PHYTOCHEMICAL CONSTITUENTS AND PHARMACOLOGICAL USE OF ETHANOLIC STEM EXTRACT OF *Sida acuta* AS AN ANALGESIC

BY

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UNIVERSITY OF BENIN

JUNE, 2025

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A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF POSTGRADUATE DIPLOMA IN SCIENCE LABORATORY TECHNOLOGY (PGDSLT)

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CERTIFICATION

This is to certify that this research work was carried out by Teddy **ERIAMIATOE** with Matriculation Number PG/LSC2216094 for the partial fulfilment of requirements for the award of Postgraduate Diploma in the Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin.

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DEDICATION

This work is dedicated God Almighty

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The Head of Department, Science Laboratory Technology, Professor E. O. Oshomoh is greatly appreciated and the entire staff of the Department, thanks for the words of encouragement.

To my amiable wife, Dr (Mrs) Eriamiatoe Imuetinyan, thank you for your support.

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ABSTRACT

The present study investigated the phytochemical profile and analgesic potential of the ethanolic stem extract of *Sida acuta,* a plant traditionally used in folk medicine for pain relief. The primary objectives were to identify and characterize the major phytochemicals present in the extract, evaluate its analgesic activity using established *in vivo* models, and compare its efficacy to standard analgesics, aspirin and pentazocine.

Phytochemical screening revealed the presence of phenolic compounds, glycosides, saponins, flavonoids, steroids, terpenes, tannins, and alkaloids—bioactive constituents widely associated with therapeutic effects. Thin layer chromatography (TLC) confirmed the diversity of these metabolites across different solvent systems, with a chloroform–ethanol (9:1) mixture yielding the highest number of detectable compounds, emphasizing the role of solvent polarity in extraction efficiency. Analgesic activity was evaluated using acetic acid-induced writhing and hot plate models, representing peripheral and central nociception, respectively. The extract showed a dose-dependent inhibition of abdominal constrictions, achieving 100% inhibition at 400 mg/kg, comparable or superior to aspirin at 100 mg/Kg of mice. Similarly, in the hot plate model, the extract significantly increased reaction times to thermal stimuli, with effects at 400 mg/kg comparable to pentazocine at 0.1 mg/Kg of mice, a standard opioid analgesic. These findings suggest that the extract exerts dual analgesic effects, likely involving suppression of prostaglandin synthesis peripherally and modulation of central nociceptive pathways possibly via opioid or serotonergic mechanisms. FTIR spectral analysis confirmed the presence of functional groups characteristic of phenolic compounds, including O–H (3418.74-3571.33 broad stretch), C–O (1045.39 short C-C), and C=C bonds (1644.67 sharp long C=C conjugation), supporting the identification of flavonoids, tannins, and phenolic acids. GC-MS analysis further identified key compounds with known analgesic or anti-inflammatory properties such as Eugenol (RT:10, P.A: 0.30), Phytol (RT: 42.07, P.A: 6.13), Squalene (RT: 44.86 P.A: 10.85), and various fatty acid esters, supporting the observed pharmacological activity.

Overall, the study highlights *Sida acuta* stem extract as a promising source of natural analgesics with both peripheral and central mechanisms of action, warranting further investigation and potential pharmaceutical application.

CHAPTER ONE

INTRODUCTION

Since the dawn of civilization, humans have relied on medicinal plants not only for nourishment but also for their therapeutic benefits (Amanpreet and Rattan, 2022). Over time, a significant number of pharmaceutical agents have been derived from plant sources. For millennia, nature has served as a vital reservoir of medicinal compounds (Tcheghebe *et al*., 2017). Natural products offer a wealth of bioactive molecules capable of treating various human ailments, and many of these serve as lead compounds in the development of novel drugs (Amanpreet and Rattan, 2022). Examples include aromatic polyketides, polyethers, coumarins, flavonoids, terpenoids, alkaloids, and aminoglycosides, all of which are well-known natural product-derived drugs (Alka, 2012). Traditional medicine, often utilizing plant-based remedies, remains one of the oldest and most widespread methods for addressing both infectious and non-infectious diseases across different cultures. Various parts of medicinal plants—such as leaves, roots, stems, seeds, and bark—are employed in treating diverse illnesses. Their healing properties are largely attributed to bioactive constituents contained within them. Compounds such as terpenes, flavonoids, bioflavonoids, benzophenones, xanthenes, along with secondary metabolites like tannins, saponins, cyanates, oxalates, and anthraquinones, are considered the key phytochemical agents responsible for their medicinal effects (Ezeabara, and Egenti, 2018). Distinct parts of plants have also been used to treat various diseases and infections (Ekpo and Etim, 2009).

In Cameroon, *Sida acuta* is traditionally recognized for its wide range of medicinal properties, including anti-inflammatory, anti-rheumatic, diaphoretic, febrifuge, sedative, anti-ulcer, and anthelmintic effects. Preparations made from the entire plant, particularly decoctions, are commonly used in folk medicine to treat fever, relieve rheumatic conditions, and eliminate intestinal parasites. The same decoction is also used to treat malaria (Olivier *et al.,* 2016).

Overall, *Sida acuta* has been scientifically studied for its numerous pharmacological profiles such as antioxidant, antimicrobial, antibacterial, antimalarial, cardiovascular, antiulcer, analgesic, anti-inflammatory, antipyretic, hepatoprotective, hypoglycemic, insecticidal and anticancer (Olivier *et al.,* 2016). Previous studies have shown that various naturally occurring fatty acids play an active role in supporting optimal health. In addition to its major role in cardio-protection, these fatty acids possessed anti-cancer and free-radical scavenging effects, and hence the extract might be used as a promising natural source of anticancer substance (Nalini *et al.,* 2021). The plant-based sterols have been reported to contain various roles in the prevention of human pathologies (Han *et al.,* 2016).

1.1 Background of the Study

Sida acuta (family: Malvaceae) is a shrub found in tropical and subtropical regions and is commonly used in ethnomedicine for treating various ailments including fever, inflammation, pain, and wounds. Preliminary phytochemical screenings have indicated the presence of flavonoids, alkaloids, tannins, and other bioactive compounds. However,

scientific validation of its traditional analgesic use and comprehensive analysis of its bioactive components remain limited. Advancing research on *Sida acuta* may uncover novel compounds that could serve as lead candidates for the development of new analgesic drugs.

1.2 Problem statement

Despite widespread traditional use of *Sida acuta* for pain relief, there is a paucity of scientific evidence supporting its analgesic efficacy and identifying the active phytochemicals responsible. Furthermore, the pharmacological pathways through which its analgesic effects are mediated are not well understood. The lack of validated scientific data limits its integration into modern pharmacotherapy and may hinder the discovery of novel analgesic agents from this plant.

1.3 Aim and Objectives

Aim of the study:

This study aims was to investigate the chemical constituent and pharmacological use of *Sida acuta* ethanolic stem extract as analgesic shedding light on its potential as a natural remedy for pain management

Objective of study:

The following specific objectives were focus on to achieve this aim:

1. Identify and characterize the major phytochemical constituents present in the extracts of *Sida acuta* stem extract

2. Evaluate the analgesic activity of *Sida acuta* stem extracts using appropriate *in vivo* models. Acetic acid induced writhing and hot plate method in mice.

3. Compare the analgesic efficacy of the stem extracts with standard analgesic drugs; aspirin and pentazocine

4 Characterization of *Sida acuta* stem extract with organic solvent using infrared (IR) spectroscopic technique and its interpretation

5. Separation and purification of extract using gas chromatography-mass spectroscopy(GC-MS) and Thin layer chromatography

1.4 Scope of study

To achieve the specific objectives, this study covers the following scope as outlined.

1. Sample collection and preparation of *Sida acuta* stems.

2. Ethanolic isolation of *Sida acuta* stems using soxhlet apparatus

3. Comprehensive identification and analysis of principal phytochemical compounds in the ethanolic stem extract

4. Thin layer chromatoghaphic profiling of the ethanolic stem extract to separate and visualize the phytochemical components.

5. Analgesic Activity Evaluation using Acetic acid-induced writhing test and Hot plate method in mice to assess peripheral analgesic activity and central

analgesic activity. Use of appropriate controls and standard drug (aspirin and pentazocine respectively).

6. Fourier Transform Infrared (FTIR) Spectroscopic analysis of the ethanolic stem isolate and the alkaloid fraction to identify functional groups present.

7. Gas Chromatography-Mass Spectrometric (GC-MS) analysis of the ethanolic stem isolate to identify volatile bioactive compounds based on retention time and mass spectra.

8. Compilation, statistical analysis, and interpretation of phytochemical and pharmacological data.

1.5 Significance of the study

This study was aimed to examine the chemical compositions and analgesic potentials of ethanolic stem extract of *Sida acuta* which could have significant implication for:

- Valiudation of traditional use of *Sida acuta* for pain relief by providing evidence for its efficacy and safety.
- Identification of potential analgesic compounds through phytochemical screening, FTIR, and GC-MS analysis.
- Addition of valuable data to the growing body natural product research on plant-derived pharmacologically active compounds.
- Basis for the development of plant-based analgesics that may serve as alternatives to synthetic drugs with adverse side effects.

- Demonstration of both peripheral and central analgesic activities, supporting the versatility of the ethanolic stem isolate of *Sida acuta* in pain management.

CHAPTER TWO

LITERATURE REVIEW

2.1 Active Ingredients in Medicinal Plants

The most important types of active ingredients in medicinal plants are terpenoids and alkaloids (Eriamiatoe *et al.*, 2020). Others such as fatty acids (e.g., chaulmoogra oil), carbohydrate, proteins, steroids, saponin glycosides, tannins are also involved however.

2.1.1 Alkaloids

Alkaloids are Biogenically produced chemical compounds which are organic bases containing basic nitrogen atoms. About 12000 alkaloids are known and characterised, 95% of them are from the higher plants and others are produced from a large variety of organisms, including bacteria, fungi, and animals and are part of the group of natural products (also called secondary metabolites) (Cushnie *et al.*, 2014). Many alkaloids can be purified from crude extracts by acid - base extraction (Cushnie *et al.*, 2014). Many alkaloids exhibit toxicity toward bacteria, fungi, and other organisms, making them biologically active and pharmacologically significant. Due to their diverse physiological effects, they are widely used as medications, psychoactive agents, and in traditional entheogenic practices. Notable examples include cocaine, a local anesthetic and stimulant; caffeine and nicotine, both central nervous system stimulants; morphine, a potent analgesic; and quinine, an antimalarial compound. Several alkaloids, such as chloroquine and quinine, are known for their distinctly bitter taste. Their chemical structures are illustrated in Figures 1 and 2. (Madziga *et al*., 2010).

2.1.1.1 Medicinal uses of Alkaloids

In the 17th century, Jesuits in South America discovered that a native remedy for other diseases made from an infusion of the bark of cinchona (Cinchona spp., Rubiaceae) coincidentally controlled malaria contains quinine. (Eriamiatoe *et al.*, 2020).

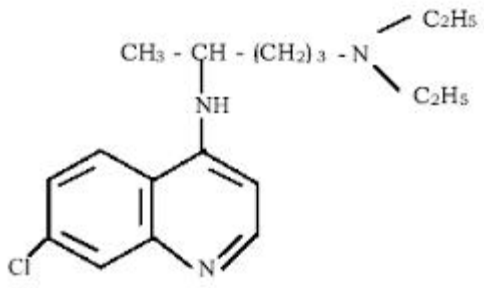

Figure 2.1: Chemical structure of chloroquinine

Also artemisinin from *Artemisia annua*, has proven effective and is currently being used in Southeast Asia.(Seigler and Riggins, 2012)

Infusions of *Ephedra* species (*Ephedraceae* family), a gymnosperm, have been used in native Chinese medicine for thousands of years, where the plant is commonly known as "ma huang." In the 1920s, it was "rediscovered" by Western medicine, leading to the identification of its active compound, ephedrine leading to the isolation of quinine (Seigler and Riggins, 2012).

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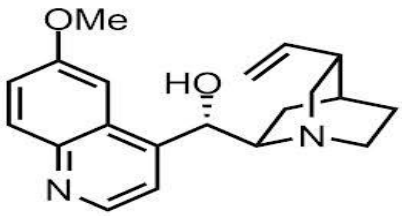


Figure 2.2: Quinine

Ephedrine, along with several structurally related compounds, is still widely used today as a nasal decongestant (found in medications such as Sudafed and Robitussin) and for the treatment of hypotension (low blood pressure) (Seigler and Riggins, 2012).

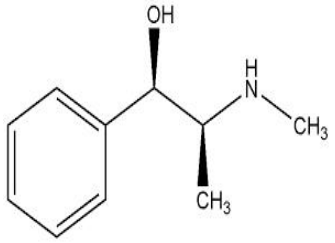


Figure 2.3: Ephedrine

Rauvolfia alkaloids

Rauvolfia serpentina (Apocynaceae) is rich in alkaloids known for their strong hypotensive effects. One of its primary alkaloids, reserpine, is widely utilized in the treatment of hypertension and certain mental disorders, with relatively high doses often administered to patients with schizophrenia (Sharma *et al.*, 2012).

During the time of Dioscorides, it was already recognized that isolates from leaves and willow bark could relieve pain. The active compound responsible for this effect is salicin, which, however, is too harsh for internal use. In the late 1800s, a German chemist developed a new compound, acetylsalicylic acid, which shared salicin's beneficial

properties but could be safely taken orally. This substance, better known as aspirin, became a highly effective anti-inflammatory, analgesic, and antipyretic, and remains one of the most widely used drugs worldwide. Interestingly, the detailed mechanism of aspirin's action was only uncovered in the past two decades (Eriamiatoe *et al.*, 2020).

In the Andean regions of South America, Indigenous peoples have traditionally chewed coca leaves (Erythroxylum coca, Erythroxylaceae) combined with lime to release the active alkaloids, which helped to suppress hunger and alleviate pain. When these alkaloids were later isolated, they were found to possess potent local anesthetic properties. Although cocaine itself has been applied in surgical procedures, particularly in dental surgery, synthetic analogues of these alkaloids are now more commonly used (Sharma *et al.,* 2012).

Table 2.1: Methods for detection of alkaloids

Reagent	Composition	Result (Indication of Alkaloids)
Mayer's Reagent	Potassiomercuric iodide solution	Cream-colored precipitate
Wagner's Reagent	Iodine in potassium iodide solution	Reddish-brown precipitate
Tannic Acid	Tannic acid solution	Formation of a precipitate
Hager's Reagent	Saturated solution of picric acid	Yellow precipitate
Dragendorff's Reagent	Solution of potassium bismuth iodide and potassium chlorate; treated with HCl, evaporated to dryness, and exposed to ammonia vapors	Orange or reddish-brown precipitate (except for caffeine and a few other alkaloids)

2.1.2 Flavonoids

The flavonoids belong to one of the most bioactive compounds which naturally exist in the plant kingdom (Okwu and Ukanwa, 2010). Different naturally derived flavonoids have been described and subcategorized into flavones, anthocyanidines flavanones, chalcones, isoflavonoids, aurones and flavans, (Okwu and Ukanwa, 2010).

They are phytonutrients and the general names comes from the colour they carry, flavus is a latin word which means yellow and these colours carried by flowers comes from the

flavonoid. They are polyphenols because they have hydroxyl functional group (Chukwumah *et al.,* 2009).They play diverse roles in the health of plants, animals and especially man.

Presently over 6000 flavonoids has been discovered this makes them very unique among phytonutrients in which it outweighs the nutrients obtained from animal meals. For example, catechins are available in tea. The major function of flavonoid is to prevent the body against diseases. Another example is tangeretin which also repair damaged tissues and their intake has been cited in their decrease risk of certain typed of cancer which include lung, prostate e.t.c. they act as antioxidants, anti-inflammatory, radical scavenging, anti-depressant (Marchand, 2002).

Bridelia ferruginea benth leaves extract contains two flavonoids bioactive compounds 6, 4 dihydroxy-3-propen chalcones and 4 propenoxy-7-hydroxy anthocyanidines (Okwu and Nnamdi, 2011) and are ingredients of the yoruba 'agbo' and are used in the preparation of a popular mouth wash and as a remedy for thrush in children, it is also used to cure piles, diarrheal and dysentery (Marchand, 2002).

Alchornea cordifolia commonly called Christmas bush (Yoruba- Akoyin; Benin- Ebe-Uhosa) has been reported the presence of tannins, phenolic acids, gallic acid, flavonoids, quercetin, hyperin and guaijaverin and an alkaloid tri-isopentenyl guanidine (Maniyar *et al.*, 2010) in Edo and Ondo States of Nigeria, it is used in the preparation of remedies for urinary, respiratory and gastro- intestinal disorders and in the treatment of malarial fever (Eriamiatoe *et al.,* 2020).

2.1.2.1 Detection of Flavonoids in Plant Samples

Three methods were employed to test for the presence of flavonoids in plant extracts:

a. Ethyl Acetate-Ammonia Test:

A portion of the plant extract was heated with 10 mL of ethyl acetate for approximately 3 minutes. The mixture was then filtered, and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia (NH_3) solution. The appearance of a yellow coloration indicated the presence of flavonoids (Eriamiatoe *et al.,* 2020).

b. Sodium Hydroxide–Hydrochloric Acid Test:

To a portion of the extract solution, 5 mL of 20% sodium hydroxide (NaOH) was added, followed by the addition of dilute hydrochloric acid (HCl). A colorless solution was taken as a positive indication of flavonoids (Eriamiatoe *et al.,* 2020).

c. Ferric Chloride Test:

Approximately 0.1 g of the extract was dissolved in 5 mL of 90% ethanol. One drop of 10% ferric chloride ($FeCl_3$) solution was added to the mixture. The formation of a pale yellow precipitate was considered indicative of flavonoid content (Nuhu, *et al.,*2025).

Many modern techniques has also been employed to characterize and identify flavonoids in natural product. Although UV detector has been commonly used, its detection limit is still unsatisfactory due to weak adsorbance of flavonoids under UV detection (Wittig *et al.,* 2001). Furthermore, it can hardly provide any information for unknown samples. All these reasons have prompted the hypenation of high performance liquid chromatography with mass spectrometry (Carbone *et al*., 2004). Presently, different kinds of ionization methods have been developed, among which, electrospray ionization has been widely used for the analysis of natural product in either positive or negative ion mode (Hughes *et al.*, 2001)

2.1.3 Saponins

Saponins are natural high-molecular-weight glycosides of triterpenes or steroids, found widely across plants and some marine animals like starfish. They were traditionally recognized for their surface-active properties and foam-forming ability. Beyond these, saponins exhibit a range of biological activities, including anti-cholesterolemic effects by forming complexes with cholesterol in the gastrointestinal tract, thus reducing absorption (Wang *et al.*, 1999). Other activities include anti-inflammatory, antiparasitic, and antiviral effects (Cheung, 2007).

Recent studies show that saponins can induce tumor cell death through pathways involving death receptor activation, mitochondrial targeting, and oxidative stress (Sofowora, 1993). Their multiple apoptotic actions suggest they are promising candidates for anticancer therapies, including against drug-resistant cancers. Although hundreds of saponins have been described, many novel anticancer saponins likely remain undiscovered (Elango and Jadhav, 2010).

2.1.3.1 Test for Saponins

Saponins can be hydrolyzed with mineral acid into sugars and sapogenins; the sugars are detected using Fehling's solution, which changes from blue to green, then produces a brown precipitate (Sofowora, 1993).

Analytical methods for saponin quantification include spectrophotometry, TLC-densitometry, GC, and HPLC. More advanced hyphenated techniques like LC–MS, LC–NMR, and CE allow rapid screening of crude extracts, aiding the identification of new bioactive compounds and minimizing unnecessary isolation of known constituents (Baccou *et al*., 1977; Hostettmann, 1999).

2.1.4 Tannins

Tannins (tannic acids) are water-soluble polyphenols found in various plant foods and are traditionally used in leather tanning, fabric dyeing, ink making, and medicine (Catherine et al., 2009). They are also present in galls, abnormal growths caused by insects.

Tannin solutions are acidic and astringent, contributing to the taste and color of tea. Medicinally, tannins have been used to treat ulcerative colitis, tonsillitis, pharyngitis, hemorrhoids, and skin eruptions, and internally to control diarrhea, intestinal bleeding, and poisoning by metals, alkaloids, and glycosides through precipitate formation (Sartor *et al.,* 2013).

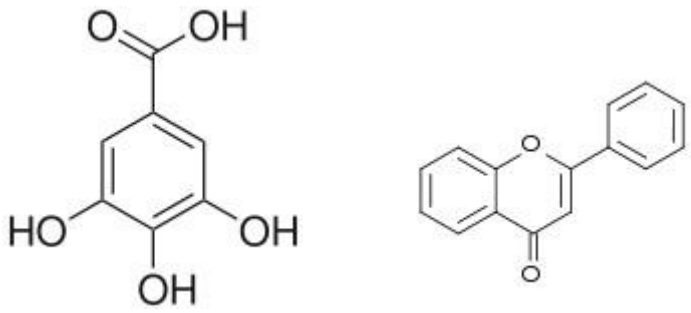


Figure 2.4: Hydrolyzed and condensed tannins

Hydrolyzed tannins are compounds formed from gallic acid or epigallic acid units condensed to a central sugar molecule. When hydrolyzed, these tannins produce gallic or epigallic acids and sugar (Catherine *et al.,* 2009).

Hydrolyzed tannins have a very low bioavailability following oral ingestion, due both to their poor lipid solubility and their ability to form strong complexes with proteins (Clinton, 2009). Hydrolysis of tannins occurs mostly in the large bowel at neutral to

alkaline pHs. Hydrolyzed tannins inhibit the absorption of iron, which may, if prolonged, lead to anemia. Tannins are metal ion chelators, which render the iron unavailable to the body (Catherine *et al.,* 2009). Tannins only reduce the bioavailability of plant sources of iron; animal sources of iron are left available for absorption. Tannic acid does not affect absorption of other trace minerals such as zinc, copper, and manganese in rats (Clinton, 2009).

Condensed tannins do not interfere with iron absorption. They are preferable for human consumption and therapeutic treatment (Catherine *et al.,* 2009). Condensed tannins are dimers or oligomers of catechin, epicatechin, or similar units. These units are polymers of 2 to 50 or more flavonoid units that are joined by carbon-carbon bonds, which are not susceptible to being cleaved by hydrolysis. Mixtures of these oligomers are powerful antioxidants known as oligomeric proanthocyanidins (Clinton, 2009).

Patients with ulcerative colitis (UC) lack the protective advantage provided by normal mucin production. This deficiency compromises the intestinal barrier, making the gastrointestinal tract more susceptible to damage caused by oxidized molecules. These reactive oxidative species are thought to exacerbate inflammation and contribute to mucosal injury, which are hallmarks of UC pathology (Clinton, 2009).

Tannins, particularly the procyanidins found in condensed tannins, have demonstrated protective properties against oxidative stress-induced cell death. These compounds are

believed to interact with the plasma membranes of intestinal epithelial cells, thereby stabilizing cellular structures and mitigating oxidative damage (Catherine *et al*., 2009).

2.1.4.1 TEST FOR TANNINS

Tannins are soluble in water, dilute alkalis, ROH, glycerol but only sparingly soluble in other organic solvent. Solution of tannins precipitate heavy metals, alkaloids, glycosides and gelatin. Tannins give blue colour with ferric salts and condensed tannins give brownish precipitate (Sofowora, 1993).

2.1.5 Eugenol

Eugenol is an allyl-substituted derivative of guaiacol (2-methoxyphenol) naturally present in various aromatic plants, including basil, cinnamon, lemon balm, and nutmeg, though it is most abundantly sourced from clove (*Syzygium aromaticum*). In its extracted form, eugenol is a clear, pale yellow liquid with a characteristic clove-like aroma. Beyond its medicinal applications, eugenol is widely utilized in the fragrance industry and is a key ingredient in products such as perfumes and clove-flavored cigarettes (Lyu and Ishida, 2019). Although it is generally considered safe, eugenol may be harmful to people if it is used above the recommended doses (Kannan and Vincent, 2012).

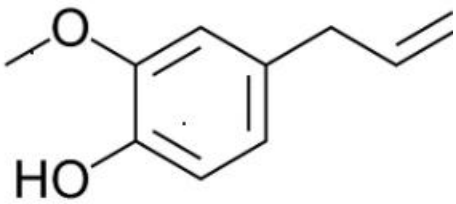


Figure 2.5: Chemical structure eugenol.

In medicine, eugenol is used as an antiseptic and an anesthetic. It is believed to relieve pain when applied to skin or to other injured body parts as well. Some men even apply the liquid to their genitals to prevent premature ejaculation. In dentistry, it is often applied to cavities, used during restorative procedures, and rubbed on the gums to numb them before dentures are inserted (Lyu and Ishida, 2019).

Eugenol also is commonly used as an additive in a particular kind of cigarette, called clove cigarettes. It is also used to attract insects, such as bees, for research purposes (Lyu and Ishida, 2019). Some people value the compound because it absorbs ultraviolet rays as well (Kannan and Vincent, 2012).

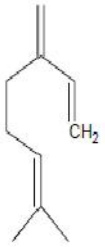
2.1.6 Terpenoids

Terpenoids (also called “isoprenoids”) constitute one of the largest families of natural products accounting for more than 40,000 individual compounds of both primary and secondary metabolisms (Yadav *et al.*, 2014).

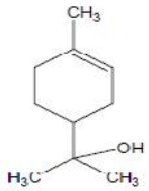
Terpenoids are the hydrocarbons of plant origin of the general formula $(C_5H_8)n$ as well as their oxygenated, hydrogenated and dehydrogenated derivatives.” Thermal

decomposition of terpenoids give isoprene as one of the product. Otto Wallach pointed out that terpenoids can be built up of isoprene unit (Clinton, 2009).

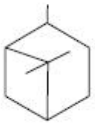
Examples of terpenoids is shown in figure 6.



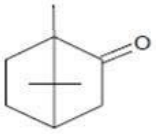
Myrcene



a-Terpineol



Pinane



camphor

Figure 2.6: Terpenoids (Yadav *et al.*, 2014).

Plant terpenoids are widely utilized for their aromatic properties and have long played a role in traditional herbal medicine. In recent years, they have attracted scientific interest due to their potential antibacterial, anti-neoplastic, and other pharmacological activities. While plants synthesize and accumulate terpenes primarily as a defense mechanism against herbivores, they also emit complex volatile blends in response to both biotic and abiotic stresses. These volatile terpenes serve ecological functions, such as attracting the natural predators or parasitoids of herbivorous pests. However, because of the chemical complexity of these blends, assigning specific biological roles to individual terpene compounds remains a challenge. Nonetheless, the significance of terpenes in both ecological interactions and human applications is substantial (Yadav *et al*., 2014).

In terms of extraction, less polar hydrocarbon terpenoids, such as hydrotetraterpenoids, are typically extracted using non-polar solvents like petroleum ether. In contrast, more polar compounds such as xanthophylls are more effectively extracted using polar solvents such as ethanol, or solvent mixtures combining ethanol with moderately non-polar solvents like chloroform (Eriamiatoe *et al*., 2020).

2.1.7 Steroids

Plant steroids constitute a diverse group of natural products and among the plant steroids, phytosterols are ubiquitous in the plant kingdom. It is significant that some phytosterols have been reported to possess hypocholesterolemic activity (Ezeabara, and Egenti, 2018).

Steroidal alkaloids are nitrogen-containing plant steroids with an array of biological activities such as for replacement therapy (male and female), athletes (glucocorticoids), skin conditions (hydrocortisone), antifertility pill (oestrogens and progesterones), cancer (breast, testes, prostrate) and rheumatoid arthritis (Han *et al.,* 2016).

The analysis of plant-derived steroids primarily relies on advanced chromatographic techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), and gas-liquid chromatography (GLC). These methods are often coupled with various detection systems, including photodiode array (PDA), evaporative light scattering (ELS), fluorescence (FL) detectors, and mass spectrometry (MS), which enhance the accuracy of detection, quantification, and structural identification of steroidal compounds. In addition

to these widely adopted approaches, less conventional techniques such as immunoassays and biological assays are also employed for the analysis of plant steroids, particularly in bioactivity-guided studies (James *et al*., 2012).

Most glycosides can be extracted with polar solvents such as acetone, ethanol, methanol, water or mixtures of these. Since Glycosides are relatively polar in nature and its polarity depends on both number and type of sugar. Cardiac glycosides have bulky steroidal aglycone which shows an appreciable solubility in chloroform.

In some cases it may be aglycone rather than the glycoside is to be extracted so then it requires hydrolytic separation of aglycone and sugar before or after extraction. (Eriamiatoe *et al*., 2020)**.**

To test for O-glycosides, plant samples are boiled with a mixture of hydrochloric acid (HCl) and water to hydrolyze the anthraquinone glycosides into their corresponding aglycones. After hydrolysis, an aqueous base such as sodium hydroxide (NaOH) or ammonium hydroxide (NH_4OH) is added. The development of a pink or violet coloration in the base layer indicates the presence of O-glycosides (Saha *et at.,* 2020).

For the detection of C-glycosides, plant samples are hydrolyzed using a mixture of ferric chloride ($FeCl_3$) and hydrochloric acid (HCl). Similar to the O-glycoside test, the addition of an aqueous base (NaOH or NH_4OH) leads to the appearance of a pink or violet color in the base layer, confirming the presence of C-glycosides (James *et al*., 2012).

2.1.8 Phenols

Broadly, phenolics are distributed in plant kingdom and are the most aabundant secondary metabolites of plants. Phenol are sometimes called Phenols and are the compounds containing a hydroxyl group (—OH) directly attached to an aromatic ring. The term phenol is commonly used in the context of hydroxybenzene, which is a liquid at room temperature when contaminated with a little water (Ruangsang *et al.,* 2010).

These phenolics are over 8,000 structural variants and generally are categorized as phenolic acids and analogs, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others based on the number of phenolic rings and of the structural elements that link these rings (Fresco *et al*., 2008).

Phenolic compounds provide essential functions in the reproduction and growth of plants; act as defense mechanisms production and growth of plants; act as defense mechanisms against pathogens, parasites, and predators; as well as contribute to the color of plants (Ruangsang *et al.,* 2010).

In addition, phenolics abundant vegetables and fruits are reported to play an important role as chemopreventive agents; for example, the phenolic components of apples have been linked with inhibition of colon cancer *in vitro* (Veeriah, 2008). Many phenolic compounds have been reported to possess potent antioxidant activity and to have anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory activities to a greater or lesser extent (Ruangsang *et al.,* 2010)..

Their physiological and pharmacological functions may originate from their antioxidant and free radical scavenging properties and function of regulating detoxifying enzymes (Surh, 2003). Further, these antioxidant activities are related to the structures of phenolic compounds, generally depending on the number and positions of hydroxyl groups and glycosylation or other substituents (Cai *et al.*, 2006).

These compounds can exits as free phenols or glycosidic form. Phenol are said to be relatively polar because of the multiplicity of the hydroxyl functions, they are weak acid and therefore can be extracted or partitioned into aqueous alkali as phenolate salts (Sartor *et al.*, 2013).

2.2 Description of *Sida acuta*

Sida acuta belongs to a family: Malvaceae, order: Malvales genus: Sida and species: *Sida acuta* in the Plantae kingdom. It is a tropical weed that belongs to crops, meadows, roadsides, and wastelands. Globally, *Sida acuta* is dispensed in pantropical areas (Karou, 2007). Locally, it is called “isekotu” in Yoruba, “Udu” in Igbo and “Ugbasan” in Bini (Murray, 2021). It has a woody taproot, hairy, upright, branched perennial shrub up to 1m high and is reproduced from its seeds. When the plant is young, the stem appears to be rounded, slender, woody, fibrous, and hairy. It has simple alternating leaves with stems that are 1.3 cm long, about half of which are articulated. Mallow plants are noted for their principal economic purposes such as a source of natural textiles, food, beverages, wood, traditional medicine, and gardening. Studies undertaken at indigenous locations have indicated that the plant has several traditional uses that vary from place to region

(Benjumea *et al*., 2016). The medicinal plant *Sida acuta*, also referred to as wireweed, has a number of possible advantages. Because it contains bioactive substances such as alkaloids, flavonoids, tannins, saponins, and glycosides, it is frequently utilized in traditional medicine.

Plate 1: *Sida acuta* plant (Amanpreet and Rattan, 2022).

2.2.1 SOME PHYTOCHEMICAL CONSTITUENTS OF *Sida acuta*

Alkaloids

Alkaloids occur in the plants belonging to the indoloquinolines family. Cryptolepine and its derivatives include quindoline, quindolinone, cryptolepinone, and 11-methoxy-quindoline are the major alkaloids. Among these compounds, Cryptolepine-5-methylindolo-(2-3b)-quinoline has been used widely for studies because of its clinical properties against malaria, colic, and stomach ulcers (Karou *et al*., 2005). Cryptolepine-5-

methylindolo-(2-3b)-quinoline has also been known for its cytotoxic activity which ultimately gives rise to an idea for production of anticancerous drugs (Nalini *et al.,* 2021).

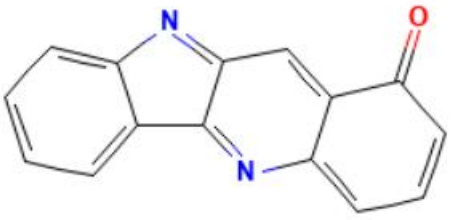
Steroids

Ecdysterone, beta-sistosterol, stigmasterol, and ampesterol are the plant's major steroids (Amanpreet and Rattan, 2022).

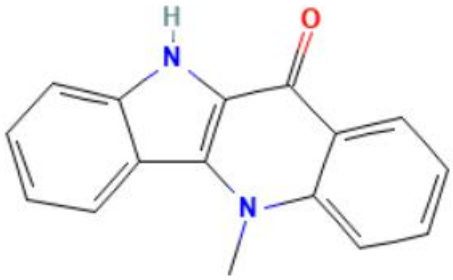
Phenols

Phenolic compounds such as evofolin-A, and B, scopoletin vomifoliol, Loliolide, and 4-ketopinoresinol have already been isolated and studied (Tcheghebe *et al*., 2017).

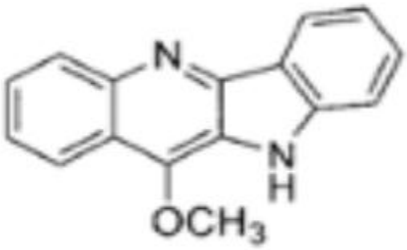
SOME BIOACTIVE CHEMICAL STRUCTURES
Sida acuta **(**Jang *et al.,* 2015**).**



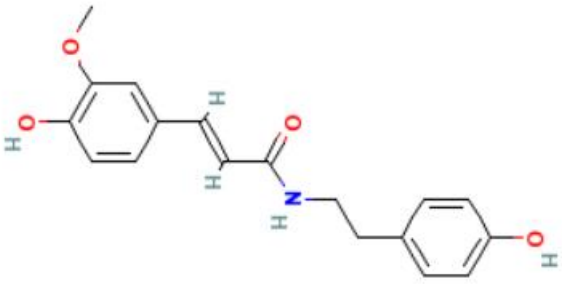
Quindolinone



Cryptolepinone

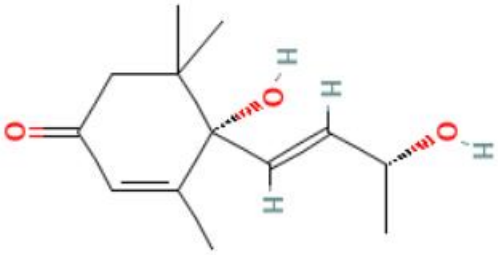


11 - methoxyquindoline

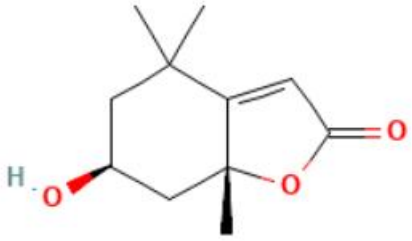


N - trans - feruloyltyramine

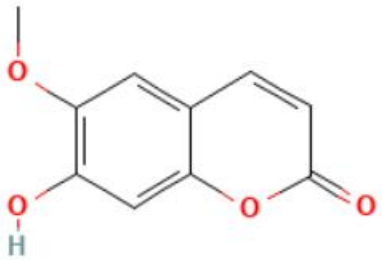
Figure 2.6: Chemical structure of some Alkaloids



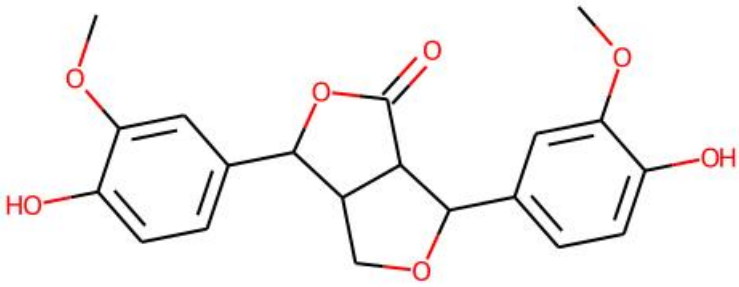
Vomifoliol



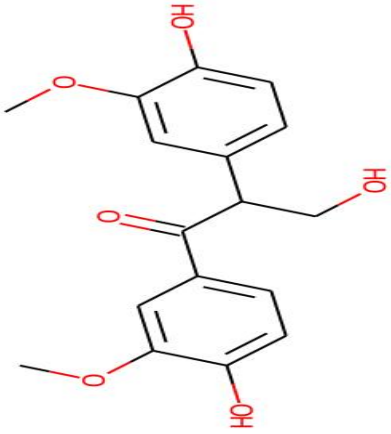
Loliolide



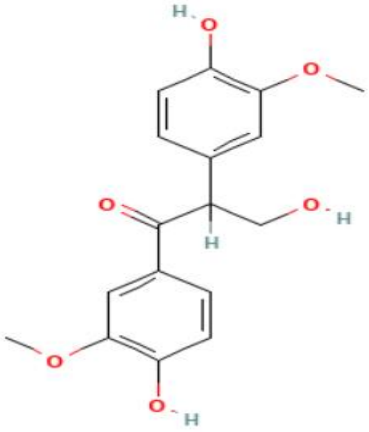
Scopoletin



4-ketopinoresinol



Evofolin-A



Evofolin-B

Figure 2.7: Chemical structure of some Phenolic compounds **(**Jang *et al.,* 2015**).**

2.2.2 MEDICINAL BENEFITS OF *Sida acuta* TO MAN

1. Birth Control

The leaves are used to induce abortions or as a birth control method in Africa and some regions of Asia. From day 1 to day 7 of pregnancy, female albino rats were used to test plant extracts for abortifacient effects at a safe dosage of up to 100 mg/kg body weight (Sharangouda *et al*., 2009). The ethanolic extract was the most successful in causing miscarriage or abortion, according to the researchers. However, when tested on female albino rats that were young and had had their ovaries removed, the petroleum ether extract showed the highest estrogenic activity. Women may potentially have temporary infertility as a result of *Sida acuta*. Because the sterility may be reversed by stopping the herb, some people utilize it as a natural birth control method (Sharangouda *et al*., 2009).

2. **Anti-impotence**

The aphrodisiac properties of *Sida acuta* are well recognized in several African and Asian nations. A decoction made from its leaves and roots is utilized as a natural remedy for erectile dysfunction. Sexual issues are prevalent among men over the age of 40, with the likelihood of occurrence increasing as one grows older. A review from 2018 indicated that approximately one-third of men face some degree of impotence. If you belong to this group, and Viagra has proven ineffective, or if you prefer a natural alternative, you might want to try *Sida acuta* extract or tea (Daniel and Lydia, 2024).

Additionally, when boiled in sesame oil, it can be applied to inflamed testicles and elephantiasis to help reduce inflammation. Extracts from this plant are also employed to combat bacterial and protozoal infections. (Daniel and Lydia, 2024).

3. **Anti-Fungi Activities**

The flavonoid extract of *Sida acuta* has exhibited antifungal properties, as evidenced by its inhibition zone and activity index, with eight different plant extracts tested against *Candida albicans*; seven of these showed significant antifungal activity. Notably, the free flavonoids demonstrated greater potency against Candida compared to the bound flavonoids (Alka, 2012). *Sida acuta* has effectively shown its ability to inhibit *Aspergillus fumigatus*, producing notable partially clear zones; however, some sporadic fungal growth was still evident (Hoffman *et al*., 2004). In Africa, it is common for individuals to mix the powdered leaves with coconut oil and regularly apply the resulting paste to their hair to eliminate dandruff and strengthen it (Pooja *et al*., 2015).

4. **Anti-inflammatory**

In a study carried out in 2005 involving mice*, Sida acuta* was found to have anti-inflammatory effects. The animals were administered extracts of the plant for several weeks. At the conclusion of the study, the isolates significantly diminished the inflammatory response in the mice without causing any adverse effects (Konate and Souza, 2010). The powdered aerial parts of *Sida acuta* displayed notable anti-inflammatory activity. (Tcheghebe *et al*., 2017).

5. Analgesic

Sida acuta is utilized as a pain reliever in various regions around the globe. In 2012, a research team examined the pain-relieving properties of the plant's leaves by utilizing petroleum ether, acetone, and distilled water (Konate *et al.,* 2012). The acetone extract of the leaves exhibited the highest analgesic activity compared to the other extracts. The pain relief effects of *Sida acuta* were assessed using the tail immersion and hot plate techniques at three different doses in mice. The highest dosage of 500mg/ml demonstrated significant pain management capabilities. (Tcheghebe *et al*., 2017).

6. Antiulcer

Cancer is often referred to as a *"silent killer"* and remains one of the leading causes of mortality worldwide. Among various types, breast cancer has emerged as the most prevalent form of cancer affecting women. Globally, it accounts for approximately 2.3 million new cases and 685,000 deaths, making it a significant public health concern (Amanpreet and Rattan, 2022).

The report: Race, Ethnicity and Breast Cancer by Susan G. Komen revealed that a high rate of breast cancer can be seen among white and black women as compared to American, Indian, and Alaska Native women. (Uysal *et. al*., 2021).

Chemical agents used for the treatment of cancer cells may cause harm to normal cells of the body (Uysal *et. al*., 2021). So, due to this concern, the role of *Sida acuta* extracts was studied against MDA-MB-231(cancer cells) (Amanpreet and Rattan, 2022). After 48

hours of treatment, it was found that *Sida acuta's* methanolic extract played a vital role in defeating cancer cells with an IC_{50} value of 102.4µg/ ml (Uysal *et. al*., 2021).

Involved Constituents: According to the study, the elements of *Sida acuta* (methanolic extract) that have potential against cancer cells are 3, 4, 5- tricaffeoylquinic acid, O-deohyhexosyl-hexosylluteolin, N-coumaroyl tyramine (+) ESI, and vomifoliol (-) ESI (Akilandeswari *et al*., 2010). The operative component that has been observed is Vomifoliol which was cultured in Hepa lclc7 cells of mice (Uysal *et. al*., 2021).

Another study was conducted demonstrated that the inhibitory effect of *Sida acuta* (methanolic extract) became notable after 48 hours of incubation i.e. 51.62% against HepG-2 cells (liver cancer cell lines in humans) (Pieme *et al*.,2010).

The ethanol extract from the leaves of *Sida acuta* demonstrated a considerable level of antiulcer activity in various internal organs. Another study conducted in 2010 further validated the antiulcer properties of the entire plant's ethanol extract. Researchers concentrated on the plant's effectiveness against the gastrointestinal side effects of aspirin and the occurrence of gastric ulcers, utilizing HCl-ethanol induced ulcers and water immersion stress-induced ulcers in rats. The study revealed that the ethanol extract significantly reduced the incidence of ulcers in the subjects treated with *Sida acuta* extract (Akilandeswari *et al*., 2010). The antiulcer activity of ethanol extract of whole plant of *Sida acuta* was also supported by (Malairajan *et al.,* 2006).

7. **Anti-diabetic**.

The hypoglycemic activity of *Sida acuta* was evaluated through experimental studies involving both normal and diabetic rabbits. The effects of the plant's isolates on blood glucose levels were assessed in glucose-overloaded normal rabbits as well as in alloxan-induced diabetic rabbits (Sharma *et al*., 2012). The results indicated that the extracts were effective in both models: they significantly reduced blood glucose levels in diabetic rabbits and enhanced glucose tolerance in normal rabbits subjected to glucose loading. However, the glucose-lowering effect of the extract was comparable, but not superior, to that of glibenclamide, a standard antidiabetic drug used in the treatment of type II diabetes mellitus (Tcheghebe *et al*., 2017).

8. **Antipyretic properties**

In 2012, scientist Sharma carried out a modest study to assess the health advantages of *Sida Acuta*, namely its antipyretic properties. A variety of solvents, including ether, acetone, ethanol, and water, were utilized to produce the extracts. All of the extracts were found to reduce participants' body temperatures, however the acetone extract exhibited superior antipyretic qualities. In comparison to other extracts, the ethanol extract was found to have faster and more potent antipyretic action; its effects were noticeable in less than two hours. (Sharma *et al*., 2012).

9. **Anti-liver Damage**

Ferulic acid, a plant-based antioxidant frequently found in anti-aging treatments, is found in *Sida acuta*. Additionally, it is utilized as a natural remedy for diabetes, hypertension, and a few other illnesses. To ascertain whether *Sida acuta* can indeed stop liver damage, its positive effects on the liver were examined. Two rat groups were used: mice with liver damage from an overdose of paracetamol and rats in good health. Through examination of alterations in the liver organ's tissues, the liver protective effect was further validated in rats fed *Sida acuta*. (Sredeve *et al.,* 2009).

10. Wound Healing

In two different rat wound models excision and incision—scientists applied tropical methanol extract of *Sida acuta* ointment. The *Sida acuta* ointment cured the wound in the excision group more quickly than the control group, and it also caused a higher rate of wound contraction. The researchers came to the conclusion that the ointment's ability to heal wounds was noticeably better in both evaluated wound types than in the control group. (Akilandeswari et al., 2010)

The effects of *Sida acuta* (ethanolic extract) on wounds was also studied using *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa*, and *Bacillus subtilis* as test models (Adetutu *et al*., 2011). Their study demonstrated that ethanolic extract of *Sida acuta* (leaves) displays low wound healing activity against gram-positive species (Amanpreet and Rattan, 2022). It has also been stated that the wound healing potential of plants like *Sida acuta* can also be achieved by anti-inflammatory, antioxidant, or

antibacterial mode of action (Adetutu *et al*., 2011) In addition to ointment, you can clean wounds with a leaf decoction to hasten healing (Adetutu *et al*., 2011).

11. **Antioxidant**

Health depends on antioxidants. They stop free radicals from damaging cells, which may be a crucial factor in the emergence of numerous illnesses like cancer and heart disease. *Sida acuta* can do all of this and more in a single serving. While you cannot stop your body from being exposed to free radicals, you can counteract their effects. Six kinds of mallows were discovered to have the plant's antioxidant activity, with *Sida acuta* showing notable antioxidant qualities. Using an MTI assay, the researchers also find that the extract had cytotoxic action on human and murine cancer cells. (Uysal *et al.,* 2021).

12. **Anti-cancer**

Alkaloids, tannins, flavonoids, saponins, terpenes, and phenolics are among the medicinal substances found in *Sida acuta*. They shield the body from several forms of cancer. An analysis of *Sida acuta* in vitro cytotoxicity and antioxidant properties was carried out in 2010. HepG-2 cells were used to test *Sida acuta's* possible antiproliferation of cancer cells and antioxidant properties against cellular damage *in vitro*. Five Cameroonian medicinal plants *Sida acuta*, *Urena lobata, Viscum album*, *Sida cordifolia* and *Sida rhombilifolia* were isolated and used (Konate and Souza, 2010).

According to the findings, S*ida acuta* extracts exhibited strong anti-proliferative properties among the five. It had a concentration-dependent effect on cancer cell viability

and proliferation. The antioxidant property data revealed a significant rise in Glutathione S-Tranferase, Superoxide Dismutase, and Catalase (Pieme *et al*., 2010).

13. **Anti-Cardiovascular**

For the majority of racial and ethnic groups in the US, heart disease is the leading cause of mortality. Every year, it kills over 655,000 Americans, or one out of every four people. Approximately one person worldwide passes away from cardiovascular disease every 36 seconds. The World Health Organization estimates that 17.9 million people died from cardiovascular causes in 2016, with heart attacks accounting for 85% of these deaths (Kannan and Vincent, 2012). This represents 31% of all fatalities worldwide. With *Sida acuta*, you can strengthen your heart. The plant may support healthy heart functions and assist control heartbeat, according to the few research that have been done on it. (Kannan and Vincent, 2012).

14. Anti-Headache and Respiratory Illnesses

Alkaloids, tannins, flavonoids, saponins, terpenes, and phenolics are among the phytochemicals found in *Sida acuta* leaves, as previously mentioned. These substances have analgesic, diuretic, antispasmodic, and anti-inflammatory properties. Alkaloids can prevent or lessen headaches brought on by high blood pressure. They are also utilized in complementary therapies for colds, fevers, and persistent diarrhea. (Ogunlemoyole *et al*., 2022).

15. **Anti-degenerative Diseases**

Many other medical illnesses, including many degenerative diseases, are covered by *Sida acuta* benefits. For example, saponin is used for the treatment or management of high

blood sugar, high cholesterol, asthma symptoms, and other conditions in addition to cancer. Terpenes are powerful substances that have been utilized to cure a variety of illnesses. Among their many other properties are anti-inflammatory, antioxidant, antiviral, antidiabetic, and anticancer (Karou *et al*., 2007).

16. **Anti-venom**

It has been discovered that *Sida acuta* extract neutralizes snake venom. *Bothrops atrox* venom's hemorrhagic effect can be moderately neutralized by an ethanol extract of the entire plant. (Karou *et al*., 2007).

17. **Enzyme Inhibitory Activity**

A study conducted showed that water and methanolic extracts of *Sida acuta* have the potential to harmonize the action of enzymes that are objected to cope with mental deterioration i.e. Alzheimer's disease (acetylcholineste rase and butyrylcholinesterase), hyperpigmentation of skin (tyrosinase) and diabetes type II i.e. adult-onset diabetes (α-amylase and α- glucosidase) (Uysal *et al.,* 2021).

18. **Anti-Mycobacterial Activity**

Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis, an acid-fast bacillus known for its resistance to acidic environments. According to Papitha *et al*. (2013), the Luciferase Reporter Phage (LRP) assay serves as a rapid, cost-effective, and simple method for assessing the antimycobacterial activity of test compounds. In this assay, a compound is considered to exhibit antitubercular activity only if it results in a

reduction of at least 50% in relative light units (RLU), indicating significant inhibition of mycobacterial viability.

The hydroalcoholic extract of *Sida acuta* demonstrated 99% antimycobacterial activity, outperforming the standard antibiotics isoniazid and rifampicin, which are commonly used in combination to treat tuberculosis (Amanpreet and Rattan, 2022).

19. Antimicrobial Activities

This study investigated the quantitative chemical constituents of plant origin and *in vitro* antimicrobial activities of *Sida acuta* at varying concentrations using standard analytical procedures. Antimicrobial activity was analyzed using the disc diffusion method, while Minimum Inhibitory Concentrations (MICs) of the crude isolates were determined via the agar well diffusion technique. Statistical analysis was performed using Duncan's Multiple Range Test to assess significance (Ezeabara and Egenti, 2018).

Phytochemical screening confirmed the presence of key secondary metabolites—alkaloids, tannins, saponins, flavonoids, cardiac glycosides, anthraquinones, terpenoids, and steroids—across the stem, leaf, and root samples, though in varying concentrations. Notably, the leaf contained the highest concentration of alkaloids (2.31 ± 0.03 mg/100 g). All plant parts demonstrated dose-dependent antimicrobial activity against the test microorganisms. At 500 mg/mL, the leaf extract showed the most potent inhibition against *Pseudomonas aeruginosa, Micrococcus varians*, and *Candida albicans*, while the root isolate was most effective against *Salmonella typhi*, *Escherichia coli* and *Aspergillus flavus* (Ezeabara and Egenti, 2018).

These findings indicate that all parts of S. acuta, particularly the leaf and root, possess significant antimicrobial properties, highlighting their potential for medicinal applications (Ezeabara and Egenti, 2018).

2.2.3 SIDE EFFECTS OF *Sida acuta*

1. Toxicity Effect

Data indicate that for acute toxicity *Sida acuta* can be weakly poisonous with LD_{50} higher than 3.2g/kg body weight by oral route is consider safe or practically non-toxic for an acetone extract. (Konate and Souza, 2010). Concurring the subchronic toxicity study also show same, no significant difference however, the presence of tannins which is usually high in *Sida acuta* extract and the lack of food often lead to the depression in growth of test subject (Konate *et al*., 2012).

2. Sedative and hynotic effects

Neuropharmacological reseearch on the effect of ethanolic isolate of *Sida acuta* is indicative of sedative and hypnotic properties. The extract reduced dormancy time to sleep and increased the duration of sleep induced by pentobarbital indicating depressant effect on the central nevous system of the test subjects. (Benjumea *et al.,* 2016).

3. Laxative Activities

A research into the laxative effect of an aqueous leaf extract of *Sida acuta* in constipated rats induced with loperamide demonstrated that the extract normalized fecal output and water content in a dose dependent manner, indicative of its laxative properties, which

when consume in excess could gastrointestinal disorder such as diarrhea (Nweje *et al*., 2019).

4. Heamatological effects

A study evaluating the effects *Sida acuta* ethanolic leaf isolates on certain hematological parameters in rats found significant changes in parameters such as hemoglobin concentration and white blood cell count. These alterations indicate that the extract may influence blood physiology, though the clinical significance of these findings requires further investigation. (Oduwegwu *et al*., 2017).

2.2.4 Methods of Extraction and Isolation of Therapeutic Plants

In pharmaceutical practice, extraction refers to the process of isolating the bioactive constituents of plant or animal tissues from their inactive or inert components using selective solvents through standardized procedures (Cannell, 2008). This process gives rise to various forms of preparations, including decoctions, infusions, fluid extracts, tinctures, semisolid (pilular) isolates, and powdered isolates (Cannell, 2008). The extraction process generally involves three fundamental stages of handling plant material, which include:

(i) Initial Extraction Phase

This phase involves the penetration of the solvent into plant cells or tissues, leading to the solubilization and release of secondary metabolites into the extraction medium. Solvents of varying polarity are employed either individually or in combination, based on the

target phytochemicals. This stage also facilitates the removal of a significant portion of unwanted materials. Common techniques at this stage include maceration, digestion, decoction, Soxhlet extraction, supercritical fluid extraction (SFE), hydrodistillation, enfleurage, and expression (écuelle method) (Cannell, 2008; Patil and Shettigar, 2010).

(ii) Fractionation and Preliminary Purification

The second stage focuses on fractionating the crude extract to isolate components based on their physicochemical properties. This may be achieved using open-column chromatography (e.g., silica gel columns) or liquid-liquid partitioning techniques, such as counter-current distribution. Other methods used for preliminary purification include distillation, sublimation, evaporation, fractional crystallization, and fractional distillation (Cannell, 2008; Swami *et al*., 2008).

(iii) Final Purification and Characterization

This stage involves the isolation of desired compounds in a sufficiently pure form using advanced chromatographic and spectroscopic methods. These include High-Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), Gas Chromatography (GC), Gas-Liquid Chromatography (GLC), and hyphenated techniques such as GC-MS, HPLC-MS, LC-NMR, as well as UV spectroscopy and fluorimetry (Oleszek and Marston, 2000; Daffre *et al*., 2008; Philipson, 2007; Cannell, 2008).

1. **Maceration**

In this process, coarsely powdered crude drug is soaked in a solvent at room temperature for at least three days with frequent agitation. After solubilization of the active constituents, the mixture is strained, the marc is pressed, and the combined liquids are filtered or decanted (Cannell, 2008).

2. Infusion

Infusions are prepared by steeping plant material in cold or boiling water for a short time. They result in dilute extracts of easily soluble components and are commonly used in traditional herbal medicine (Patil and Shettigar, 2010).

3. Digestion

This is a warm maceration process where the solvent is heated gently during extraction. It enhances the solvent's efficiency and is used when elevated temperatures do not degrade the phytochemicals. The extract is usually reduced in volume by boiling and then filtered (Cannell, 2008).

4. Fractionation

All separation techniques rely on the partitioning of a mixture into distinct fractions. When a sample is successively treated with solvents such as dimethyl ether, chloroform, and ethyl acetate, specific flavonoid subclasses—such as flavanones, flavonols, methoxylated flavonoids, and flavonoid monoglycosides—are effectively extracted (Cannell, 2008). Meanwhile, more polar compounds like di- and polyglycosylated

flavonoids typically remain in the aqueous residue due to their limited solubility in non-polar organic solvents (Cannell, 2008). The crude saponins compounds may be obtained since they are water soluble compounds (Patil and Shettigar, 2010). The alkaloids are basic compounds that may be extracted either into aqueous acid solution after removal of neutral impurities with an organic solvent, or by treating the groups wet plant material with an alkaline substance such as $CaCO_3$ powder and extracting with diethyl ether after standing overnight. (Yalavarthi and Thiruvengadarajan, 2013).

5. Sublimation

Sublimation is used to obtain the substances from dried plant material or a dry crude isolate (Patil and Shettigar, 2010). Caffeine of high purity can be isolated from dry tea leaves (Nweje *et al* 2019).

6. Percolation

This method is one of the most commonly employed techniques for extracting active constituents in the preparation of tinctures and fluid extracts (Patil and Shettigar, 2010). It involves the use of a percolator—a conical, narrow vessel open at both ends. Initially, the powdered plant material is moistened with a suitable quantity of menstruum and left to stand in a well-sealed container for approximately four hours to allow thorough saturation. Afterward, the moistened mass is packed into the percolator and sealed at the top (Cannell, 2008). More menstruum is added to form a shallow layer above the packed material, and the mixture is macerated in the sealed vessel for 24 hours. Following this,

the percolator outlet is opened, and the liquid is allowed to flow slowly under gravity (Cannell, 2008). Additional menstruum is added incrementally until the percolate reaches approximately three-quarters of the final required volume (Patil and Shettigar, 2010). The remaining marc is then pressed, and the expressed liquid is combined with the initial percolate. The final volume is adjusted by adding more menstruum if necessary, and the mixture is clarified either by filtration or by allowing it to stand before decantation (Cannell, 2008; Patil and Shettigar, 2010).

7. Solvent Extraction

Different solvents are commonly employed to isolate specific phytochemical compositions from plant materials (Cannell, 2008). Freshly harvested plant parts are either dried under controlled low-temperature conditions (50–60°C) using artificial means or, preferably, air-dried in the shade to reduce moisture content, thereby enhancing shelf life (Patil and Shettigar, 2010). Once adequately dried, the plant material—such as berries—is ground into a fine powder using mechanical grinders, and oil content is extracted using appropriate organic solvents (Cannell, 2008). The resulting defatted plant material is then subjected to further extraction using a Soxhlet apparatus or by maceration in water or 95% ethanol (Patil and Shettigar, 2010). The obtained alcoholic extract is filtered and concentrated under reduced pressure (in vacuo) or by evaporation. To hydrolyze certain compounds, the concentrate is treated with 12N hydrochloric acid and refluxed for a minimum of six hours (Patil and Shettigar, 2010). This processed extract is

then concentrated again and used for the detection and analysis of various phytoconstituents (Yalavarthi and Thiruvengadarajan, 2013).

Saponins are generally characterized by their high molecular weight, which often presents challenges in obtaining them in a highly purified form (Patil and Shettigar, 2010). According to Sharma *et al*. (2012), plant materials such as tubers, roots, stems, and leaves are thoroughly washed, sliced, and then extracted with either hot water or 95% ethanol over several hours. The resulting extract is subsequently filtered and concentrated under reduced pressure (Sharma *et al.*. 2012). The target saponin compounds are then precipitated by the addition of ether (Cannell, 2008).

To maximize the extraction of bioactive compounds with potent biological activity, exhaustive extraction (EE) is often employed. This technique involves the sequential use of solvents with increasing polarity, allowing for the efficient recovery of a wide range of phytoconstituents (Sharma *et al*. 2012).

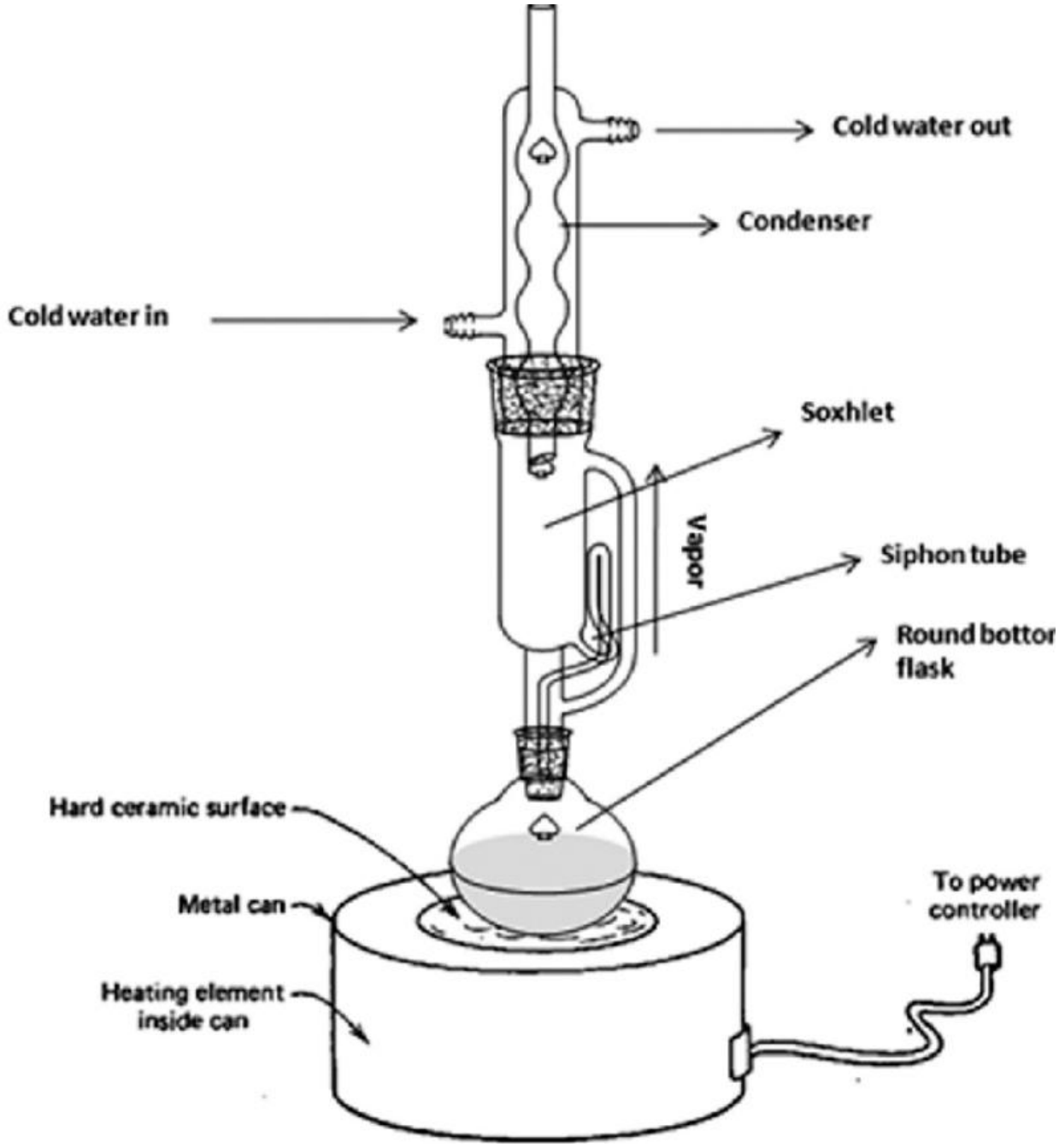


Figure 2.8: Soxhlet extraction (Dasari and Goud, 2014).

8 Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction (SFE) is regarded as one of the most advanced and efficient extraction techniques available today (Patil and Shettigar, 2010). It serves as a modern alternative to traditional extraction methods, aiming to minimize the use of organic solvents while enhancing extraction efficiency and sample throughput (Sharma *et al.,* 2012 ; Patil and Shettigar, 2010).

In SFE, gases—most commonly carbon dioxide (CO_2)—are subjected to conditions above their critical temperature and pressure, transforming them into a supercritical fluid with both gas-like diffusivity and liquid-like solvating properties (Cannell, 2008). This supercritical fluid is then passed through a chamber containing the plant material, dissolving the desired components (Cannell, 2008). The extract-laden fluid is subsequently transferred into a separation vessel, where the extract is isolated, and the CO_2 is recovered and recycled for reuse (Cannell, 2008).

Carbon dioxide is particularly advantageous for extracting essential oils because it is fully miscible with oil constituents such as aldehydes, ketones, esters, and alcohols, while being insoluble in non-target substances like proteins, starches, minerals, and water (Yalavarthi and Thiruvengadarajan, 2013). The essential oils obtained via supercritical CO_2 extraction are often superior in quality compared to those obtained through steam distillation or conventional solvent extraction (Patil and Shettigar, 2010).

One of the key benefits of SFE is the ability to fine-tune the solvent properties of CO_2 by adjusting pressure and temperature, allowing selective extraction of specific compounds. Additionally, SFE leaves behind virtually no solvent residues, as CO_2 evaporates completely (Cannell, 2008). However, the high cost of equipment and operation remains a significant drawback (Cannell, 2008). Despite this, other gases and liquids have also been explored as supercritical solvents due to their efficiency under pressurized conditions (Patil and Shettigar, 2010).

9. Solid Phase Extraction

This process involves the separation of dissolved and suspended component from liquid mixture by using another component in the mixture according to the physical and chemical property (Eriamiatoe *et al*., 2020)

2.2.5 Distillation

Distillation may be defined as separation of components of a mixture of two or more liquids by virtue of difference in their vapour pressure (Cannell, 2008). There are three system of distillation, hydro distillation, hydro-steam distillation and steam distillation (Cannell, 2008).

2.2.5.1 Hydrodistillation

Hydrodistillation is one of the oldest and most traditional techniques employed for the extraction of essential oils from plant materials (Eriamiatoe *et al*., 2020). In this method, the plant material is immersed in water and boiled within a basic metallic distillation

apparatus (Cannell, 2008). The process relies on the principle of osmotic pressure to facilitate the diffusion of essential oils from the oil glands into the surrounding medium (Cannell, 2008).

As the mixture boils, volatile components—including essential oils—are carried upward with the steam and directed through a coiled condenser immersed in a water bath (Eriamiatoe *et al*., 2020). The vapors condense upon cooling, and the resulting distillate is collected at the base of the condenser, where the oil and water separate (Eriamiatoe *et al*., 2020). Despite its simplicity and historical use, hydrodistillation has several limitations. Controlling the temperature is often challenging, and the efficiency of distillation can vary due to the complex composition of essential oils, many of which contain compounds with boiling points ranging from 150°C to 300°C (Cannell, 2008). Moreover, there is a risk of localized overheating and charring of the plant material, which can degrade sensitive constituents and negatively affect the quality of the extracted oil (Patil and Shettigar, 2010).

2.2.5.2 Hydro-Steam Distillation

This technique is a modified version of hydrodistillation. In steam distillation, the plant material is placed on a perforated grid or mesh platform situated above the base of the distillation vessel (Cannell, 2008). Water is filled below the grid and heated to generate saturated steam. As the steam rises, it passes through the plant material, causing the volatile essential oils to vaporize (Patil and Shettigar, 2010). These vapors—consisting of

steam and essential oil—are then directed into a condenser, where they cool and separate into oil and water layers (Cannell, 2008).

2.2.5.3 Steam Distillation

Steam distillation is a widely used technique for extracting essential oils from plant materials through the application of heat and vaporization (Eriamiatoe *et al*., 2020). This method involves exposing the plant material to steam, which facilitates the release of volatile oils (Cannell, 2008). One common approach is to mix the plant material with water and heat the mixture to boiling (Cannell, 2008). The resulting vapors—comprising both water and essential oils—are then collected and condensed, allowing the oil to separate from the aqueous layer (Patil and Shettigar, 2010). Although similar to hydro-steam distillation, steam distillation differs in that no water is retained at the bottom of the still; instead, only dry steam is introduced (Sharma *et al.,* 2012). This technique is known for its high efficiency and improved yield of essential oils (Eriamiatoe *et al*., 2020). It is also considered faster and more energy-efficient, making the charging and emptying of the distillation unit easier (Cannell, 2008). Furthermore, the shorter distillation time reduces the risk of thermal degradation of heat-sensitive components such as esters (Sharma *et al*., 2012). However, this method is not suitable for essential oils containing hydrolyzable compounds, as they may break down under these conditions (Patil and Shettigar, 2010).

2.2.5.4 Counter-Current Extraction (CCE)

Counter-current extraction (CCE) is a highly efficient technique that involves the movement of wet, pulverized plant material in one direction while it is simultaneously exposed to a solvent flowing in the opposite direction (Patil and Shettigar, 2010). The raw material is initially processed into a fine slurry using toothed disc disintegrators and introduced into a cylindrical extractor (Cannell, 2008). As the material advances through the extractor, it comes into contact with fresh solvent, promoting thorough isolation (Cannell, 2008). The farther the material travels, the more concentrated the isolate becomes (Patil and Shettigar, 2010). Optimal extraction is achieved by adjusting the flow rates and quantities of both the solvent and the plant material (Swami *et al*., 2008). This method is not only time-efficient but also avoids exposure to high temperatures, making it ideal for thermolabile compounds (Eriamiatoe *et al*., 2020). At the end of the process, a concentrated isolate is collected from one end, while the exhausted residue (marc), largely free of solvent, is discharged from the opposite end (Swami *et al*., 2008).

2.2.5.5 Fractional Crystallization

This method is use on the difference in solubility of components of a mixture in a particular solvent. Frequently, derivatives of the particular components are used (picrates of alkaloids, osazones of sugars). (Yadav *et al.,* 2014).

2.2.6 Chromatography

This is a group of techniques for separation of compounds of mixtures by their continuous distribution between two phases, one of which is moving past the other (Patil and Shettigar, 2010).

2.2.6.1 Chromatographic Fingerprinting and Marker Compound Analysis

Chromatographic fingerprinting is a vital technique used for the identification and quality evaluation of herbal medicines (HMs) (Patil and Shettigar, 2010). It involves generating a chromatographic profile that reflects the presence of common chemical constituents with pharmacological or chemical significance (Patil and Shettigar, 2010). These fingerprints are characterized by two essential attributes: "integrity"—the overall chemical representation of the sample—and "fuzziness", referring to minor variations in constituent concentrations between different samples (Cannell, 2008). Such fingerprints allow for reliable authentication and differentiation of herbal medicines, even when individual marker compounds are present in varying quantities (Eriamiatoe *et al*., 2020). Instead of focusing on one or two marker components, this approach emphasizes evaluating multiple constituents simultaneously to capture the complete chemical landscape of the herbal extract (Sharma *et al.*. 2012 ; Patil and Shettigar, 2010). Given that herbal medicines may contain hundreds of compounds—many in trace amounts—and natural variability among samples, it becomes crucial to establish consistent and representative fingerprint profiles (Eriamiatoe *et al*., 2020).

Thin Layer Chromatography (TLC) is widely employed in the phytochemical evaluation of herbal drugs due to its simplicity and effectiveness. TLC offers:

- Rapid analysis with minimal sample preparation,
- Qualitative and semi-quantitative profiling of constituents,
- Basic quantification of resolved compounds (Rao, 2012).

In High-Performance TLC (HPTLC) fingerprinting, detailed data such as chromatograms, retardation factor (Rf) values, band colors, absorption spectra, peak wavelengths, and inflection points are recorded (Sharma *et al.*. 2012). Derivatization with specific reagents further enhances band resolution, enabling better identification (Cannell, 2008). This comprehensive data is used to authenticate herbal drugs, detect adulteration, and maintain product consistency (Sharma *et al.,* 2012 ; Patil and Shettigar, 2010).

High-Performance Liquid Chromatography (HPLC) fingerprinting involves recording chromatograms, retention times, and UV-visible absorption spectra (often via photodiode array detectors) across different mobile phase systems (Cannell, 2008). Likewise, Gas-Liquid Chromatography (GLC) is utilized for fingerprinting volatile and fixed oils in herbal preparations (Eriamiatoe *et al.,* 2020). Advanced hyphenated techniques now offer greater accuracy and structural information (Patil and Shettigar, 2010).

These include:

- HPLC–DAD (Diode Array Detection),

- GC–MS (Gas Chromatography–Mass Spectrometry),
- CE–DAD (Capillary Electrophoresis–DAD),
- HPLC–MS (Liquid Chromatography–Mass Spectrometry), and
- HPLC–NMR (Liquid Chromatography–Nuclear Magnetic Resonance) (Rao, 2012).

These combined methods not only enhance qualitative analysis but also allow real-time structural elucidation of bioactive compounds in complex herbal matrices (Rao, 2012).

2.2.6.2 Liquid chromatography

a. Preparative high performance liquid chromatography

There are basically two types of preparative HPLC. One is low pressure (typically under 5 bar) traditional PLC, based on the use of glass or plastic columns filled with low efficiency packing materials of large particles and large size distribution (Rao, 2012). A more recent form PLC, Preparative High Performance Liquid Chromatography (Prep. HPLC) has been gaining popularity in pharmaceutical industry. In preparative high-performance liquid chromatography (HPLC), where the operating pressure often exceeds 20 bar, larger stainless steel columns packed with coarse particles (typically 10–30 μm) are employed to accommodate higher sample loads and reduce back-pressure (Eriamiatoe *et al.,* 2020). For normal-phase chromatography, commonly used columns include, Kromasil 10 μm, Kromasil 16 μm, Chiralcel AS 20 μm (used for chiral separations). For reverse-phase chromatography, typical examples include, Chromasil C18, Chromasil C8, YMC C18 (Eriamiatoe *et al.,* 2020). These columns are chosen based on the nature of the

analyte, desired separation efficiency, and loading capacity in preparative-scale purification (Rao, 2012). The aim is to isolate or purify compounds, whereas in analytical work the goal is to get information about the sample (Rao, 2012). Preparative HPLC is closer to analytical HPLC than traditional PLC, because its higher column efficiencies and faster solvent velocities permit more difficult separation to be conducted more quickly (Oleszek and Marston, 2000; Philipson, 2007). In analytical HPLC, the important parameters are resolution, sensitivity and fast analysis time whereas in preparative HPLC, both the degree of solute purity as well as the amount of compound that can be produced per unit time i.e. throughput or recovery are important (Rao, 2012). This is very important in pharmaceutical industry of today because new products (Natural, Synthetic) have to be introduced to the market as quickly as possible (Oleszek and Marston, 2000; Philipson, 2007). Having available such a powerful purification technique makes it possible to spend less time on the synthesis conditions (Rao, 2012).

b. Liquid Chromatography- Mass Spectroscopy (LC-MS)

In the pharmaceutical industry, Liquid Chromatography–Mass Spectrometry (LC–MS) has become a preferred analytical technique at various stages of drug development (Patil and Shettigar, 2010). It is valued for its high sensitivity and specificity in detecting and quantifying complex compounds (Patil and Shettigar, 2010). Recent advancements in ionization techniques—such as electrospray ionization (ESI), thermospray, and ion spray—have significantly enhanced the capabilities of LC–MS, allowing the analysis of compounds with high molecular weights, including proteins and peptides (Rao, 2012).

This technique also enables precise molecular weight determination and isotope pattern analysis, which is essential in structural elucidation and metabolite identification (Oleszek and Marston, 2000; Philipson, 2007). LC–MS is especially powerful for detecting trace compounds in complex biological matrices and plays a crucial role in pharmacokinetics, metabolomics, and toxicology studies (Rao, 2012).

c. Liquid Chromatography–Nuclear Magnetic Resonance (LC–NMR)

LC–NMR is a powerful hyphenated technique that integrates chromatographic separation with the structural elucidation capabilities of nuclear magnetic resonance spectroscopy (Daffre *et al*., 2008). It is particularly effective for analyzing complex mixtures and for identifying compounds that are light-sensitive or oxygen-sensitive (Eriamiatoe *et al.,* 2020). The online LC–NMR configuration enables real-time data acquisition during chromatographic separation, enhancing both speed and accuracy. The introduction of pulsed field gradient (PFG) techniques and advanced three-dimensional NMR approaches has significantly improved resolution, sensitivity, and the ability to determine structural and molecular weight information (Daffre *et al*., 2008). LC–NMR is highly valuable in drug discovery, providing detailed insights into compound structures and enabling rapid identification of unknown constituents (Patil and Shettigar, 2010). Its applications extend to pharmacokinetics, drug metabolism, natural product research, and toxicity studies (Rao, 2012).

2.2.6.3 Gas chromatography

a. Gas Chromatography Fourier Transform Infrared spectrometry

Coupling capillary column gas chromatographs with Fourier Transform Infrared Spectrometer provides a potent means for separating and identifying the components of different mixtures (Patil and Shettigar, 2010).

b. Gas Chromatography–Mass Spectrometry (GC–MS)

Gas chromatography can be directly coupled with a rapid-scan mass spectrometer for enhanced analytical capability (Patil and Shettigar, 2010). The low flow rate of capillary columns allows their output to be introduced directly into the ionization chamber of the mass spectrometer (Rao, 2012). One of the most straightforward detectors used in GC is the Ion Trap Detector (ITD) (Rao, 2012). In this system, ions from the eluted sample are generated via electron impact or chemical ionization and retained within a radio frequency field. These trapped ions are subsequently released in a controlled manner into an electron multiplier detector, allowing for scanning based on their mass-to-charge (m/z) ratios (Daffre *et al*., 2008; Philipson, 2007). The ion trap detector is notably compact and more cost-effective compared to quadrupole mass analyzers (Oleszek and Marston, 2000). GC–MS has proven instrumental in identifying numerous compounds within natural products and biological systems (Oleszek and Marston, 2000; Philipson, 2007; Daffre *et al*., 2008).

2.2.6.4 Supercritical Fluid Chromatography (SFC)

Supercritical Fluid Chromatography (SFC) is a hybrid analytical technique that integrates key features of both gas chromatography (GC) and liquid chromatography (LC) (Daffre *et al*., 2008). As an emerging third mode of column chromatography, SFC is increasingly being adopted across industrial, regulatory, and academic laboratories (Rao, 2012). Its significance lies in its ability to separate and analyze compounds that are challenging to handle with either GC or LC (Eriamiatoe *et al.,* 2020). These include non-volatile or thermally sensitive compounds, which are unsuitable for GC, and those lacking functional groups needed for detection via common spectroscopic or electrochemical methods used in LC (Rao, 2012). SFC has demonstrated wide applicability in the analysis of natural products, pharmaceuticals, food components, and pesticides (Oleszek and Marston, 2000; Philipson, 2007; Daffre *et al*., 2008).

2.3 ANALGESIC

An analgesic, or painkiller, is a type of drug used to relieve pain, a condition known as analgesia (Tambaro *et al.,* 2013). These drugs function by acting on both the peripheral and central nervous systems, distinguishing them from anesthetics, which eliminate sensation entirely and temporarily Raja *et al*., 2020). Common analgesics include paracetamol (also called acetaminophen or APAP in the U.S.), non-steroidal anti-inflammatory drugs (NSAIDs) like salicylates, and opioid medications such as morphine and opium (Tambaro *et al.,* 2013).

The choice of analgesic depends on the severity of pain and how the patient responds to other medications (Deng *et al.*, 2011). According to the World Health Organization's (WHO) pain ladder, mild analgesics are recommended as the first line of treatment (Raja *et al.,* 2020). Pain is an unpleasant, often poorly defined sensation triggered by external or internal stimuli (Deng *et al.*, 2011). It serves as a vital warning signal, protective in nature, but can lead to significant discomfort, suffering, or even incapacitation (Amol *et al.,* 2014). When intense, pain may cause additional symptoms such as a sinking feeling, anxiety, sweating, nausea, changes in blood pressure, rapid heartbeat, and increased breathing rate (Deng *et al.*, 2011). Analgesia can be achieved by blocking the nerve-sensitizing mechanisms activated by substances like bradykinin, tumor necrosis factor-alpha (TNFα), and interleukins (ILs) (Deng *et al.*, 2011). Analgesics work by selectively relieving pain through action on the central nervous system or peripheral pain pathways, without significantly affecting consciousness (Amol *et al.,* 2014). Importantly, they alleviate the symptom of pain without addressing its underlying cause (Amol *et al.,* 2014).

2.4 PAIN MECHANISMS AND PATHWAYS

Pain is a complex sensory and emotional experience that varies in intensity and character, ranging from mild discomfort to severe, intolerable sensations Raja *et al*., 2020). It is detected by nociceptors—specialized sensory receptors with free nerve endings that respond to harmful stimuli (Basbaum *et al*., 2009). These receptors transmit pain signals via first-order sensory neurons, whose cell bodies are located in the dorsal root ganglia (Basbaum *et al*., 2009; Raja *et al*., 2020).

Pain signals travel through two main types of nerve fibers:

Aδ-fibers (myelinated; conduction speed 5–30 m/s) – Responsible for transmitting sharp, localized pain, often triggered by intense pressure or heat (Dubin and Patapoutian, 2010; Vardeh *et al*., 2016).

C-fibers (unmyelinated; conduction speed 0.2–2.0 m/s) – Carry slow, dull, and burning pain, typically associated with chemical stimuli like bradykinin, histamine, hydrogen ions (H^+), and potassium ions (K^+) released during tissue damage or inflammation (Dubin and Patapoutian, 2010; Vardeh *et al*., 2016).

Regardless of whether pain is triggered by mechanical, thermal, or chemical stimuli, its intensity can be amplified by prostaglandins, which sensitize nociceptors (Sachs *et al*., 2020). Inflammatory pain and ischemic pain, such as that seen in angina pectoris or myocardial infarction, are often mediated by these chemical signals. Visceral pain, which results from organ distension, spasms, or anoxia, is often diffuse and difficult to localize (Julius and Basbaum, 2001).

Once pain signals reach the spinal cord through the dorsal root, they travel upward via the dorsolateral funiculus and synapse on second-order neurons in the dorsal horn (Todd, 2010). These neurons send axons across the midline and ascend toward the brain through the anterolateral system (also known as the spinothalamic tract) (Todd, 2010).

Pain Pathways

Pain transmission occurs via two primary pathways:

Neospinothalamic tract – Transmits sharp, well-localized pain to the thalamus, which then relays it to the primary somatosensory cortex (postcentral gyrus) for precise localization (Tracey and Mantyh, 2007).

Paleospinothalamic tract – Carries dull, aching, or burning pain to the limbic system, frontal cortex, and other areas, contributing to the emotional and affective aspects of pain (Tracey and Mantyh, 2007).

Pain signals are further modulated by descending pain-inhibitory pathways that originate from the brainstem's reticular formation and influence pain transmission at different levels of the spinal cord (Ossipov *et al*., 2010; Kuner and Kuner, 2021). These pathways release endogenous opioids (e.g., enkephalins, endorphins) and monoamines (e.g., norepinephrine, serotonin), which act to inhibit pain transmission (Ossipov *et al*., 2010; Kuner and Kuner, 2021).

Pain Modulation Strategies

Pain perception can be modified through several mechanisms, including:

Eliminating the underlying cause of pain,

Reducing nociceptor sensitivity (e.g., NSAIDs, acetaminophen, aspirin local anesthetics),

Blocking nociceptive conduction in sensory nerves (e.g., local anesthetics),

Suppressing pain signal transmission in the spinal cord (e.g., opioids, gabapentinoids)

Inhibiting pain perception at the cortical level (e.g., opioids, general anesthetics) and

Modifying emotional and cognitive responses to pain (e.g., antidepressants, cognitive behavioral therapy) (Kuner and Flor, 2017; Vasquez *et al*., 2021).

2.5 METHODS EVALUATION OF ANALGESIC ACTIVITY

The evaluation of analgesic activity (pain-relieving properties) of compounds or natural products can be done using different experimental models (Kanarek and Homoleski, 2000). These methods are broadly classified into:

2.5.1 Thermal Methods (Heat-Induced Pain)

- Hot Plate Test: Measures reaction time (latency) to pain induced by a heated surface. Used to assess central analgesic effects (e.g., opioid-like drugs).
- Tail Flick Test: Measures the time taken for an animal (usually a rat or mouse) to flick its tail in response to radiant heat. It evaluates spinal reflexes and opioid analgesia.
- Hargreaves Test: Similar to the tail flick test but uses a focused beam of heat on the hind paw (Kanarek and Homoleski, 2000).

2.5.2 Chemical-Induced Pain Models

- Acetic Acid-Induced Writhing Test: Intraperitoneal injection of acetic acid induces abdominal constrictions (writhing) in mice (Kanarek and Homoleski, 2000). Used to evaluate peripheral analgesic effects (e.g., NSAIDs).
- Formalin Test: Injection of dilute formalin into the paw induces two phases of pain—early phase (neurogenic pain) and late phase (inflammatory pain). This test helps

distinguish central vs. peripheral analgesia (Lavich *et al.,* 2005 ; (Kanarek and Homoleski, 2000).

- Capsaicin-Induced Pain Model: Uses capsaicin to activate transient receptor potential vanilloid 1 (TRPV1) channels, simulating neuropathic pain (Kanarek and Homoleski, 2000).

2.5.3 Mechanical and Pressure-Based Methods

- Randall-Selitto Test: Measures the pain threshold by applying increasing pressure to a rat's hind paw until a withdrawal response occurs (Kanarek and Homoleski, 2000)..
- Von Frey Filament Test: Uses calibrated filaments of different forces to stimulate pain responses, mainly for neuropathic pain assessment (Kanarek and Homoleski, 2000).

2.5.4 Electrical Stimulation Methods

- Tail Clip Test: A clip is applied to a rodent's tail, and the time taken to remove it is measured (Kanarek and Homoleski, 2000)..
- Electrically Induced Paw Withdrawal: Low-voltage electrical stimulation of the paw to measure pain sensitivity (Kanarek and Homoleski, 2000).

2.5.5 *In Vivo* Models of Chronic Pain

- Complete Freund's Adjuvant (CFA)-Induced Pain Model: CFA injection into the paw induces long-term inflammation, mimicking arthritis (Kanarek and Homoleski, 2000)..
- Chronic Constriction Injury (CCI) Model: Induces neuropathic pain by tying ligatures around a nerve (Lavich *et al.,* 2005).

2.5.6 Human-Based Methods (Clinical and Behavioral Approaches)

- Visual Analog Scale (VAS): A subjective scale where patients rate their pain intensity.
- Cold Pressor Test: Measures pain tolerance when a hand or limb is immersed in ice water (Kanarek and Homoleski, 2000).
- Quantitative Sensory Testing (QST): Assesses sensory response to controlled stimuli (e.g., heat, pressure) (Lavich *et al.,* 2005) .

These methods help in screening new analgesic drugs and understanding their mechanism of action (e.g., opioid, NSAID, or neuropathic pain relief) (Kanarek and Homoleski, 2000 ; Lavich *et al.,* 2005).

2.6 Hot Plate Test

The hot plate test is a standard method for assessing the analgesic activity of drugs that act on the central nervous system (e.g.,opioids) by measuring an animal's response to thermal pain (Lavich *et al.,* 2005).

2.6.1 Principle

Pain is induced by placing a rodent (mouse or rat) on a heated surface (typically 50–55°C) (Lavich *et al.,* 2005).. The time taken for the animal to react (by licking its paws, jumping, or withdrawing) is recorded. A longer reaction time suggests stronger analgesic effects (Lavich *et al.,* 2005)..

2.6.2 Procedure

1. Pre-test Handling: Acclimate the animals to the laboratory environment for at least 30 minutes.

2. Baseline Measurement: Place each rodent on the hot plate and record the time (in seconds) until it shows a pain response (paw licking, jumping, or withdrawing). This is the baseline reaction time.

3. Drug Administration: Administer the test compound (or standard drug like morphine) intraperitoneally (i.p.) or orally (p.o.).

4. Post-Treatment Testing: Place the animal on the hot plate again at different time intervals (e.g., 15, 30, 60 minutes after drug administration).

5. Cut-off Time: To avoid tissue damage, a maximum cut-off time (usually 30 seconds) is set.

6. Results Interpretation:

If the treated group shows a significant increase in reaction time compared to the control, the drug has central analgesic activity (Lavich *et al.,* 2005). Opioids (like morphine) show a strong delay in response, while NSAIDs show little or no effect (Lavich *et al.,* 2005).

2.6.3 Advantages

Measures central analgesic effects (spinal and supraspinal modulation).

Simple and reproducible.

Sensitive to opioid analgesics (Lavich *et al.,* 2005).

2.6.4 Limitations

Not suitable for testing peripheral analgesics (like NSAIDs), stress and repeated testing can affect response (Lavich *et al.,* 2005).

2.7 Ethanoic Acid-Induced Writhing Test

This model is used to assess peripheral analgesic activity (NSAIDs, flavonoids, plant extracts, etc.). It is based on the induction of pain via chemical irritation (Kanarek and Homoleski, 2000).

2.7.1 Principle

Intraperitoneal injection of acetic acid causes pain and inflammation in mice, leading to writhing movements (abdominal constrictions, stretching of hind limbs) (Kanarek and Homoleski, 2000). Analgesics reduce the number of writhes, indicating their effectiveness (Kanarek and Homoleski, 2000).

2.7.2 Procedure

1. Pre-test Handling: Acclimate the mice to the laboratory environment.

2. Drug Administration: Give the test compound (or standard drug, e.g., aspirin, diclofenac) orally or intraperitoneally.

3. Acetic Acid Injection: Inject 0.6% acetic acid (10 mL/kg) intraperitoneally.

4. Observation Period: Place the mice in a transparent box and count the number of writhing movements (abdominal contractions) over a 10–30 min period.

5. Results Calculation:

$$\% \text{ Inhibition} = [(\text{Control Writhing} - \text{Treated Writhing}) / \text{Control Writhing}] \times 100$$

Higher inhibition means stronger peripheral analgesic activity (Kanarek and Homoleski, 2000).

2.7.3 Advantages

- Highly sensitive for NSAIDs and plant extracts with anti-inflammatory properties.
- Simple and cost-effective (Kanarek and Homoleski, 2000).

2.7.4 Limitations

Not specific for central analgesia (does not measure opioid effects).

Acetic acid causes visceral pain, which may differ from other pain mechanisms (Kanarek and Homoleski, 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Design

This present research employed an experimental laboratory design involving phytochemical screening, plant extraction, thin layer chromatographic analysis to evaluate Retention factor (Rf) values of the components in the ethanolic isolate and *in vivo* evaluation of pain-relieving activity using animal models. The pain-relieving effect of *Sida acuta* stem isolates was assessed using the hot plate method and ethanoic acid-induced writhing test in mice. The experimental groups included a control, standard drug (Pentazocine and Aspirin), and three treatment groups receiving graded doses of the isolate (100 mg/kg, 200 mg/kg, and 400 mg/kg).

3.2 Materials and Equipment

Table 3.1a Materials, Equipment, and Tools used

S/N	Glasswares and Equipment	Source	Operating characteristics
1	Soxhlet apparatus	Borosilicate	Glass
2	beaker	Borosilicate	Glass
3	Pipette	borosilicate	Glass
4	Evaporating dish	borosilicate	Glass
5	Funnel	Borosilicate	Glass
6	Conical flask	Borosilicate	Glass
7	Measuring cylinder	Borosilicate	Glass
8	Glass rod	Borosilicate	Glass
9	Reagent bottle	Borosilicate	Glass
10	Wash bottle		Glass
11	Separatory funnel	Borosilicate	Glass
12	Volumetric flask	Borosilicate	Glass
13	Rack	pyrex	wooden
14	Test tube holder	pyrex	wooden
15	Heating mantle	Korea	Model: WHM12015 Supplier: witeg laboratory. Power: 230v, 450w, 60Hz.

S/N	Glasswares and Equipment	Source	Operating characteristics
16	Water bath	China	H H-600
17	Analytical balance	England	Model: AR2130 Supplier: Ohaus Rating: 9.6v-20v, 6w Company:adventure TM
18	hot plate analgesia meter	China	Model kc-6-BS. Supplier: Shenyang Sino-King Equipment Power requirement: 160w,110/220v,50-60Hz Temperature range: 65 ^{0}C

Table 3.2 Chemicals and reagents:

S/N	**Reagents**	Sources	Physical and chemical properties
1	Ethanol	Loba chemie	Molar mass: 285.41g/mol Solubility: Soluble in alcohol, chloroform , slightly in water Colour: White crystalline powder
2	Pentazocine	Loba chemie	Molar mass: 285.41g/mol Solubility: Soluble in alcohol, chloroform , slightly in water Colour: White crystalline powder
3	Aspirin	Loba chemie	Molar mass: 180g/mol Solubility: Slightly soluble in water, Colour: White crystalline powder
4	Ferric chloride	BDH	Colour: Dark brown crystals Molar mass: 162.2g/mol Solubilty: soluble in water. Toxicity: Cause iron poisoning when ingested
5	Sulphuric aid	Loba chemie	Colour: Colourless. Odour: Odourless. Molar mass: 98.08g/mol Toxicity: Corrosive
7	Tween 80	Loba chemie	Colour: Yellow. Solubility: Soluble water, ethanol. Toxicity: Safe in regulated amount.

S/N	Reagent	Source	Physical and chemical properties
8	Acetic anhydride	Sigma aldrich	Molar mass: 102.09g/mol. Colour: Colourless liquid. Odour: Strong pungent. Solubility: React with water, soluble in ether and chloroform.
9	Picric acid	Loba chemie	Molar mass229.10g/mol Colour: Yellow crystalline solid Odour: Odourless
10	Wagner's reagent	Loba chemie	Colour: Reddish brown solution
11	Lead acetate	Spectrum chemical	Colour: White crystalline Solubility: Very soluble Molar mass: 379.33g/mol Toxicity: Very toxic
12	Distilled water	Chemistry department	Colour: Colourless pH: neutral to litmus
13	Hydrochloric acid	Loba chemie	Colour: Colourless Odour: Pungent Molar mass: 36.46g/mol Corrosive
14	Glacial acetic acid	Loba chemie	Colour: Colourless Odour: Sharp, pungent smell. Molar mass: 60.05g/mol. Flammability: Flammable

Test-based animals: Male and female albino mice (18–25 g)

Analytical tools: Heating mantle Rotary evaporator, weighing balance, hot plate analgesia meter, thin layer chromatography, fronrier transforn infrared spectroscopy and Gas chromatography mass spectroscopy (GC-MS)

Animal housing tools: Cages, bedding, feeding accessories

3.3 Collection and Preparation of Plant Material

Fresh *Sida acuta* stem were obtained from a local botanical source, authenticated by a taxonomist, washed, and shade-dried. The dried stems were pulverized into powdery using a mechanical grinder and stored in airtight containers.

3.4 Isolation of Plant Material

About 500g of the powdered plant material was isolated using 99.7% ethanol in a Soxhlet apparatus for 6–8 hours. The isolate was concentrated under vacuum using a rotary evaporator and subsequently dried on a water bath maintained at 40°C. The resulting dried isolate was stored at 4°C in a refrigerator until further use.

3.5 Phytochemical Screening

Qualitative phytochemical screening was conducted to detect the presence of alkaloids, flavonoids, saponins, tannins, phenols, glycosides, and terpenoids using standard procedures (Harborne, 1998).

3.5.1 Test for alkaloid

To detect the presence of alkaloids, Dragendorff's reagent, Wagner's reagent, and picric acid were employed. A 1 mL portion of the ethanolic stem extract of *Sida acuta* was dispensed into three separate test tubes labeled A, B, and C.

- **Tube A:** 2 mL of Dragendorff's reagent (potassium bismuth iodide solution) was added. A reddish-brown precipitate formed, indicating a positive test for alkaloids Ghoshal *et al.,* 2022).
- **Tube B:** 2 mL of Wagner's reagent (iodine in potassium iodide solution) was added, also resulting in a reddish-brown precipitate, confirming the presence of alkaloids Ghoshal *et al.,* 2022).
- **Tube C:** 2 mL of picric acid solution was added, and the appearance of a yellow precipitate further confirmed the presence of alkaloids (Jonathan *et al.,* 2018 ; Ghoshal *et al.,* 2022).

3.5.2 Test for Flavonoid

To detect flavonoids, 2 mL of the extract was boiled in 5 mL of distilled water and then filtered. A few drops of 10% lead acetate solution were added to the filtrate. The formation of a yellow precipitate indicated the presence of flavonoids (Harborne, 1998).

3.5.3 Test for Glycosides (Confirmatory Test – Keller-Killiani Test)

A measured volume of the extract (1 mL) was mixed with 1 mL of glacial acetic acid containing a trace amount of ferric chloride. Carefully, 1 mL of concentrated sulfuric acid was added along the wall of the test tube (Eriamiatoe *et al.,* 2020). The formation of a

distinct brown ring at the interface indicated a positive result for the presence of cardiac glycosides (Jonathan *et al.,* 2018).

3.5.4 Test for Phenols

A measured volume of the extract (1 mL) was added to 5 ml 90% ethanol; also a drop of 10% ferric chloride solution was added. Formation of a deep a pale yellow coloration indicates the presence of phenolic compounds (Eriamiatoe *et al.,* 2020).

3.5.5 Test for Saponins

A measured volume the extract (5 mL) was diluted with 10 mL of distilled water in a test tube and shaken vigorously for 2 minutes. Persistent froth (for 5 minutes) shows the presence of saponins (Eriamiatoe *et al.,* 2020).

3.5.6 Test for Tannins

A measured volume of ethanolic stem extract (2 mL) was diluted with 10 ml of distilled water, allowed to boil for 5 minutes and filtered. The filterate treated with few drops of 1% ferric chloride solution. A bluish precipitate indicates the presence of tannins (Aletan and Kwazo, 2019).

3.5.7 Test for Terpenoid (Salkowski Test)

To detect terpenoids, 2 mL of the extract was mixed with 2 mL of chloroform in a test tube. Then, 2 mL of concentrated sulfuric acid was carefully added along the inner wall of the tube to form a separate layer (Eriamiatoe *et al.,* 2020. The appearance of a reddish-

brown coloration at the interface between the two layers indicated the presence of terpenoids (Eriamiatoe *et al.,* 2020).

3.6 SEPARATION OF ACIDIC/BASIC ORGANIC COMPOUNDS

The plant extract was initially dissolved in diethyl ether and transferred into a separating funnel. To this, 20 mL of 2 M hydrochloric acid (HCl) was added. The mixture was gently shaken and then allowed to separate into layers. The lower aqueous (acidic) layer was collected into a conical flask. This extraction process was repeated twice using two additional portions of 20 mL of 2 M HCl to ensure complete transfer of basic compounds into the aqueous phase. The acidic fraction was then neutralized to pH 7 using aqueous solution of Na_2CO_3. The organic compound was then separated from the aqueous mixture by extracting with diethyl ether. The ether was evaporated to obtain the isolate.(Eriamiatoe *et al.,* 2020)

The diethyl fraction from acid extraction was again extracted with 3 portions of 2M NaOH. The basic portion was then neutralized to pH 7 with basic HCl acid extracted with diethyl ether (Eriamiatoe *et al.,* 2020).

3.7 THIN LAYER CHROMATOGRAPHIC (TLC) ANALYSIS

At a temperature of 100^0C pre-coated TLC plate (CDC-Alufolien Kielselgel 60 F254; 25 folien 20 x20cm, 0.2mm thickness) were activated for 2-3 hours and stored in closed desiccators (Eriamiatoe *et al.,* 2020). The samples were spotted at intervals of about 0.5cm at marked edge. To minimise the disturbance of solvent-saturated atmosphere, the plates were placed squarely in the tank and the lid replaced quickly (Eriamiatoe *et al.,* 2020).

The developing solvent was allowed to travel up the plate until it reaches desired level. The plate was carefully taken out of the developing chamber, and the solvent front immediately marked with pencil were treated in iodine tanks to develop and spots marked (Eriamiatoe *et al.,* 2020). The solvent used are;

Chlorofom 100%

Hexane 100%

Chloroform : Ethanol (9:1)

Hexane : Chloroform (1:1) (Patil and Shettigar, 2010).

3.8 Test-based Animals

Albino mice of both sexes were acclimatized for 21days under standard laboratory conditions.

3.9 Analgesic Activity Tests

The animals were maintained on a standard pellet diet with unrestricted access to water (ad libitum). Ethical approval for the study was obtained in compliance with institutional guidelines for the care and use of laboratory animals (Wang *et al.,* 2019).

I. **Hot Plate Method**:

Mice were placed on a heated metal surface (55 ± 1°C), and the reaction time (paw licking or jumping) was recorded. Baseline readings were taken before administration. Animals were administered the extract orally at 100, 200, and 400 mg/kg, while the standard group received Pentazocine (0.1 mg/kg) intraperitoneally. Reaction times were

recorded at 0, 30, 60, 90, 120, 150 and 180 minutes post-administration (Deng *et al.*, 2011 ; Lavich *et al.*, 2005 ; Raja *et al.,* 2020 ; Wang *et al*., 2019).

II. **Ethanoic Acid-Induced Writhing Test**:

Pain was induced by intraperitoneal injection of 0.6% ethanoic acid (10 mL/kg) (Lavich *et al.*, 2005). The number of abdominal constrictions (writhes) was counted for 30 minutes post-injection (Deng *et al.*, 2011). Reduction in writhing compared to control was used to calculate percentage inhibition (Deng *et al.*, 2011 ; Lavich *et al.*, 2005).

% Inhibition Formula:

$$\text{Inhibition} = \frac{\text{writhes}_{\text{control}} - \text{writhes}_{\text{test}}}{\text{writhes}_{\text{control}}} \times 100$$

3.10 Data Collection and Statistical Analysis

All data were expressed as mean ± standard error of mean (*sem*) (Deng *et al.*, 2011).

CHAPTER FOUR

RESULT AND DISCUSSION

4.1 Phytochemical Screening of Ethanolic Stem Isolate of *Sida acuta*

Preliminary phytochemical screening of the ethanolic isolate of *Sida acuta* revealed the presence of several bioactive constituents (Rajesh *et al*., 2010). The isolate tested positive for alkaloids, flavonoids, saponins, tannins, glycosides, terpenoids, and phenolic compounds (Rajesh *et al*., 2010).

Table 4.1: Phytochemical Constituents of ethanolic stem extract of *Sida acuta*

Phytochemical test	Observation	Presence (+)
Alkaloids		
Dragendoff's reagent	Reddish brown precipitate	+
Wagner's reagent	Reddish brown precipitate	+
Picric reagent	Yellow precipitate	+
Flavonoids	Yellow	+
Glycosides	Brown ring	+
Phenolics	Pale yellow	+
Saponins	Foam formation	+
Tannis	Bluish precipitate	+
Terpenoids	Reddish brown coloration	+

These constituents are known to contribute to various pharmacological activities. For instance, flavonoids and alkaloids are implicated in modulating pain perception by

inhibiting cyclooxygenase and lipoxygenase pathways, while saponins and phenols possess anti-inflammatory and antioxidant properties (Rajesh *et al*., 2010).

4.2 The hot plate test result

The dataset in table 4.2, represents the latency time (in seconds) before a pain response (e.g., paw licking or jumping) in the hot plate analgesic test, which evaluates central analgesic activity. Higher latency times indicate stronger analgesic (pain-relieving) effects. is a brBelow eakdown and interpretation of the datas

Table: 4.2 Time-Dependent Analgesic Effect of Plant Extracts and Pentazocine in Mice Using the Hot Plate Method (Latency in Seconds, Mean ± *sem*)

Time (minutes)	Control mean (±*sem*)	Extract 100 mg/Kg	Extract 200 mg/Kg	Extract 400 mg/Kg	Pentazocine 0.1 mg/Kg
0	6.22 ±0.05	6.10±0.29	5.20±0.52	4.42±0.04	4.45±0.37
30	5.78±0.26	10.06±1.38	8.50±0.82	10.4±1.91	10.44±1.02
60	6.98±0.51	10.50±1.45	7.64±0.41	12.02±0.63	10.56±1.45
90	6.40±0.27	7.50±0.79	7.36±0.77	9.92±1.23	8.18±0.85
120	6.34±0.49	7.08±0.40	6.20±0.44	7.80±0.64	9.54±1.26
150	6.80±1.04	7.08±1.48	5.02±0.019	10.78±2.22	7.12±0.99
180	7.12±0.60	5.86±0.88	7.98±0.55	12.24±1.08	8.62±0.16

1. **Baseline (0 min):**

All groups started with similar latency times (approx. 4.4–6.2 sec), indicating no pre-treatment difference in pain response.

2. **Control Group:**

Latency times stayed relatively stable (6.22 to 7.12 sec), showing no significant analgesic effect over time.

3. **Extract at 100 mg/kg:**

- Peak effect: 60 min (10.50 sec), close to Pentazocine (10.56 sec).
- Shows significant increase from baseline at 30 min (10.06 sec).
- Effect drops after 60 min, indicating moderate and short-lived analgesic effect.

4. **Extract at 200 mg/kg:**

- Peak effect: 30 min (8.50 sec) and again at 180 min (7.98 sec).
- Less consistent than 100 mg/kg.
- Indicates weaker and less stable analgesic effect than the 100 mg/kg and 400 mg/kg doses.

5. **Extract at 400 mg/kg:**

- Peak effect: 180 min (12.24 sec) – strongest analgesic response overall.
- Shows sustained elevation from 30–180 min, especially at 60 min (12.02 sec) and 150 min (10.78 sec).
- Indicates dose-dependent analgesic activity and better efficacy at higher dose.

6. **Standard (Pentazocine 0.1 mg/kg):**

- Consistent strong analgesic response throughout (up to 10.56 sec at 60 min).

- Serves as a positive control.

Key Interpretation:

- All doses of the extract show analgesic activity, especially 100 mg/kg and 400 mg/kg.
- 400 mg/kg dose shows dose-dependent, strong, and sustained analgesia, close to or surpassing Pentazocine at some time points.
- 200 mg/kg shows some effect but is less consistent and less potent.
- The extract demonstrates central analgesic properties, supporting further exploration.

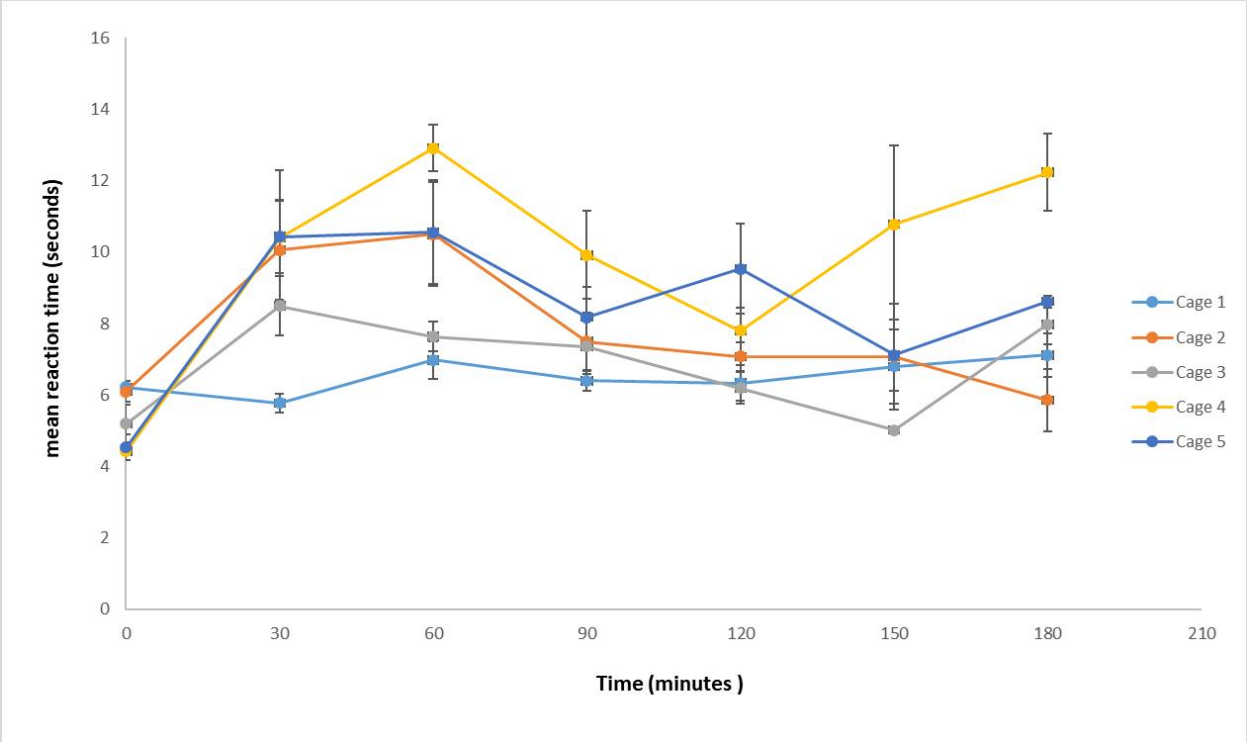


Figure 4.1: A line graph illustrating the analgesic effect of *Sida acuta* ethanolic stem extract over time in the hot plate test.

"Analgesic Effect Over Time in Hot Plate Test (Mean Latency ± *sem*)"

X-Axis (Time in minutes):

- Shows the observation time points post-administration (0, 30, 60, 90, 120, 150, and 180 minutes).

Y-Axis (Latency Time in seconds):

- Indicates how long (in seconds) the mice delayed reacting to the hot plate (e.g., paw licking, jumping). **A** longer latency means greater analgesic (pain-relieving) effect**.**

Group-by-Group Interpretation:

1 **Control Group (cage 1):**

- Maintains a relatively steady latency of around 6–7 seconds across all time points.
- Indicates no analgesic effect; pain response remains normal.

2 **Pentazocine 0.1 mg/kg (cage 5):**

- Shows a significant increase in latency after 30 minutes, peaking around 60 minutes (10.56 s), then stabilizing.
- Confirms the strong analgesic action of Pentazocine, a known opioid analgesic.

3 **Extract 100 mg/kg (cage 2):**

- Shows a noticeable increase in latency at 30–60 minutes (peaks around 10.5 s), then declines slightly.

- Indicates moderate analgesic activity, but less sustained than higher doses.

4 **Extract 200 mg/kg (cage 3):**

- Latency increases at 30 and 60 minutes but is less consistent than 100 or 400 mg/kg.
- This dose shows variable analgesic response—effective, but not as pronounced.

5 **Extract 400 mg/kg (cage 4):**

- Shows the strongest and most sustained analgesic effect among all extract doses.
- Latency increases steadily, peaking at 180 minutes (12.24s)—even surpassing Pentazocine.
- Demonstrates a clear dose-dependent effect and possibly central analgesic action.

Conclusion:The extract exhibits dose-dependent analgesic activity.

400 mg/kg of the extract is comparable or superior to Pentazocine, especially in sustained response. Suggests potential for central analgesic mechanisms, which is what the hot plate test primarily measures.

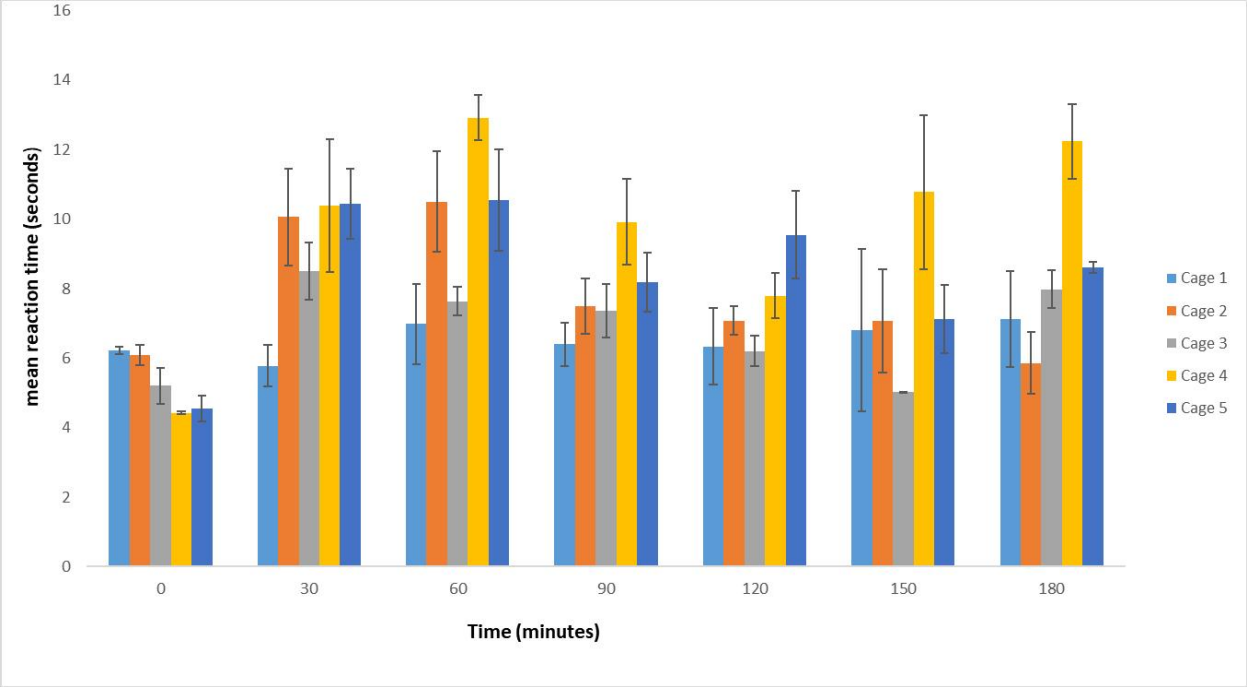


Figure 4.2: A bar chart showing the reaction times at the 0-180-minutes mark for all treatment groups in the hot plate test.

Figure 4.3: A bar chart showing the reaction times at the 60-minute mark for all treatment groups in the hot plate test.

From figure 4.3,

- Extract at 400 mg/kg shows the highest latency (12.02s), indicating the strongest analgesic effect at this time point.
- Pentazocine and Extract 100 mg/kg also show significant analgesic activity.
- Control group has the lowest latency, confirming no analgesic effect.

4.3 Ethanoic acid-induced writhing test result

Purpose of the Test:

The ethanoic acid-induced writhing test in mice is a standard *in vivo* model for assessing peripheral analgesic (pain-relieving) activity. The test quantifies the number of writhing movements (abdominal constrictions) induced by acetic acid. A reduction in writhes indicates analgesic activity (Deng *et al.*, 2011 ; Lavich *et al.*, 2005).

Table: 4.3 Writhing Responses and Statistical Analysis of Analgesic Activity in Mice Using the Acetic Acid-Induced Writhing Test

Cage	No of mice	No of writhing	Mean number of writhing	Deviation (d^2)	Standard deviation (s)	Standard error of mean(*sem*)
1	4	145,125, 100, 110	120	1150	19.60	±9.80
2	5	10,19,16, 19, 15	15..80	83.60	3.55	±1.58
3	5	11, 0, 5, 2, 0	3.60	85.20	4.60	±2.06
4	5	0	0	0	0	0
5	5	12, 14, 20, 15, 18	15.8	40.80	3.10	±1.43

Table:4.4 Effect of Extract on Acetic Acid-Induced Writhing in Mice

Cage	Dosage	mean ± *sem*	% inhibition
1	Control 0.3%v/v/50 tween 80	120 ± 9.80	-
2	100mg/Kg/50 extract	15.80 ± 1.43	86.80
3	200mg/Kg/50 extract	3.60 ± 2.06	97.00
4	400mg/Kg/50 extract	0 ± 0	100.00
5	Aspirin 100mg/Kg/50	15.80 ± 1.58	86.80

% Inhibition Formula:

$$\text{Inhibition} = \frac{\text{writhes}_{\text{control}} - \text{writhes}_{\text{test}}}{\text{writhes}_{\text{control}}} \times 100$$

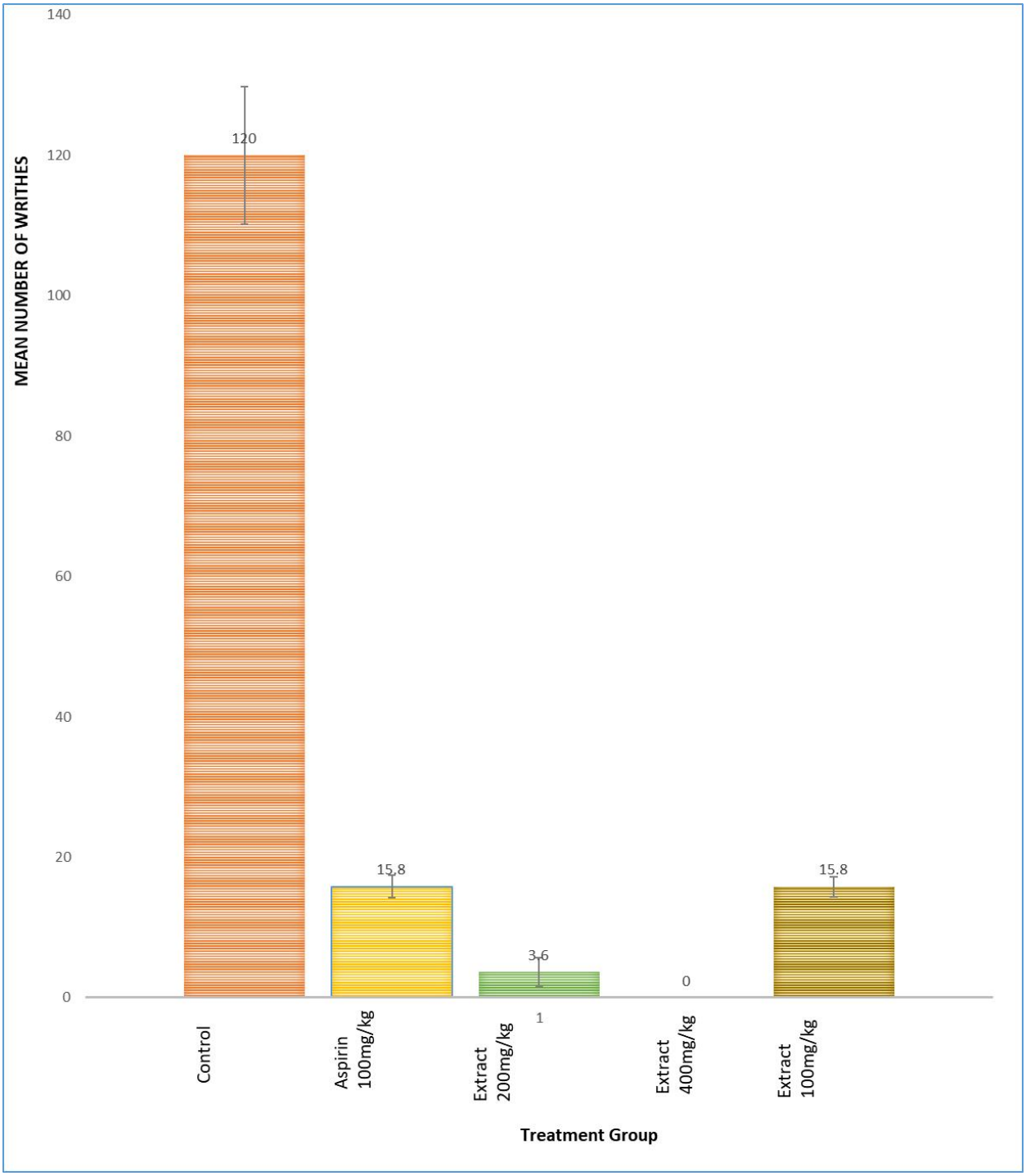


Figure 4.4: The graph is a bar plot showing the average number of writhes observed in each treatment group during the ethanoic acid-induced writhing test in mice

Y-axis:

- Represents the average number of writhes (± *sem*) recorded for each group.

X-axis:

- Treatment groups:
  - ❖ Control (no treatment)
  - ❖ Standard drug (Aspirin 100 mg/Kg)
  - ❖ Test isolate at 100, 200, and 400 mg/Kg

Error bars:

- The Standard Error of the Mean (±*sem*), indicating the variability of writhing counts in each group is represented

Interpretation:

- The lower the bar, the greater the analgesic (pain-relieving) effect.
- Aspirin (standard) shows the most significant reduction in writhes.
- The extract shows a dose-dependent reduction, meaning higher doses reduce writhes more effectively.

Interpretation:

1. Control Group: Mice exhibited a high number of writhes (120), as expected when exposed to acetic acid without any analgesic intervention.

2. Aspirin (Standard Drug):

- At 100 mg/kg, Aspirin significantly reduced writhes to 15.80, corresponding to 86.80% inhibition.
- This serves as a benchmark for comparing the plant extract's analgesic potential.

3. Extract-Treated Groups:

- 100 mg/kg: Showed a dramatic reduction in writhes (15.80 ± 1.43), identical in effect to Aspirin (86.80% inhibition). This suggests that even at a low dose, the extract has strong analgesic potential.
- 200 mg/kg: Further reduction to 3.60 writhes, 97.00% inhibition — indicating dose-dependent efficacy.
- 400 mg/kg: Complete inhibition (0 writhes, 100% inhibition) — this is exceptional and suggests the extract completely blocked the pain response at this dose

4.4 Result of thin layer chromatographic analysis of Ethanolic stem extract of *Sida acuta*

Result as shown below;

Table 4.5: Rf value and colour reaction of Ethanolic stem extract

Solvent system: Chlorofom

solvent front: 6.60

Origin	Distance travel by sample	Solvent front	Rf value	Colour change
0.00	0.30	6.60	0.045	Greenish brown

Table 4.6: Rf value and colour reaction of Ethanolic stem extract

Solvent system: Hexane

Solvent front: 6.60

Origin	Distance travel by sample	Solvent front	Rf value	Colour change
0.00	2.90	6.60	0.44	Greenish yellow

Table 4.7: Rf value and colour reaction of Ethanolic Stem extract

Solvent system: Chloroform : Ethanol (9:1)

Solvent front: 8.0

Origin	Distance travel by sample	Solvent front	Rf value	Colour change
0.00	3.50	8.00	0.43	yellow
0.00	2.00	8.00	0.25	blue
0.00	0.80	8.00	0.10	brown
0.00	4.20	8.00	0.52	Greenish yellow

Table 4.8: Rf value and colour reaction of ethanolic stem extract

Solvent system: Hexane : Chloroform 1:1

Solvent front: 6.0

Origin	Distance travel by sample (cm)	Solvent front (cm)	Rf value	Colour change
0.00	0.50	6.00	0.08	brown
0.00	2.30	6.00	0.38	blue

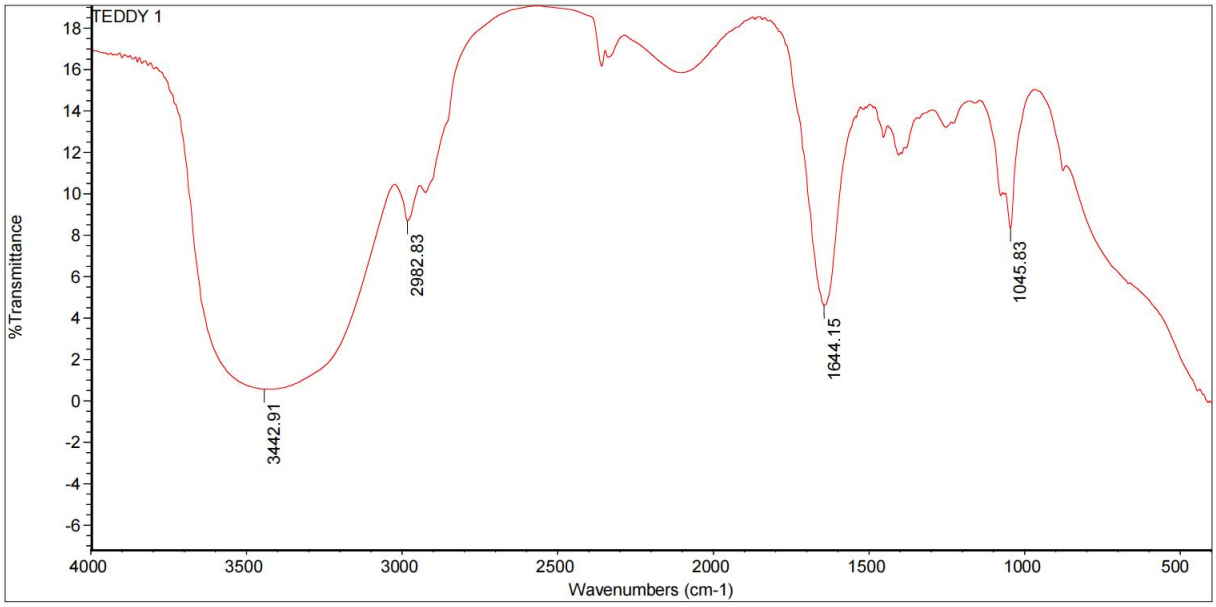
4.5 FTIR Analysis

IR Analysis Result

The I.R band were observed at 1045.83/cm (C-O, long, sharp) of primary alcohol; 1644.15/cm (C=C long, sharp) of alkenes indicative of unsaturation; 2982.83/cm (O-H short) of acids, carboxylic; 3442.91/cm (N-H broad) of amine, primary.

Table 4.9a: **I.R Absorption bands of Functional Groups detected in Ethanolic stem extract**

S/N	Frequency (/cm)	Appearance	Bond	Compound or functional group
1	3442.91	Broad	N-H, O-H	Amine, R-NH_2
2	2982.83	Short	O-H	Acids, carboxylic
3	1644.15	Long, sharp	C=C/C=N or C=O	Alkene, imine
4	1045.83	Long, sharp	C-O	1^{o} alcohol



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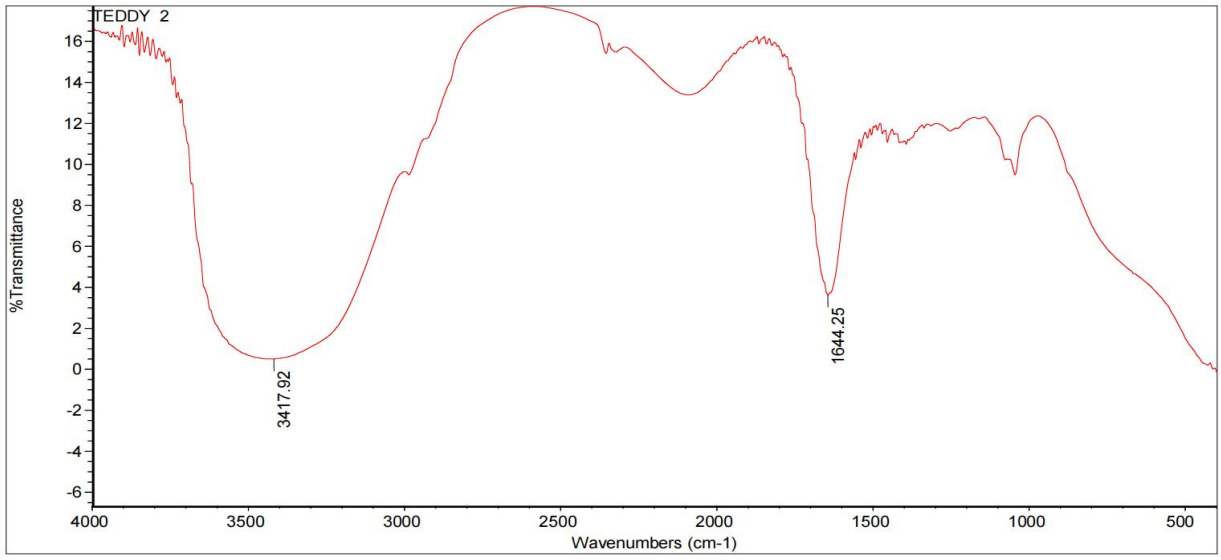
FIND PEAKS:

Spectrum:	TEDDY 1			
Region:	4000.00	400.00		
Absolute threshold:	9.467			
Sensitivity:	50			
Peak list:				
	Position:	1045.83	Intensity:	8.317
	Position:	1644.15	Intensity:	4.603
	Position:	2982.83	Intensity:	8.696
	Position:	3442.91	Intensity:	0.556

Figure 4.5a: Ft-ir of ethanolic stem extract of *Sida acuta*

Table 4.9b: **I.R Absorption bands of Functional Groups detected in Alkaloid extract**

S/N	Frequency (/cm)	Appearance	Bond	Likely compound or functional group
1	3417.92	Broad, weak	N-H stretching	Amine
2	1644.25	medium	C=C/ C=N	Likely a C=N bond in a heterocyclic ring



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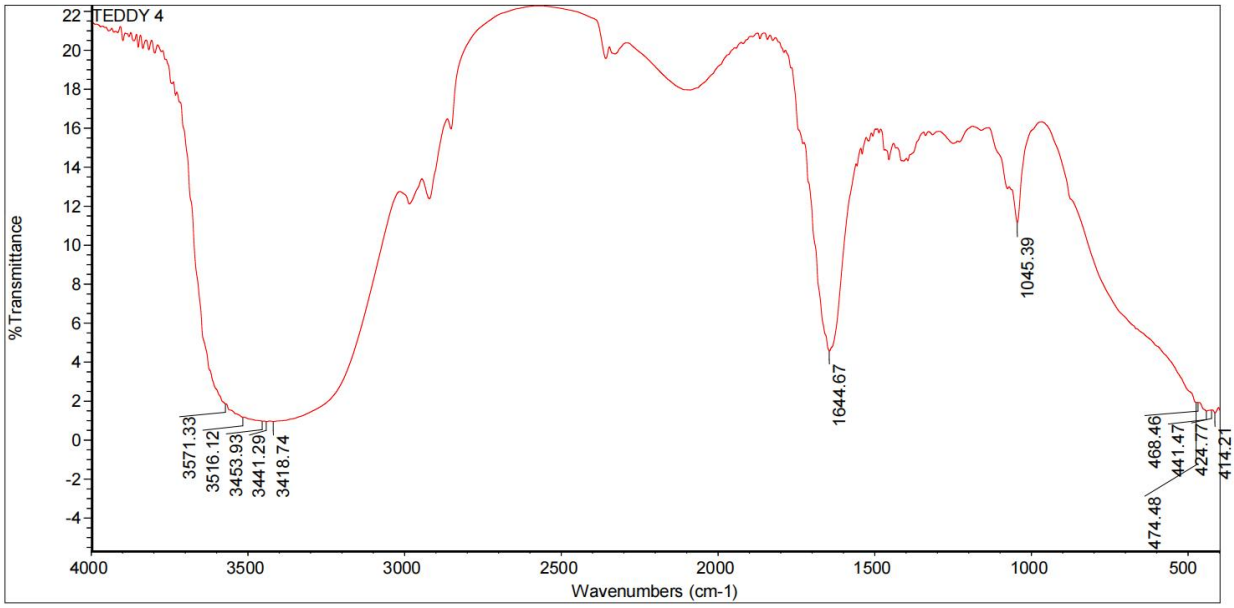
Region: 4000.00 400.00
Absolute threshold: 8.762
Sensitivity: 50
Peak list:
Position: 1644.25 Intensity: 3.606
Position: 3417.92 Intensity: 0.495

Figure 4.5b: Ft-ir of ethanolic stem extract of *Sida* acuta (alkaloid extract)

Table 4.9c: I.R Absorption bands of Functional Groups detected in phenolic extract

S/N	Frequency (/cm)	Appearance	Bond	Likely compound or functional group
1	3418.74-3571.33	Broad	O-H stretch	Likely from multiple phenolic O-H group
2	1644.67	Sharp long	C=C stretch or conjugated	Likely of aromatic ring
3	1045.39	Short	C-O stretch	Likely of phenolic ether
4	600-500		C–H out-of-plane bending vibrations	Substituted benzene rings.



Mon May 12 13:56:21 2025 (GMT+01:00)
FIND PEAKS:

Spectrum:	TEDDY 4		
Region:	4000.00	400.00	
Absolute threshold:	11.610		
Sensitivity:	100		
Peak list:			

Position:	414.21	Intensity:	1.379
Position:	424.77	Intensity:	1.533
Position:	441.47	Intensity:	1.471
Position:	468.46	Intensity:	1.920
Position:	474.48	Intensity:	1.912
Position:	1045.39	Intensity:	11.142
Position:	1644.67	Intensity:	4.551
Position:	3418.74	Intensity:	0.945
Position:	3441.29	Intensity:	0.943
Position:	3453.93	Intensity:	0.963
Position:	3516.12	Intensity:	1.164
Position:	3571.33	Intensity:	1.848

Figure 4.5c: Ft-ir of ethanolic stem isolate of *Sida acuta* (phenolics)

4.6 GC-MS ANALYSIS

The GC-MS chromatogram of the isolated stem extract given in fig 4.6a showed 20 peaks. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library using the National Institute of Standards and Technology (NIST) Search. The relative % amount of each component was calculated by comparing its average peak area to the total areas. Measurement of peak areas and data processing were carried out by chemStation software

The was subjected to chromatographic analysis using a Varian 3800/4000 gas chromatograph mass. All the peaks were identified based on mass spectral matching (≥ 90%) from both the NIST and Wiley libraries. Only compounds with 90% or greater spectral matching accuracy are reported. No response factors were calculated.

Table 4.9d : GC-MS Analysis of ethanolic stem extract of *Sida acuta*

Peak Number	Retention Time	Compound Detected	Peak Area %
1	2.73	Acetic acid	9.95
2	4.15	Hexanoic acid	1.15
3	8.00	Benzeneacetaldehyde	3.01
4	8.79	Benzene, 1,2,3,5-tetramethyl-	2.21
5	10.00	Eugenol	0.30
6	11.14	2-Propenoic acid, 3-(4-hydroxyphenyl)-, methyl ester	0.60
7	13.08	2-Pentadecanol	5.43
8	16.00	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	1.20
9	18.62	n-Hexadecanoic acid	5.52
10	19.91	Z,Z-2,13-Octadecadien-1-ol	5.13
11	23.00	9,12-Octadecadienoic acid, methyl ester	11.06
12	24.00	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	3.81

Peak Number	Retention Time	Compound Detected	Peak Area %
13	24.53	7-Tetradecenal, (Z)-	8.64
14	25.50	6-Octadecenoic acid, (Z)-	9.55
15	30.75	Hexadecanoic acid, methyl ester	0.90
16	34.86	9,12-Octadecadienoic acid, ethyl ester	2.51
17	40.45	1,2-Benzenedicarboxylic acid, butyl octyl ester	4.41
18	42.07	Phytol	6.13
19	44.86	Squalene	10.85
20	44.25	Cyclohexasiloxane, dodecamethyl-	7.63

Sample ID: DCPE/CAL/GCMS/052025/119996/01 Low Mass (m/z): 40 [redacted]
Operator: Ewere Donatus V.
Data Path: C\msdchem\data\sample-01# High Mass (m/z):800
Run Time: 45.00:00
Instrument Name: GCMS
Sample Name:gcms/data/chromatogram/sample-01# Acquisition Date: 09/05/2025 10:21:09
Comment:
Equipment: Varian 3800/4000 GCMS Client: Consults
ALS Vial: 1 Sample Multiplier: 1
Search Libraries: C:\database\NIST08 Minimum Quality: 10
Column: Agilent MS capillary column
Dimension: 30 m × 0.25 mm i.d
Carrier Gas: N_2. Flow: 1.0 ml/min
Unknown Spectrum: Apex minus start of peak
Integration Events: ChemStation Integrator.autointl.e.

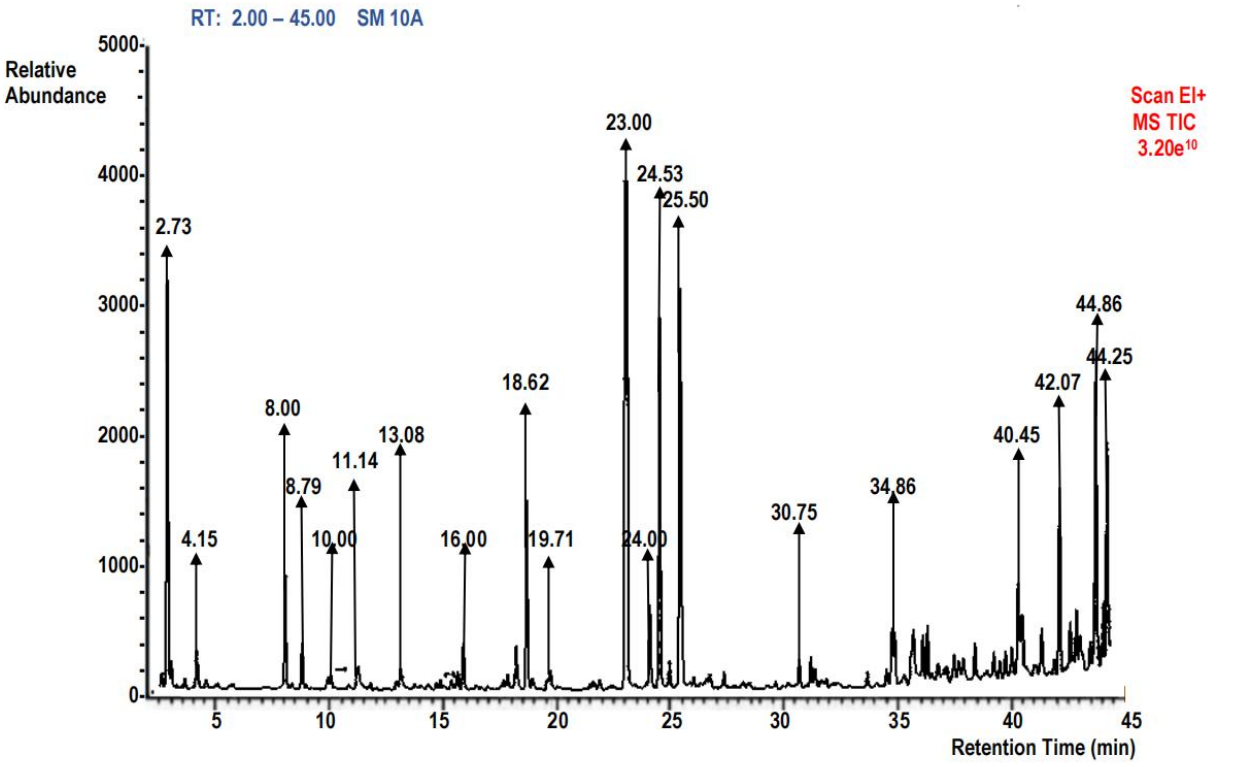


Figure 4.6: GC-MS chromatogram of ethanolic stem extract

4.7 Discussion

The phytochemical screening results presented in Table 4.1 revealed the presence of phenolic compounds, glycosides, saponins, flavonoids, steroids, terpenes, tanins and alkaloids. The presence of alkaloids, saponins, flavonoids, and other phytoconstituents in medicinal plants has been widely associated with their potential as valuable sources of therapeutic agents (Eriamiatoe *et al.,* 2020). The thin layer chromatographic (TLC) analysis revealed that various phytochemicals were detected across the different solvents used, highlighting the influence of solvent polarity on the extraction efficiency. Altering the polarity of the solvents proved effective in maximizing the detection of phytochemicals. Chloroform and ethanol extracts, in a 9:1 mixture, yielded the highest number of detectable compounds, while some phytochemicals were also identified using chloroform. TLC is commonly employed to aid in the identification of bioactive constituents in plant extracts. Among the four solvents tested—chloroform, hexane, chloroform-ethanol mixture 1 : 1 and hexane-chloroform mixture, chloroform-ethanol mixture was the most effective in extracting secondary metabolites. Table 4.7 present the Rf values obtained from the various solvents, offering insights into solvent polarity and assisting in the selection of an appropriate solvent system for further compound isolation through chromatographic and spectroscopic methods.

The present study also evaluated the analgesic potential of ethanolic stem extract of *Sida acuta* using two well-established animal models: the acetic acid-induced writhing test and the hot plate method, representing peripheral and central nociception respectively.

In the acetic acid-induced writhing test, Table: 4.3 shows writhing responses and statistical analysis of analgesic activity and Table:4.4, the extract demonstrated a profound dose-dependent reduction in the number of abdominal constrictions. At 100 mg/kg, the extract reduced writhes by 86.80%, a value identical to that of aspirin (86.80%), a standard non-steroidal anti-inflammatory drug (NSAID). At 200 mg/kg, the inhibition reached 97.00%, and at 400 mg/kg, the extract completely abolished the writhing response (100.00% inhibition), suggesting a highly potent peripheral analgesic effect as seen in figure 4.4. This model is known to be sensitive to compounds that interfere with the synthesis or action of prostaglandins and other inflammatory mediators (Barath, 2024). The significant inhibition of writhing observed implies that the extract may possess NSAID-like properties, possibly through the suppression of prostaglandin biosynthesis or modulation of inflammatory mediators.

In the hot plate test, which is primarily indicative of centrally mediated analgesia, Table: 4.2 shows time-dependent analgesic effect of plant extracts and pentazocine in mice. The extract also exhibited significant activity in a dose-dependent manner. Mice treated with 100, 200, and 400 mg/Kg of the extract showed marked increases in reaction time to thermal stimulus compared to the control group. At the highest dose (400 mg/Kg), the effect was comparable to that of pentazocine, a centrally acting opioid analgesic, suggesting that the extract may influence central nociceptive pathways. The increased latency time observed could be due to activation of central inhibitory mechanisms,

possibly involving opioid receptors, serotoninergic, or other neurotransmitter systems responsible for pain modulation (Kim *et al.*, 2025).

Taken together, the extract displays dual analgesic action, with significant peripheral and central components. The extract's efficacy at lower doses comparable to standard drugs, and its complete suppression of peripheral pain at higher doses, highlights its therapeutic potential. These findings support the presence of bioactive compounds within the extract that act through multiple mechanisms to relieve pain (Barath, 2024 ; Kim *et al.*, 2025).

From Table 4.9a, the ethanolic stem extract of *Sida acuta* showed characteristic absorption bands at 1045.83/cm (C-O stretch) of alcohols; 1644.15/cm (C= C bond) of alkenes; 2982.83/cm (O-H stretch) of carboxylic acid and 3442.91/cm (N-H bond) of primary amine. This result implies that the fraction is an organic base, the presence of N-H bond suggest that the compound might be an amide.

Phenolics are compounds characterized by multiple phenolic hydroxyl groups attached to aromatic rings. Common examples include flavonoids (e.g., quercetin, catechin), tannins, and phenolic acids (e.g., gallic acid, caffeic acid) (Konate and Souza, 2010). These compounds typically exhibit Broad O–H stretching bands in the range of 3400–3600 cm^{-1}, attributed to strong hydrogen bonding between hydroxyl groups. Multiple C–O and C=C stretching vibrations due to various aromatic and hydroxyl substitutions. A strong band around 1045 cm^{-1}, indicative of C–O stretching, typical of phenolic –OH groups. Aromatic C=C or conjugated C=O stretching vibrations around 1644 cm^{-1}, commonly observed in flavonoids and tannins. Aromatic C–H out-of-plane bending vibrations in the

region of 500–600 cm^{-1}, especially in substituted benzene rings. From Table 4.9c, the FTIR spectrum in figure : 15c aligns closely with those of phenolic compounds, particularly those that are aromatic, hydroxylated, and involved in hydrogen bonding—such as flavonoids, tannins, or phenolic acids.

The key Compounds with Potential Analgesic or Related Bioactivity includes Eugenol (Peak 5, RT 10.00) known for strong analgesic, anti-inflammatory, and anesthetic properties by inhibiting voltage-gated sodium channels and prostaglandin synthesis, reducing pain perception. Phytol (Peak 18, RT 42.07) is known for antinociceptive (pain-blocking), anti-inflammatory, and sedative effects (Benjumea *et al.,* 2016). This may be achieved through involvement in modulation of GABAergic pathways or inhibition of inflammatory mediators. Squalene (Peak 19, RT 44.86), known for antioxidant and membrane-stabilizing effects, which can support analgesia indirectly. Though not a primary analgesic, it can reduce oxidative stress associated with inflammation. Fatty acids and esters Examples: n-Hexadecanoic acid, 6-Octadecenoic acid, linolenic and linoleic acid esters known for anti-inflammatory properties which can contribute to analgesic activity by reducing the source of pain (Konate and Souza, 2010).

CHAPTER FIVE

5.1 CONCLUSION AND RECOMMENDATIONS

The findings of this study provide compelling evidence for the analgesic potential of the ethanolic stem extract of *Sida acuta*. Phytochemical screening, IR spectroscopic characterization, TLC, and GC-MS analyses revealed the presence of several bioactive compounds, including flavonoids, alkaloids, and saponins, which likely contribute to the observed pharmacological effects. The extract demonstrated significant, dose-dependent analgesic activity in both acetic acid-induced writhing and hot plate models, with comparable efficacy to standard analgesics such as aspirin and pentazocine. These results support the traditional use of *Sida acuta* in pain management and suggest its potential as a source of novel analgesic agents.

Recommendations:

1. Mechanistic Studies: Further research should investigate the specific pathways— peripheral and/or central—through which the extract exerts its analgesic effects.
2. Toxicological Evaluation: Comprehensive acute and sub-chronic toxicity assessments are essential to establish the extract's safety profile.
3. Bioactive Compound Isolation: Isolation and structural elucidation of the active constituents should be pursued to identify the most potent analgesic compounds.

4. Structure-Activity Relationship (SAR) Analysis: Understanding how chemical structure relates to analgesic activity will aid in optimizing pharmacological efficacy.

5. Formulation Development: Efforts should be made to formulate the extract or its active components into stable, standardized dosage forms.

6. Clinical Trials: Following successful preclinical validation, clinical trials should be conducted to confirm the efficacy and safety of the extract in human subjects.

7. Comparative Plant Part Studies: Investigating other parts of *Sida acuta* may reveal additional pharmacologically relevant compounds and expand its medicinal applications.

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APPENDIX

CHEMICAL METHOD (ACETIC ACID INDUCE WRITHING TEST)

Cage one: 5% tween 80 positive test

Mice	Weight (g)
1	24.90
2	24.40
3	23.60
4	22.20
5	25.40

Cage two 100mg/kg/50 Aspirin

Mice	Weight (g)	Volume of aspirin (ml)
1	-	
2	22.5	0.05
3	18.60	0.04
4	22.80	0.05
5	29.50	0.06

Cage three extract 200mg/Kg/100

Mice	Weight (g)	Extract volume(ml)	Acetic acid 10ml/kg
1	26.40	0.05	0.26
2	28.60	0.06	0.29
3	26.60	0.05	0.27
4	29.40	0.06	0.29
5	19.20	0.04	0.19

Cage four extract 400mg/Kg/100

Mice	Weight (g)	Extract volume(ml)	Acetic acid 10ml/kg
1	23.80	0.10	0.24
2	21.50	0.09	0.22
3	19.40	0.03	0.20
4	26.60	0.11	0.27
5	26.60	0.11	0.27

Cage five extract 100mg/Kg/100

Mice	Weight (g)	Extract volume (ml)	Acetic acid 10ml/Kg
1	23.00	0.02	0.23
2	25.40	0.03	0.25
3	26.60	0.03	0.27
4	27.50	0.03	0.28
5	27.3	0.03	0.27

Thermal Methods (Heat-Induced Pain) Hot plate test

Control Group (cage 1):

NO	WT(g)	0 (min)	30(min)	60(min)	90(min)	120(min)	150(min)	180(min)
1	28.8	6.4 s	6.6 s	7.0 s	6.8 s	5.6 s	8.2 s	9.2 s
2	22.7	6.2 s	5.8 s	7.5 s	5.4 s	7.5 s	9.9 s	7.1 s
3	16.6	6.2 s	4.9 s	8.6 s	7.0 s	6.2 s	6.8 s	7.5 s
4	20.5	6.2 s	5.8 s	5.7 s	6.4 s	7.4 s	4.8 s	5.6 s
5	29.1	6.1 s	5.8 s	6.1 s	6.4 s	5.0s	4.3 s	6.2 s
MEAN	23.54	6.22	5.78	6.98	6.4	6.34	6.8	7.12
SD		0.109	0.602	1.152	0.616	1.099	2.335	1.381
		5	5	5	5	5	5	5
±sem		0.049	0.269	0.515	0.276	0.492	1.045	0.618

Key: standard deviation (SD), standard error of mean (***±sem***)

Extract 100 mg/kg (cage 2):

NO	WT(g)	Doses(Ml)30	0(min)	30(min)	60(min)	90(min)	120(min)	150(min)	180(min)
1	38	0.038	5.9	6.7	6.4	5.9	8.6	4.8	5
2	37.6	0.038	5.8	10.1	10.8	10.5	6.4	12.9	9.1
3	23.7	0.024	7.2	15.1	15.4	6.5	7.2	5.1	3.8
4	29.9	0.03	5.5	9.5	9.4	7.4	6.7	6.3	5.7
5	29	0.03	6.1	8.9	10.5	7.2	6.5	6.3	5.7
Mean	31.64	0.032	6.1	10.06	10.5	7.5	7.08	7.08	5.86
±sem		0.003	0.292	1.384	1.451	0.795	0.404	1.486	0.881

Extract 200 mg/kg (cage 3):

N0	WT(g)	Dose(ml)	0(min)	30(min)	60(min)	90(min)	120(min)	150(min)	180(min)
1	31.8	0.07	4.5	10.8	7	10.3	6.2	5	9.9
2	33.2	0.07	7.2	7	7	6.2	6.2	5	6.5
3	34.5	0.07	4.9	10.2	8.6	7.5	7.6	5	7.7
4	31.7	0.06	5.2	7.1	8.7	6.2	6.2	5	7.9
5	29.8	0.06	4.2	7.4	6.9	6.6	4.8	5.1	7.9
SD	32.2	0.066	5.2	8.5	7.64	7.36	6.2	5.02	7.98
±sem			0.52	0.82	0.41	0.77	0.44	0.02	0.55

Extract 400 mg/kg (cage 4)

NO	WT(g)	Doses(Ml)	0(min)	30(min)	60(min)	90(min)	120(min)	150(min)	180(min)
1	25	0.1	4.5	10.4	11.3	8.8	7.8	8.2	12.2
2	24.8	0.1	4.5	7.9	12.6	9.9	10	14	14.3
3	22.6	0.9	4.4	17.8	15.3	14.6	8.1	11.6	15
4	22.5	0.9	4.3	8.1	12.9	8.8	6.8	9.3	10.1
5	21.3	0.9	4.4	7.8	12.5	7.5	6.3	10.8	9.6
SD	23.24	0.58	0.084	4.274	1.463	2.751	1.430	4.972	2.419
MEAN	23.24	0.58	4.42	10.4	12.92	9.92	7.8	10.78	12.24
±sem			0.037	1.91	0.65	1.23	0.64	2.22	1.08

CAGE 5: PENTAZOCINE 0.1mg/Kg STANDARD DRUG

NO	WT(g)	Dose(Ml)	0(min)	30(min)	60(min	90(min)	120(min)	150(min)	180(min)
1	24.8	0.25	4.4	9.2	8.8	11.3	6.4	7.2	9.1
2	23.1	0.23	4.5	9.1	15.5	7.9	11.2	10.7	8.7
3	18.3	0.18	3.3	9.1	10.5	6.2	7.3	5.2	8.6
4	23.3	0.23	4.9	10.4	11.2	7.3	13.3	5.4	8.1
5	20.1	0.2	5.6	14.4	6.8	8.2	9.5	7.1	8.6
Mean	21.92	0.218	4.54	10.44	10.56	8.18	9.54	7.12	8.62
SD	2.646	0.027	0.838	2.281	3.242	1.904	2.818	2.206	0.356
±sem			0.37	1.02	1.46	0.85	1.26	0.99	0.16

Gas Chromatography-Mass Spectrometry (G C-MS) Analysis of Bioactive Compounds

Introduction

Chromatography is an important analytical tool that allows for the separation of components in a gas mixture. GC is a common type of chromatography used to separate and analyze compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance or separating the different components and relative amounts of different components of a mixture. GC can also be used to prepare pure compounds from a mixture. GC-MS uses two techniques that are combined into a single method for analyzing mixtures of chemicals. Gas chromatography separates the components of a mixture, and mass spectroscopy characterizes each of the components individually. Combining the two techniques helps to analyze the samples both qualitatively and quantitatively. As the sample is injected into the chromatograph, the sample mixture gets separated into individual components due to different flow rates. This results in quantitative analysis of the components, along with a mass spectrum of each component.

Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. Strengths of GC/MS analysis are (a) identification of organic components from complex mixtures, (b) quantitative analysis, and (c) determination of traces of organic contamination.

Gas chromatography mass spectroscopy (GC-MS), a hyphenated system which is a very compatible technique and the most commonly used technique for the identification and quantification purpose was used. The unknown organic compounds in the complex mixture can be determined by interpretation and also by matching the spectra with reference spectra.

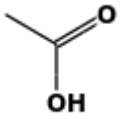
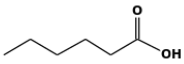
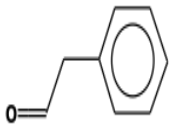
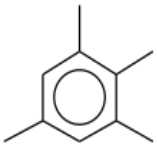
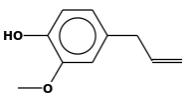
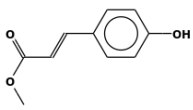
Instrumentation and methodology of GC-MS analysis

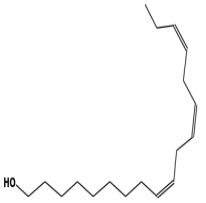
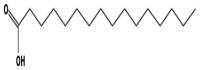
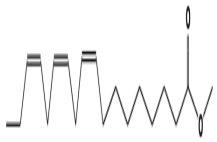
GC-MS is a unique analysis technique used for identification and quantification which is limited to analytes that are not only volatile and thermally labile but can also withstand the harsh partitioning conditions of the gas chromatograph. A representative spectral output of all the ascertainable compounds from the empirical sample is displayed by this technique. The Gas-chromatography device has an injection port from where the process is initiated by injecting the sample to that port. After this, evaporation and separation of the components take place one by one and finally this equipment identifies the components present in the corresponding sample solution then transferred in GCMS vial for analysis.

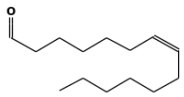
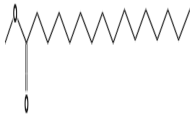
GC-MS analysis was carried out on a GC system using a Varian 3800/4000 gas chromatograph mass spectrometer equipped with an Agilent capillary column employing

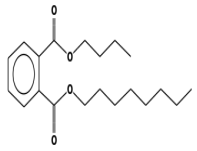
the following conditions: Column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) as carrier gas at a constant flow of 1ml/ minute and a sample injection volume of 1 µl which was employed (split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 minutes), with an increase of 10°C/minute, to 200°C, then 5°C/minute to 280°C, ending with a 9 minutes isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 800 Da. Total run time was 43 min. The compounds were then identified from the GC-MS peaks, using library data of the corresponding compounds. GC-MS was analyzed using electron impact ionization at 70 eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library using NIST Search. The relative % amount of each component was calculated by comparing its average peak area to the total areas. Measurement of peak areas and data processing were carried out by chemStation software

The was subjected to chromatographic analysis using a Varian 3800/4000 gas chromatograph mass All the peaks were identified based on mass spectral matching (≥ 90%) from both the NIST and Wiley libraries. Only compounds with 90% or greater spectral matching accuracy are reported. No response factors were calculated.

Peak #	RT	Compound Detected	Mol. Formula	MW	Peak Area %	Comp %wt	m/z	Structures
1	2.73	Acetic acid	$C_2H_4O_2$	60	9.95	3.84	43, 45, 60	
2	4.15	Hexanoic acid	$C_6H_{12}O_2$	116	1.15	2.73	41, 60, 116	
3	8.00	Benzeneacetaldehyde	C_8H_8O	122	3.01	3.41	65, 91, 122	
4	8.79	Benzene, 1,2,3,5-tetramethyl-	$C_{10}H_{14}$	134	2.21	1.90	77, 119, 134	
5	10.00	Eugenol	$C_{10}H_{12}O_2$	164	0.30	1.21	55, 77, 164	
6	11.14	2-Propenoic acid, 3-(4-hydroxyphenyl)-, methyl ester	$C_{10}H_{10}O_3$	178	0.60	1.18	65, 147, 178	
7	13.08	2-Pentadecanol	$C_{15}H_{32}O$	228	5.43	4.42	43, 45, 228	

Peak #	RT	Compound Detected	Mol. Formula	MW	Peak Area %	Comp %wt	m/z	Structures
8	16.00	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	$C_{18}H_{32}O$	264	1.20	1.62	41, 79, 264	
9	18.62	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.52	4.19	43, 73, 256	
10	19.91	Z,Z-2,13-Octadecadien-1-ol	$C_{18}H_{34}O$	266	5.13	4.21	41, 55, 266	
11	23.00	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	11.06	14.72	41, 79, 294	
12	24.00	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	292	3.81	4.41	41, 79, 292	

Peak #	RT	Compound Detected	Mol. Formula	MW	Peak Area %	Comp %wt	m/z	Structures
13	24.53	7-Tetradecenal, (Z)-	$C_{14}H_{26}O$	210	8.64	9.26	41, 56, 210	
14	25.50	6-Octadecenoic acid, (Z)-	$C_{18}H_{34}O_2$	282	9.55	12.53	41, 55, 282	
15	30.75	Hexadecanoic acid, methyl ester	$C_{18}H_{32}$	270	0.90	1.41	41, 67, 270	
16	34.86	9,12-Octadecadienoic acid, ethyl ester	$C_{20}H_{36}O_2$	308	2.51	3.22	41, 67, 308	

Peak #	RT	Compound Detected	Mol. Formula	MW	Peak Area %	Comp %wt	m/z	Structures
17	40.45	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	334	4.41	5.46	41, 149, 334	
18	42.07	Phytol	$C_{20}H_{40}O$	296	6.13	6.10	43, 71, 296	
19	44.86	Squalene	$C_{30}H_{50}$	410	10.85	7.31	41, 69, 410	
20	44.25	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	444	7.63	6.24	73, 341, 444