociception caused by either DAE (300 mg/kg, p.o.) or morphine (3 mg/kg, i.p.) in the hot plate test.

![Figure 4.10: Effect of pretreatment of mice with naloxone (2 mg/kg, i.p.), theophylline (10 mg/kg, i.p.), L-NAME (10 mg/kg, i.p.), glibenclamide (8 mg/kg, p.o.), ondansetron (0.5 mg/kg, i.p.), atropine (5 mg/kg, i.p.), yohimbine (3 mg/kg, p.o.), nifedipine (10 mg/kg, p.o.) and prazosin (1mg/kg) on the antinociceptive effect of DAE (300 mg/kg, p.o.) in the assessment of analgesia using the hot plate test.

Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with the vehicle group (one-way ANOVA, followed by a Dunnett’s multiple comparison post hoc test).
Figure 4.11: Effect of pretreatment of mice with naloxone (2 mg/kg, i.p.), theophylline (10 mg/kg, i.p.), LNAME (10 mg/kg, i.p.), glibenclamide (8 mg/kg, p.o.), ondansetron (0.5 mg/kg, i.p.), atropine (5 mg/kg, i.p.), yohimbine (3 mg/kg, p.o.), nifedipine (10 mg/kg, p.o.) and prazosin (1mg/kg) on the antinociceptive effect of morphine (3 mg/kg, i.p.) in the assessment of analgesia using the hot plate test.

Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett’s multiple comparison post hoc test).
4.5 PACLITAXEL-INDUCED NEUROPATHIC PAIN

4.5.1 THERMAL HYPERALGESIA

Neuropathic pain was confirmed in thermal hyperalgesia models on the 16th day post paclitaxel injection. DAE (100-1000 mg/kg) produced significant analgesic properties from day one to day five (P<0.0001 and F3,64=10.95; Fig 4.12A), when nociceptive score was measured as %MPE on alternate days. Pregabalin (10, 30, 100 mg/kg) similarly produced analgesic properties in this model (P<0.0001, F3,64=16.11;Figure 4.12C).

Furthermore, when nociceptive score was measured as AUC, there was a significant difference between the DAE (100, 300, 1000 mg/kg) and the vehicle values with all the doses significantly reversing thermal hyperalgesia in the model used (P=0.0016, F3,12=9.7; Figure 4.12B). PGE (10, 30, 100 mg kg-1) also significantly produced similar analgesic properties when the nociceptive score was measured as AUC (P=0.0039, F3,12=7.7; Figure 4.12D).
Figure 4.12: The effects of daily dosing of DAE (100-1000 mg/kg)(A,B), pregabalin (PGE 10-100 mg/kg)(C,D) and vehicle on established paclitaxel-induced thermal hyperalgesia as a time-course curve in the hot plate test and nociceptive score measured as AUC respectively.

Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett’s multiple comparison post hoc test).
4.5.2 COLD ALLODYNIA

There was no significant difference between the vehicle and the treatment groups except for DAE at the dose of 100 mg/kg and 300 mg/kg. DAE, at these doses significantly reversed cold allodynia only on the 5th day (P=0.0024, F_{3,64}=5.36; Fig 4.13A). In the PGE-treated group, a dose 30 mg/kg only reversed cold allodynia on the 5th day, but PGE at 100 mg/kg significantly reversed cold allodynia from day 1 to 5.

Furthermore, when nociceptive scores were measured as AUC, PGE (10, 30, 100 mg/kg), the cold allodynia significantly reversed in the model used (P=0.002, F_{3,12}=8.9; Fig 4.13D).

However, when nociceptive scores were measured as AUC, only DAE at 300 mg/kg significantly reversed cold (P=0.075, F_{3,12}=2.9; Fig 4.13B) Also, PGE at doses of 30 mg/kg and 100 mg/kg significantly reversed cold allodynia in the model used (P<0.0001, F_{3,64}=17.45; Fig 4.13C).
Figure 4.13: The effects of daily dose of DAE (100-1000 mg/kg)(A&B), pregabalin (10-100 mg/kg)(C&D) and vehicle on established paclitaxel-induced cold allodynia as a time-course curve, and nociceptive scores measured as AUC.

Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett’s multiple comparison post hoc test).
4.6 TOXICITY

4.6.1 Acute Toxicity

In the acute toxicity studies, the LD$_{50}$ of orally administered DAE in Sprague Dawley rats was found to be greater than 3000 mg/kg. No toxidromes or mortality was observed in any animals within the first 6hrs after 24 hrs and after 48 hrs. Both the vehicle- and DAE-treated groups were normal and did not exhibit any significant change in behavior, skin effects, breathing, pupil, postural abnormalities or fur. There was pronounced sedation within the first 3 hours in the treated groups but these animals returned to normal by the 24$^{th}$ hour. These observations from the oral acute toxicity study suggest that the DAE is practically safe.

4.6.2 Sub-acute toxicity

4.6.2.1 Survival, body weights and clinical observations

All animals survived throughout the 14-days until the scheduled necropsy. Both the physical and behavioral examinations did not reveal any dose-related adverse effects. Compared to the control group, no biologically significant increase in body weight was observed in the treated groups, though there was a little drop in weight in the group treated with DAE at 1000 mg/kg. However, this was not statistically significant (Table 4.3). Necropsy at the end of the study did not reveal any gross pathological abnormalities in the rats. These results suggest that administration of DAE at dose levels up to 1000 mg/kg for 14 days has no adverse effects on survival, body weights and clinical observations.
Table 4.3: The effects of DAE (100, 300 and 1000 mg/kg) on the change in body weights of rats in a 14-day sub-acute toxicity study

<table>
<thead>
<tr>
<th>Day</th>
<th>Vehicle</th>
<th>DAE 100 mg/kg</th>
<th>DAE 300 mg/kg</th>
<th>DAE 1000 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>246.00 ± 6.26</td>
<td>275.83 ± 4.05</td>
<td>266.50 ± 18.10</td>
<td>272.66 ± 13.46</td>
</tr>
<tr>
<td>5</td>
<td>255.33 ± 5.88</td>
<td>280.00 ± 3.54</td>
<td>270.50 ± 18.20</td>
<td>270.00 ± 14.67</td>
</tr>
<tr>
<td>9</td>
<td>263.33 ± 6.66</td>
<td>283.83 ± 3.45</td>
<td>274.00 ± 18.93</td>
<td>268.66 ± 15.27</td>
</tr>
<tr>
<td>14</td>
<td>270.83 ± 7.73</td>
<td>287.16 ± 4.15</td>
<td>277.16 ± 19.29</td>
<td>267.00 ± 15.93</td>
</tr>
</tbody>
</table>

4.6.2.2 Organ weights

There were no significant changes in the absolute and relative organ weights in DAE-treated groups compared to the control (Table 4.4)

These results strongly suggest that the extract (DAE) had no significant adverse effects on organ weights.

Table 4.4: The effects of DAE (100, 300 and 1000 mg/kg) on the weights of major organs (g) isolated from rats in a 14-day sub-acute toxicity study

<table>
<thead>
<tr>
<th>Organs</th>
<th>Vehicle</th>
<th>DAE 100 mg/kg</th>
<th>DAE 300 mg/kg</th>
<th>DAE 1000 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.8683 ± 0.035</td>
<td>0.9317±0.036</td>
<td>0.9817±0.089</td>
<td>1.085±0.032</td>
</tr>
<tr>
<td>Liver</td>
<td>7.755 ± 0.2915</td>
<td>8.983 ± 0.3319</td>
<td>7.960 ± 0.6865</td>
<td>8.555 ± 0.338</td>
</tr>
<tr>
<td>Lung</td>
<td>1.765 ± 0.1209</td>
<td>2.075 ± 0.07343</td>
<td>1.993 ± 0.1028</td>
<td>2.200 ± 0.11</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.5517 ±0.0249</td>
<td>0.5267 ± 0.03040</td>
<td>0.5300 ± 0.01238</td>
<td>0.5517 ± 0.045</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.677 ± 0.1388</td>
<td>1.722 ± 0.07600</td>
<td>1.798 ± 0.08010</td>
<td>1.630 ± 0.191</td>
</tr>
</tbody>
</table>
4.6.2.3 Clinical pathology

- Urinalysis
There were no dose-related adverse effects on urinalysis parameters in the rats in both the control and treated groups. The urine analysis parameters, such as specific gravity, pH, albumin, glucose, red blood cells, white blood cells, epithelial cells and ketones, did not show any significant differences. Minor variations were observed but not toxicologically significant. These results implies that administration of DAE at dose levels up to 1000 mg/kg each day to rats does not affect urine parameters.

- Hematology
In the parameters investigated, there was no apparent dose-related adverse effects in the groups studied (P= 0.1-0.9; Table 4.5). Although there were some variations, they were not statistically significant in comparison to the control. These results demonstrate that administration of DAE to rats at doses up to 1000 mg/kg per day does not negatively affect the hematology.

- Serum chemistry
There were no dose-related biologically significant adverse effects of DAE on serum chemistry parameters in the extract-treated groups compared to the control groups in the renal function test (P=0.027- 0.61; Table 4.6).

In the lipid profile test, there was a significant decrease in the total cholesterol (P= 0.002; Table 4.6) and LDL (P<0.0001) in DAE at a dose of100 mg/kg and a significant increase in HDL in all three DAE-treated doses (P=0.0458; Table 4.6). However, triglyceride levels between the control and treated groups was not statistically significant (P= 0.49).
DAE at doses of 100mg/kg and DAE 300mg/kg statistically reduced Total Protein levels (P<0.0001), Albumin levels (P<0.0001), Globulin levels (P=0.0004), AST (P=0.01569) and ALP levels (P=0.034; Table 4.6) in the liver function test when compared with the control group.

However, other parameters between the control group and the DAE-treated group, were not statistically significant. (P= 0.15 - 0.566; Table 4.6)

Results from the serum chemistry analysis from the DAE-treated group, therefore, revealed that daily administration of DAE at doses of up to 1000 mg/kg to rats for 14 days did not adversely affect the serum chemistry.
Table 4.5: Hematological analysis of DAE (100, 300 and 1000 mg/kg) after a 14-day observation period

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle DAE 100mg/kg</th>
<th>DAE 300mg/kg</th>
<th>DAE 1000mg/kg</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (103 µL-1)</td>
<td>10.34±1.953</td>
<td>9.528±1.953</td>
<td>11.15±1.953</td>
<td>10.85±1.953</td>
</tr>
<tr>
<td>RBC (106 µL-1)</td>
<td>8.277±0.3942</td>
<td>8.938±0.1307</td>
<td>8.948±0.1307</td>
<td>9.307±0.1520</td>
</tr>
<tr>
<td>HGB (g dL-1)</td>
<td>14.28±1.031</td>
<td>15.68±0.2120</td>
<td>15.10±0.1390</td>
<td>15.72±0.3609</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>45.27±0.7551</td>
<td>46.54±0.5480</td>
<td>44.95±0.6610</td>
<td>46.24±1.212</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>51.67±1.085</td>
<td>52.00±0.4472</td>
<td>50.50±0.4282</td>
<td>49.50±0.6191</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>17.58±0.3953</td>
<td>17.57±0.1229</td>
<td>16.95±0.1432</td>
<td>16.88±0.2197</td>
</tr>
<tr>
<td>MCHC (gdL-1)</td>
<td>33.98±0.1869</td>
<td>33.73±0.1585</td>
<td>33.60±0.3152</td>
<td>34.23±0.1054</td>
</tr>
<tr>
<td>PLT (10³ µL-1)</td>
<td>802.7±76.59</td>
<td>744.5±114.3</td>
<td>815.3±82.45</td>
<td>591.7±50.51</td>
</tr>
<tr>
<td>LYM (%)</td>
<td>5.768±1.137</td>
<td>6.018±0.6458</td>
<td>6.483±0.5559</td>
<td>6.087±1.448</td>
</tr>
<tr>
<td>LYM# (103 µL-1)</td>
<td>53.72±9.844</td>
<td>62.53±2.424</td>
<td>58.68±1.891</td>
<td>50.93±7.660</td>
</tr>
<tr>
<td>RDW_SD (fl)</td>
<td>20.52±0.1579</td>
<td>20.05±0.2825</td>
<td>20.80±0.4973</td>
<td>21.37±0.5655</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>6.333±0.1764</td>
<td>6.067±0.1706</td>
<td>5.883±0.1249</td>
<td>6.117±0.1302</td>
</tr>
</tbody>
</table>

Data is presented as Mean± SEM. **WBC**= White blood cells, **RBC**= Red blood cells, **HGB**= Hemoglobin, **HCT**= Hematocrit, **MCV**= Mean corpuscular volume, **MCH**= Mean corpuscular hemoglobin, **MCHC**= Mean corpuscular hemoglobin concentration, **PLT**= Platelet. **LYM** = lymphocytes. Values obtained were not significantly different from the control (One-way ANOVA follow by Dunnet’s post hoc test).
### Table 4.6: Biochemical analysis of DAE (100-1000 mg/kg) in 14-day sub-acute study in SD male rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle</th>
<th>DAE 100 mg/kg</th>
<th>DAE 300 mg/kg</th>
<th>DAE 1000 mg/kg</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Renal function test (mmolL⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>6.855±0.4760</td>
<td>5.350±0.6756</td>
<td>5.500±0.5842</td>
<td>5.733±1.270</td>
<td>0.2211</td>
</tr>
<tr>
<td>Creatinine</td>
<td>54.82±5.316</td>
<td>56.20±3.756</td>
<td>59.38±6.645</td>
<td>46.62±4.346</td>
<td>0.1389</td>
</tr>
<tr>
<td>Potassium</td>
<td>5.217±0.09098</td>
<td>4.967±0.1801</td>
<td>4.900±0.07746</td>
<td>5.150±0.1258</td>
<td>0.3242</td>
</tr>
<tr>
<td>Sodium</td>
<td>141.4±1.504</td>
<td>139.1±1.081</td>
<td>139.3±1.256</td>
<td>140.5±1.535</td>
<td>0.6134</td>
</tr>
<tr>
<td>Chlorine</td>
<td>109.9±1.973</td>
<td>105.7±1.973</td>
<td>108.1±1.973</td>
<td>106.6±0.6796</td>
<td>0.2960</td>
</tr>
<tr>
<td><strong>Lipid Profile (mmolL⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>2.117±0.09069</td>
<td>1.435±0.1738**</td>
<td>2.330±0.1738</td>
<td>2.285±0.1738</td>
<td>0.0002</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.7767±0.03955</td>
<td>0.8200±0.06938</td>
<td>0.8550±0.06938</td>
<td>0.9000±0.06938</td>
<td>0.4977</td>
</tr>
<tr>
<td>HDL</td>
<td>0.7500±0.03916</td>
<td>0.8233±0.09763*</td>
<td>0.8933±0.04145*</td>
<td>1.023±0.03916**</td>
<td>0.0458</td>
</tr>
<tr>
<td>LDL</td>
<td>1.013±0.1107</td>
<td>0.2233±0.06042***</td>
<td>1.125±0.07766</td>
<td>1.082±0.1994</td>
<td>0.0001</td>
</tr>
<tr>
<td>HDL/LDL</td>
<td>0.7867±0.09759</td>
<td>3.557±0.09759**</td>
<td>0.8183±0.06447</td>
<td>1.239±0.3948</td>
<td>0.0030</td>
</tr>
<tr>
<td><strong>Liver function test</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein (gL⁻¹)</td>
<td>81.65±2.424</td>
<td>69.67±0.4944***</td>
<td>65.32±1.195***</td>
<td>77.12±1.993</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Albumin (gL⁻¹)</td>
<td>33.50±0.8079</td>
<td>28.50±0.4282***</td>
<td>31.27±0.4856*</td>
<td>32.47±0.5818</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Globulin (gL⁻¹)</td>
<td>48.08±2.462</td>
<td>41.17±0.4773*</td>
<td>34.40±1.235***</td>
<td>44.65±2.502</td>
<td>0.0004</td>
</tr>
<tr>
<td>Total Bilirubin (µmolL⁻¹)</td>
<td>8.550±0.0</td>
<td>6.867±0.4447</td>
<td>8.550±0.0</td>
<td>10.53±2.704</td>
<td>0.1451</td>
</tr>
<tr>
<td>ALT (UL⁻¹)</td>
<td>69.57±2.951</td>
<td>66.50±1.928</td>
<td>68.183±0.6457</td>
<td>61.82±3.313</td>
<td>0.1567</td>
</tr>
<tr>
<td>AST (IUL⁻¹)</td>
<td>216.2±28.87</td>
<td>126.7±6.157**</td>
<td>160.6±9.085*</td>
<td>177.7±19.60*</td>
<td>0.0195</td>
</tr>
<tr>
<td>ALP (UL⁻¹)</td>
<td>146.3±2.348</td>
<td>217.2±2.348*</td>
<td>217.2±11.83*</td>
<td>194.7±18.85</td>
<td>0.0341</td>
</tr>
<tr>
<td>GGT</td>
<td>12.67±1.308</td>
<td>10.00±0.0</td>
<td>11.83±2.442</td>
<td>13.17±1.869</td>
<td>0.5669</td>
</tr>
</tbody>
</table>

Values quoted are means ± SD.

Data are presented as mean ± SEM (n=5). *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 compared with vehicle group (one-way ANOVA followed by a Dunnett’s post hoc test).
4.6.2.4 Macroscopic Examinations

There were no dose-related macroscopic findings at the scheduled necropsy after daily administration of DAE to the rats. Minor macroscopic changes were observed and considered to be spontaneous and/or related in nature, but not to the treatment. These results suggest that administration of DAE at dose levels up to 1000 mg/kg to rats for 14 days has no adverse macroscopic effects.

4.6.2.5 Microscopic Examinations

The histopathological findings for the various organs isolated were as follows:

- Liver

Sections revealed tissues from liver with regular hepatocytes. There was no ballooning and giant cell formation. The portal tracts were intact with mild chronic inflammation with lymphocytes and occasional eosinophils. There was no significant cholestasis. There was little to negligible portal-to-portal and central-to-portal fibrosis. The features were consistent with normal liver. (Fig 4.14A - Fig 4.14D)

- Kidney

Sections revealed tissues from kidney showing regular glomeruli. There were neither mesangial cell proliferations nor basal membrane activity. There was no sclerosis and no inflammatory activity in the glomeruli. There was no fibrin deposition in the glomeruli. The proximal and distal tubules were normal. The interstitium was normal. Neither fibrosis nor increased inflammatory activity was seen in the interstitium. The vessels were also normal. (Fig 4.15A - Fig 4.15D)
All findings observed were consistent with normal related lesions in clinically normal rats of the age and strain used in this study. These results propose that daily administration of DAE at dose levels up to 1000 mg/kg to rats for 14 days has no adverse histological effects.

Fig 4.14: Micrograph of Liver (x10)
Sample photomicrographs of the liver of Sprague-Dawley rats after treatment with various doses of DAE: (A) control group showed normal hepatocytes, nuclei, sinusoids (S) and central vein (CV), (B) 100 mg/kg DAE treatment showed hepatic tissue with normal hepatocytes, nuclei, sinusoids and central vein; (C) 300 mg/kg DAE treatment showed normal hepatocytes with nuclei, central vein and portal vein with slight dilatation of sinusoids (D) 1000 mg/kg DAE treatment showed normal hepatocytes with nuclei, central vein and portal vein. Sinusoids appear slightly dilated; H and E staining. Objective magnification: ×10
Fig 4.15: Micrograph of Kidney (x 40)

Sample photomicrographs of the kidney of Sprague-Dawley rats after treatment with various doses of DAE: (A) control group showed normal kidney tissue appearance, with normal of glomerular (G), urinary space (US), Bowman’s capsule (BC), proximal convoluted tubule (PT) and distal convoluted tubules (DT); (B) 100 mg/kg DAE treatment showed normal sized glomerulus, nuclei, Bowman’s capsule, distal and proximal tubules. (C) 300 mg/kg DAE treatment showed normal renal architecture with normal cell distribution and cellular integrity; (D) 1000 mg/kg DAE treatment showed normal renal architecture i.e. renal corpuscles and renal tubules. H and E staining. Objective magnification: x40
CHAPTER FIVE

5.1 DISCUSSION

Desmodium adscendens, popularly known as sweetheart, is used traditionally for the treatment of asthma, epilepsy, pain and inflammatory conditions. However, very little information exists in scientific literature on the effect of this plant on pain. Therefore, the study sought to determine the analgesic effect of the ethanolic extract of the Desmodium adscendens plant in murine models. The findings of the present study revealed that ethanolic extract of Desmodium adscendens acts on the CNS, possesses analgesic, anti-nociceptive and anti-neuropathic activities without affecting motor coordination in the animal models. The extract also has a wide safety profile with lipid-reducing effects.

The efficacy of most herbal remedies may be ascribed to several secondary metabolites. The presence of these biologically active phytochemicals, such as saponins, flavonoids, alkaloids, steroids, tannins and glycosides, in various plant extracts are mainly responsible for their respective pharmacological properties, and they act via different mechanisms to produce their effect (Singh et al., 2002; Gomes et al., 2009). Earlier phytochemical tests on an aqueous extract of Desmodium adscendens by Addy, (1989) showed the presence of triterpenoid saponins, tetrahydroisoquinolones, phenylethylamines, polyphenols, flavonoids, anthocyanins, alkaloids and tannins from the leaf extract (Addy, 1989). These metabolites were also detected in the phytochemical screening of the DAE in addition to steroids and glycosides being present. The presence of these phytochemicals could, therefore, be responsible for the observed pharmacological activities of the extract in the various animal models used. (Bhattacharya and Satyan, 1997; Kennedy and Wightman, 2011).
Before investigating a new substance for its pharmacological activities, a procedure earlier described by Irwin is usually employed (Irwin, 1968a). This test is used to evaluate the general effects of a drug or drug candidate on the central nervous system (CNS) activity, the minimum lethal or toxic dose of a test substance, and the primary effects on behaviour and physiological functions. Findings from this test are also used to assess the safety pharmacology of drugs (Irwin, 1968; Porsolt et al., 2002; Roux et al., 2004).

The results of the present study suggested that mice treated with DAE showed initial signs of excitation at doses of 100 mg/kg and 300 mg/kg, followed by sedation as well as analgesia, altered reactivity to touch and Straub tail effects. The Straub tail effect in rodents is often assessed in a response to opioids, and has been extensively shown to be mediated by the μ receptor (Nath et al., 1994; Houshyar et al., 2000). Many agents that act via this pathway have also been shown to produce this said reaction by the activation of other mechanistic pathways, such as serotoninergic pathways (Zarrindast et al., 2001; Diaz and Maroteaux, 2011). This observation at all doses (100 mg/kg, 300 mg/kg, 1000 mg/kg) of the extract used highly postulates the possible involvement of opioidergic or serotoninergic pathways in its mode of action.

The acetic acid-induced abdominal writhing model in mice has been proven to be reliable, easy to perform, sensitive, and particularly suitable for evaluating even weaker analgesics (Le Bars et al., 2001). In this study the analgesic effect of DAE was initially evaluated using this model. The peritoneum is an example of a visceral organ that has silent nociceptors attached to C-polymodal fibers which are only activated in inflammation. Thus, acetic acid, a noxious chemical stimulus
could trigger the synthesis and/or release of prostaglandins, which subsequently cause the production of bradykinin, a noxious endogenous substance, within the peritoneum (Azi et al., 2014; Lalrinzuali et al., 2016). Furthermore, intraperitoneal administration of acetic acid induces a characteristic and quantifiable overt pain-like behavior, described as a writhing response or abdominal contortions, characterized by abdominal distention and outstretching of hind limbs. Related studies have also reported the implications of interleukins IL-1β, IL-8 and tumour necrosis factor –alpha (TNF-α) in mast cells and resident macrophages within the peritoneum during inflammation (Ribeiro et al., 2000). Data obtained from this study indicated that DAE significantly inhibited acetic acid-induced nociception, suggesting that DAE inhibited the release and or synthesis of inflammatory mediators and pro-inflammatory cytokines, partial or complete blockade of these silent nociceptors present in the peritoneum. These results corroborate with the investigation of analgesia by N’gouemo et al., (1996), on the inhibition of writhing by DAE in the acetic acid test.

The formalin test is a more specific and particularly useful test, for the assessment of new analgesics, since it encompasses neurogenic and inflammatory mechanisms of nociception (Tjølsen et al., 1992; Ellis et al., 2008). Opioids are known to inhibit both phases, whereas NSAIDs mostly affect the inflammatory phase (Le Bars et al., 2001). The extract, DAE, exhibited an obvious anti-nociceptive activity in all phases of the test. This implies DAE may have a direct effect on nociceptors associated with the early phase of the test or on pain mediators, such as prostaglandins, substance P and bradykinin. Additionally, the inhibition of pain in the second phase may be due to a modulatory effect on the release and/or synthesis of inflammatory and pro-
inflammatory mediators through peripheral and/or central mechanisms (Boakye-Gyasi et al., 2017) or an ongoing spinal activity post activation of the nociceptors.

To further investigate the involvement of central pain pathways in the analgesic effect of DAE, the hot plate test was used. The significant increase in latency time in the hot plate test by DAE (p < 0.05) at different doses suggests the involvement of higher centers (Julius, 2013) in the antinociceptive activity of DAE. This reveals the activity of the extract possibly on nociceptor nerve endings, thereby inhibiting the release of neurotransmitters and peptides from both peripheral and central nerve terminals. This, eventually, reduces pain generation and perception and the activation of the descending inhibitory pathway of pain (Reddi et al., 2013; Chahl, 1996).

To study and further characterize the possible mechanism(s) of action DAE, the analgesic effect of DAE was assessed in the presence of various antagonists of notable mediators of the pain pathway, including naloxone, theophylline, L-NAME, glibenclamide, atropine, ondansetron, yohimbine, prazosin and nifedipine. The hot plate test was used because the extract showed pronounced activity in this test. Naloxone, a nonselective opioid antagonist, significantly reversed the analgesic effect of DAE in the test, suggestive of a possible opioidergic involvement in the actions of DAE. The reversal effect was as effective as that against morphine in the hot plate test. This strongly suggests that the analgesic effect of DAE may be via interaction at/with opioid receptors at the supra-spinal level, confirming the straub tail effect earlier observed in the Irwin’s test. In agreement with this suggestion, it has been demonstrated that μ-opioid receptors may mediate mainly supra-spinal analgesia (Khatun et al., 2015). It can, therefore, be anticipated that the central effect of DAE may be prominent on μ-opioid receptors. This finding clearly suggests
that activation of opioid receptors and/or an increment of endogenous opioids, either centrally or peripherally, inhibition of neurotransmitter release from the primary afferent terminals in the spinal cord, and activation of descending inhibitory controls in the midbrain (Chahl, 1996), might be involved in the analgesic effect of DAE (Bilger et al., 1990).

Glibenclamide is an ATP-sensitive K\(^+\) channel blocker which blocks the analgesic activity of both DAE and morphine. The opening of ATP-sensitive K\(^+\) channel has been reported to participate in opioid-mediated antinociception at the level of K\(^+\) and not opioid receptor activation. It is well established that glibenclamide specifically blocks ATP-sensitive K\(^+\) channels, with no effect on Ca\(^{2+}\) or voltage-dependent K\(^+\) channels (Amoroso et al., 1990; Edwards and Weston, 1993). The present data suggest that opening of ATP-sensitive K\(^+\) channels plays a significant role in the analgesic action of DAE. This pharmacological action of DAE have been evaluated and described by several researchers as a potent K\(^+\) channel opener (Barreto, 2002; Addy et al., 1989 and McManus et al., 1993).

Neurotransmitter release from neurons is normally preceded by depolarisation of the nerve terminal and Ca\(^{2+}\) entry through voltage-sensitive Ca\(^{2+}\) channels. Drugs may inhibit neurotransmitter release by a direct effect on Ca\(^{2+}\) channels to reduce Ca\(^{2+}\) entry, or indirectly by increasing the outward K\(^+\) current, thus shortening repolarisation time and the duration of the action potential (Ocaña et al., 2004), which may be the possible case in the analgesic effect exhibited by DAE, since it its effect was reversed by glibenclamide but not nifedipine. Opioidergic drug produce both of these effects, because opioid receptors are apparently coupled via G-proteins directly to K\(^+\) channels. It is likely that compounds that open K\(^+\) channels by direct activation, like
DAE, may gain importance as effective pain relievers, since these have been shown to be very effective in models of acute and chronic pain (Ocaña et al., 2004).

The noradrenergic receptor system is involved greatly in descending modulation of pain pathways. Clonidine, an alpha 2 (α2)-adrenergic agonist acting on the nerve endings of primary afferent fibers, will inhibit the release of norepinephrine, glutamate and substance P, as well as proinflammatory cytokines, resulting in sedative and analgesic actions (Lavand'homme et al., 2003; Bantel et al., 2007). The current findings suggest and corroborate an earlier study of the possible involvement of the adrenergic system (Addy and Awumey, 1984; Addy and Dzandu, 1986; Addy and Burka, 1987, 1988, 1989, 1990; Addy, 1992; McManus et al., 1993; Barreto, 2002), since DAE activity was significantly reversed, when pre-treated with yohimbine (α2-adrenergic antagonist) and prazosin (α1-adrenergic antagonist) in this study. In addition, serotonergic receptor pathway correlates with that of noradrenergic system. Activation of serotonergic receptor will cause the release of noradrenaline which activate postsynaptic α2-adrenergic in the spinal cord leading to antinociception (Sawynok et al., 1995). Pre-treatment with yohimbine (which also inhibits the 5-HT2A receptor) significantly reversed the analgesic activity of DAE, indicating its role in the serotonergic system. However, the analgesic effect of DAE was not significantly affected in the presence of ondansetron, a 5HT3 antagonist, implying a selective serotonin pathway via the 5HT2A receptor pathway but not 5HT3 (Fredholm et al., 1999).

Since the analgesic effect of DAE was not significantly affected by theophylline (an adenosine antagonist), atropine (a muscarinic antagonist), nifedipine (L-type VGCa++ channel blocker), L-
NAME (NO synthase inhibitor) and ondansetron (5HT3 antagonist), it is suggested that DAE may not exert its analgesic activity via these pathways it to any lesser extent at all.

Chemotherapy-induced peripheral neuropathy (CIPN) is a common disturbing adverse side that has led to poor compliance and, in some cases, the discontinuation of chemotherapy (Fidanboylu et al., 2011). Paclitaxel, a widely used chemotherapeutic agent for the treatment of solid tumours, (Pourmohammadi et al., 2012) pharmacokinetically distributes in the central and peripheral nervous system following its administration in rats (Cavaletti et al., 2000) and accumulates mainly in the dorsal root ganglia and the brain at very low concentrations. Accumulation has also been reported in the sciatic nerve and spinal cord at higher concentrations (Cavaletti et al., 2000).

Though the precise mechanism by which paclitaxel causes peripheral neuropathy is yet to be established, current studies explain that continuous administration of paclitaxel causes severe peripheral neuropathy, characterized by thermal and mechanical hyperalgesia, as well as allodynia, possibly due to atypical (swollen and vacuolated) mitochondria in peripheral sensory axons of both the C-fiber and myelinated axons, nerve damage by disruptive formation of microtubules needed for axonal transport in the dorsal root ganglia, axons and Schwann cells (Vuorinen et al., 1988; Ballatore et al., 2011; Pourmohammadi et al., 2012), and a loss of intra-epidermal nerve fibers leading subsequently to loss of cellular function (Fidanboylu et al., 2011).

In the present study, DAE significantly attenuated paclitaxel-induced hyperalgesia, as evidenced by increased tail withdrawal and reaction latencies in the tail flick and hot plate tests when compared with vehicle- and paclitaxel- treated groups and this was comparable to the effect produced by pregabalin. Pregabalin is the drug of choice for the pharmacological management of
CIPN (Borgi *et al.*, 2008; Mangaiarkkarasi *et al.*, 2015). The analgesic and anti-epileptic actions of pregabalin are associated with its antagonistic effect on α2–δ1 subunit of N-type voltage-dependent calcium channels (Mangaiarkkarasi *et al.*, 2015). Studies have shown that inhibition of calcium channels significantly reduce neuronal excitability by attenuating neuronal calcium influx, thereby causing inhibition of the release of neurotransmitters, including noradrenaline, substance P and glutamate, the inhibition of synaptic transmission and other cellular enzymatic cascade reactions that lead to pain sensation (Kumar *et al.*, 2010; Mangaiarkkarasi *et al.*, 2015). Though not established, it is highly likely that DAE attenuated the sensitivity in rats to heat and cold exposure induced by paclitaxel, by acting via adrenergic receptors to inhibit the release and action of noradrenaline at the receptor site, thus reducing neuronal excitation at the post-synaptic cleft. In addition, opioids, such as morphine, that act via the opioidergic nociceptive pathway, have been shown to inhibit paclitaxel-induced neuropathic pain (Ami *et al.*, 2012). DAE has been known to suppress pain via the opioidergic nociceptive pathway, and this may partly contribute to the anti-neuropathic properties of DAE in this model. Several reports indicate that paclitaxel causes the release of pro-inflammatory pain mediators and cytokines, including bradykinin and TNF-α, as well as the activation of microglial and astroglial cells (Manthey *et al.*, 1992; Burkhart *et al.*, 1994; Costa *et al.*, 2011; Muñoz *et al.*, 2011; Li *et al.*, 2012). Therefore, the effect of DAE on pro-inflammatory pain mediators and cytokines, as a possible mechanism, cannot be ruled out. These mechanisms and, perhaps, more may be responsible for the highly significant attenuation of pain induced by paclitaxel in all the neuropathic pain models.

Plant-based medicinal agents or alternative medicines have become globally accepted in primary healthcare, particularly in developing countries, and some have been erroneously regarded as safe,
just because they are derived from natural sources. This has resulted in self-medication, compromising the life expectancy of such patients due to adverse and toxic effects from these agents (Vaghasiya et al., 2011).

International Opinion and Regulations relating to human health instructs that every new therapeutic agent be tested for its safety before it is administered to human volunteers and patients (Klaassen et al., 1991). Safety studies in appropriate animal models are therefore commonly used to assess the potential health risk to humans. (Klaassen et al., 1991).

Generally, from the study, DAE was found to possess a high safety profile in rodents. The hematological and most of the biochemical parameters assayed were within the normal ranges, with minimal changes compared to the controls. No histopathological changes in the isolated organs from the rats treated with the extract were observed. The minor differences observed were not statistically significant, and may be due to biological variations in the animals. On the other hand, significant differences seen in this study were suggestive of a possible protective and/or beneficial effects. These, however, require further tests to confirm or settle the ambiguity of these variations.

Acute toxicity is usually associated with pronounced, marked and/or changes in physiological, behavioral or biochemical processes. This may lead to toxic syndromes, such as changes in skin, fur, eyes, mucus membrane, and autonomic activity like lacrimation, piloerection, pupil size and unusual breathing pattern, abnormal gaiting, posture, response to handling, presence of convulsive
movements and tremors; stereotypic activities such as excessive grooming, repetitive circling, morbidity and or mortality in some cases (Pramyothin et al., 2006; Patel et al., 2008).

In the present study, the absence of these signs of toxicity at a dose of 3000 mg/kg implies that the no-observed-adverse-effect-level (NOAEL) of DAE is above 3000 mg/kg. This suggests that DAE is relatively safe, since substances with an LD$_{50}$ value of 1000 mg/kg by the oral route are regarded as safe or of low toxicity (Obici et al., 2008)

The extract, did not adversely alter the weight of the animals during the 14-day continuous administration, since there was no significant difference between the average weights of the animals treated with the vehicle and that treated with DAE, except with the highest dose of 1000 mg/kg where there was a slight drop in weight over the period. This was, however, not alarming low. Change in body weight either due to anorexia as well as severe weight loss or gain due to metabolic derangement, are worth noting, since they are also considered signs of toxicity (Bernstein, 1987; Zimmermann et al., 2003; Yuet Ping et al., 2013). Therefore, comparable body weight differences between DAE-treated groups and control group observed in the study may suggest negligible or no negative effects of DAE on body weight (Zimmermann et al., 2003).

Ideally, the changes in body weight and internal organ weights of rodents reflect the toxicity after exposure to the toxic substances (Auletta, 1995). Organ weight is also an essential index of physiological and pathological states in animals (Raza et al., 2002; Teo et al., 2003). The relative organ weight is a basic diagnostic feature as to whether the organ has been exposed to the injury or not. The heart, liver, kidney, spleen and lungs which are highly perfused organs, are the key
organs affected by metabolic reactions caused by these toxic substances (Dybing et al., 2002). No significant changes were observed when the organs were macroscopically observed in the control group compared to the treated groups, thus, it can be said that DAE did not adversely affect the organ weight of all the key organs assessed.

There were no extract-related adverse effects on urinalysis parameters in the 14 day period of the study. The parameters in the urine that were analyzed was not significantly different from compared to the control groups. Certain bodies present in urine are indicators and markers of a disease state of the liver or kidneys. Previous studies have shown that there is a correlation between site of toxic lesion and its corresponding bodies present in urine (Gartland et al., 1989; Anthony et al., 1994). For example, increased urinary excretion of glucose, amino acids and organic acids indicates damage to the renal proximal tubule (Gartland et al., 1989). Therefore, minimal variations in the urine parameters between DAE-treated groups and control groups may imply low or minimal toxicity and possibly minimal organ damage. This suggests that the administration of DAE up to 1000 mg/kg/day has negligible adverse effects on urine parameters.

The hematopoietic system serves as an essential index of physiological toxicity in both humans and animals, as it is extremely susceptible and easily affected by toxic compounds (Adeneye et al., 2006). This test is known as FBC or full blood count, where key blood components are assessed and analyzed. These values are used to partly establish the presence of infections (bacteria, viral), inflammation (trauma or autoimmune disease), anemia (iron deficiency, blood loss, nutrient deficiency and/or renal defects), cancers (leukemia) and clotting abilities in both animals and humans (Ajeigbe et al., 2013). Hematological parameters that were measured in this study
included WBC count and differential RBC count, Hemoglobin concentration, Hematocrit and Platelet count, etc. After a 14 day treatment with DAE, the changes in hematological parameters between the control and treatment groups were marginal and not statistically significant. It can, therefore, be said that DAE does not adversely affect or derange the hematopoietic system when given over a short period. However chronic studies must be done to firmly verify this assertion.

The liver, the largest of the viscera and the second largest organ in the body, is invaluable in detecting the toxicity of drugs (Seeley et al, 2008; Kenneth et al 2004). Liver function tests (LFTs) carried out in this current study measured and analyzed liver enzymes (AST, ALT, GGT and ALP), total protein (albumin and globulins) and total bilirubin (indirect and direct). Hepatocellular injury, and not cell death is the major triggering factor that causes the release of these intracellular liver enzymes into the circulatory system (Thapa and Walia, 2007).

In the present study, AST and ALT were significantly lower in the DAE-treated groups, compared to the control group, corroborating a study by Magielse et al., (2013), that indicated a decrease in AST and ALT after pre-treatment of an aqueous decoction of *D. adscendens* leaves and twigs in rats against acute liver damage induced by D-galactosamine. This study, however, was in contrast to a research by Quaye, (2001), that stated a significant increase in AST and ALT and direct bilirubin concentration dose-dependently of an orally administered freeze dried powder from an aqueous crude extract of *D. adscendens* leaves. ALP was elevated in the DAE-treated groups in comparison to the control group. ALP originates mainly from the liver and bone, albeit it is also present in other tissues as well. Thus, an elevated ALP could either be of liver or bone origin (Limdi and Hyde, 2003). However, to differentiate the likely origin of this elevation, is a
corresponding elevation in the enzyme gammaglutamyl transferase (GGT), which is a source of abnormality of hepatobiliary source, that strongly indicates hepatocellular but not osteocellular disease or damage (Goldberg et al.; 1975; Pratt et al.; 2000). In the present study, though ALP was elevated in the DAE-treated groups, GGT was, however, unaffected. This certifies that the source of the elevation may not be as a result of a liver disorder or abnormality. Also, the elevation of ALP may have been more physiological than pathological. Some studies have shown, though not confirmed, that production of ALP increases in tissues undergoing metabolic stimulation and not necessarily as a result of a pathological condition (Bardella MT et al.; 1999; Pratt DS et al.; 2000). Further tests such as specific bone tests, may have to be performed to clearly distinguish and assess this elevation.

Total protein which comprises albumin and globulin tests are routinely included in the battery of biochemical tests for toxicity. It usually assesses the overall health status of the organism (DiBonaventura et al., 2012). Total protein was significantly low in the DAE-treated (100 mg/kg and 300 mg/kg) groups in comparison with the control group. This was as a result correspondingly low levels of albumin and globulin recorded in these groups. Hypo-albuminaemia and hypo-globulinaemia may arise as a result of severe malnutrition, malabsorption, such as inflammatory bowel disease (IBD), and hepatocellular or renal disorder (Mintzer et al., 2009; Piazza et al., 2011).

However, in the current study, possible causes of low protein, albumin and globulin levels were not clear in the animals in these groups. There was no apparent sign of malnutrition, as body weights were not affected in these groups. Hepatic enzymes (AST & ALT) were not elevated and renal parameters were within the stipulated ranges. This has left the possible reason(s) for this low
level recorded for further studies. Follow-up tests, such as protein electrophoresis and quantitative immunoglobulins (Chang et al., 2007), may be required to evaluate the abnormal protein levels recorded.

Creatinine, urea and electrolytes (Na+, K+, Cl-) were all within the postulated clinical ranges, both in the control and the DAE-treated groups, indicating that the excretory systems of the animals were functioning properly and that DAE had little or no adverse effect on these biochemical parameters.

Cholesterol plays a key role in the health status of the cardiovascular system. There are “good” and “bad” cholesterol usually obtained from exogenous sources (Tabas, 2002). High-density lipoprotein (HDL) is considered as the good cholesterol, because it eliminates excess cholesterol in the blood by removing it from the blood and taking it to the liver. A high HDL level is, therefore related to lower risk of heart and blood vessel disease. Nonetheless, low-density lipoprotein (LDL) is considered as bad cholesterol and is associated with a higher risk of cardiac and coronary disease, as it is the lipoprotein responsible for mopping up cholesterol from the blood to the cells (Seeley et al, 2008; Kenneth et al 2004; Limdi and Hyde, 2003). Thus pronounced increase in the total cholesterol, triglycerides and LDL cholesterols are highly associated with possible risk of atherosclerosis, cardiovascular conditions and stroke (Hu et al., 2000; Tabas, 2002). With respect to the effect of DAE on the lipid profile, the results obtained shows that the extract significantly reduced total cholesterol, increased HDL but decreased LDL and had no effects on the triglycerides. One of the clinical reasons for hyperlipedemia is hypoalbuminemia. This is because albumin plays an important role in fat metabolism (McPherson, 1984). Albeit, hypoalbuminemia
was recorded in the extract-treated groups, and there was no corresponding adverse effect of the lipid levels. This suggests that 14-days administration of DAE may very likely have potential beneficial therapeutic effects on lipid metabolism, though a chronic toxicity study with similar data and also in a non-rodent model animal would be needed to confirm this assertion.

Histological examination is the first-rated standard for assessing treatment related pathological alterations in tissues and organs (Test, 1984). Therefore, aside the gross analysis of the organs, histological analysis was also done to further confirm the changes, if any, in cell structure of the organs. In the current study, histopathological evaluation of acute oral administration of DAE indicated that the extract did not adversely affect the morphology of rat organs. This corroborates with the body weight, organ weight and the biochemical analysis results. There was no obvious structural injury to the key organs tested. This implies low or negligible morphological toxicity of DAE up to a dose of 1000 mg/kg orally.

The liver is the major organ for the metabolism of drugs and a key target organ of acute toxicity (Rhiouani et al., 2008). In this study, liver histology revealed normal regular hepatocytes with no ballooning and giant cell formation either from the treatment groups or the control. This observation suggests that, though there were little variations some of the parameters in the LFTs, there was no corresponding change in the morphology and histology of the liver. Therefore, it can be said that from the sub-acute toxicity study, DAE does not cause any significant modifications to the structure of the liver cells, suggesting that it has minimal adverse effects on the organ.
The histological examination of the kidneys, the major organ for excretion, revealed regular glomeruli with neither mesangial cell proliferation nor basal membrane activity. There was no inflammation of the Bowman’s capsule and renal tubules. This further confirms the normal serum level of the related parameters to kidney dysfunction such as urea, creatinine and some electrolytes such as Na, K, Cl recorded in the renal function test. It can be concluded that, a 14-day administration of DAE to rats does not adversely affect the morphology and histology of the kidney.

5.2 CONCLUSION

From the findings of this study, it can be concluded that the ethanolic extract prepared from Desmodium adscendens is effective as an analgesic against induced nociceptive and neuropathic pain with a wide safety profile in murine behavioral models. DAE presents a viable traditional therapeutic alternative, and could demonstrate effectiveness in the management of the various types of pain.

The observed analgesic action of DAE could be possibly due to:

• Activation of opioid receptors particularly mu (μ) receptors
• Stimulation of ATP- sensitive K+ channels
• Activation of adrenergic system (α- 1 and 2 adrenoceptors) and serotogenic (HT2A) receptors in the locus coeruleus and the dorsal root.
The extract of *Desmodium adscendens* contains the secondary metabolites saponins, flavonoids, alkaloids, tannins, glycosides, sterols and terpenoids. These secondary metabolites could largely be responsible for the pharmacological action of DAE. *Desmodium adscendens* is considered safe with an LD$_{50}$ above 3000 mg/kg when administered orally.

These results obtained have added to the knowledge of scientific evidence of the analgesic properties of the plant and scientifically validated its traditional uses as an analgesic.

5.3 RECOMMENDATIONS

- The active compound(s) responsible for the analgesic effects of the ethanolic extract of the plant should be isolated and characterized.
- Chronic toxicity studies on the ethanolic extract should be performed to ascertain and further confirm its safety.
- Other pain/analgesia models should be used to evaluate/confirm the analgesic effects of the ethanolic extract and other possible mechanism of action evaluated.
- Secondary metabolites should be isolated and assessed for possible analgesic effects.
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