ASSESSMENT OF FREE RADICAL SCAVENGING PROPERTY, ANTIMICROBIAL ACTIVITY AND DIURETIC EFFECT OF *HEDYOTIS SCANDENS* ROXB. (RUBIACEAE)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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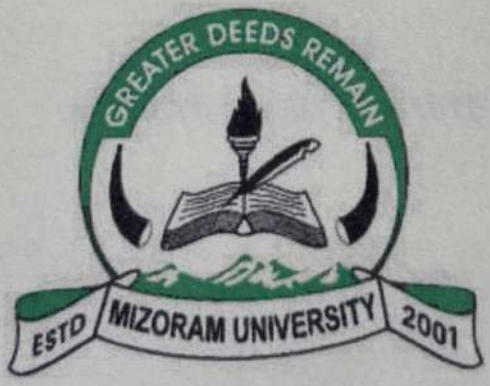

DEPARTMENT OF BOTANY
SCHOOL OF LIFE SCIENCES
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ASSESSMENT OF FREE RADICAL SCAVENGING PROPERTY, ANTIMICROBIAL ACTIVITY AND DIURETIC EFFECT OF *HEDYOTIS SCANDENS* ROXB. (RUBIACEAE)

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In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Botany of Mizoram University, Aizawl

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CERTIFICATE

This is to certify that this study **"Assessment of free radical scavenging property, antimicrobial activity and diuretic effect of *Hedyotis scandens* Roxb. (Rubiaceae)"** submitted by Elizabeth Vanlalruati Ngamlai (MZU/Ph.D/1270 of 03.09.2018) in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Botany is a record of bonafide work carried out by her under my supervision and guidance.

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I ELIZABETH VANLALRUATI NGAMLAI, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the **Degree of Doctor of Philosophy in Botany.**

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I dedicate this work to my mother Lallianmawii Haolai, my father Lalronghaka Ngamlai, my siblings Esther Vanlaldinpuii and Isaac Lalnunzira.

(ELIZABETH VANLALRUATI NGAMLAI)

CONTENTS

LIST OF TABLES

LIST OF FIGURES:

LIST OF ABBREVIATIONS:

ABTS	2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
ATCC	American Type Culture Collection
ALT	Alanine transaminase
ACE	Angiotensin-converting-enzyme BSTFA+TMCS N, O-Bis (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane
b. wt	body weight
Cl^-	Chloride
CKD	Chronic Kidney Disease
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMSO	Dimethyl sulfoxide
et al	*et alia*
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography Mass Spectroscopy
GFR	Glomerular Filtration Rate
HSPE	*Hedyotis scandens* petroleum ether extract
HSCE	*Hedyotis scandens* chloroform extract
HSME	*Hedyotis scandens* methanol extract
HSAE	*Hedyotis scandens* aqueous extract

K^+	Potassium
$Mg2^+$	Magnesium
ml	Microgram
µg	Microliter
µl	Milliliter
Na^+	Sodium
NaNO2	Sodium nitrate
NaOH	Sodium Hydroxide
NIST	National Institute of Standards and Technology
NS	Nephrotic Syndrome
Ph	Phenyl
QE	Quercetin equivalent
RAAS	Renin-angiotensin-aldosterone system
RNS	Reactive Nitrogen Species
SIDH	Antidiuretic hormone secretion
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase

CHAPTER-1

INTRODUCTION

1.1. General

Medicinal plants hold great significance for people not just as medicine, but also as fundamental constituents for the development of novel drugs and have been used in curing many diseases (Fischer and Ganellin, 2010). Many plants have been employed in traditional medicine for a long time. Although, few of these plants appear to have beneficial effects, the lack of adequate scientific data hinders the confirmation of their efficacy. Nonetheless, these plants can still be considered medicinal plants based on their traditional usage. Plants are selected for medicinal purposes for a variety of reasons, including tradition, efficacy, abundance, accessibility, doctrine of signatures, and taxonomic affiliation (Bennett and Husby, 2008; Phumthum *et al*., 2018). In some rural colonies where access to pharmaceuticals might be difficult or unavailable, reliance on medicinal plant is prominent. However, in the western communities' medicinal plants are recommended as a supplement or as substitute (Anonymous, 2013). Medicinal plants have many characteristics when used for treatment such as:

Preventive medicine- Some plants have been shown to have properties that can help prevent or treat certain diseases, and these natural remedies can be a more cost-effective and sustainable alternative to chemical therapies (Jepson *et al*., 2012)

Support of conventional medicine- Some plants contain compounds that can interact with chemical products in ways that enhance their desired effects or mitigate their negative effects. For example, some plants may contain compounds that can enhance the absorption or bioavailability of certain drugs, making them more effective at treating a particular condition. One well-known example of a plant-based compound that can enhance the effects of a chemical product is piperine, which is found in black pepper. Piperine has been shown to increase the bioavailability of a variety of drugs and nutrients, including curcumin (a compound found in turmeric),

resveratrol (found in grapes), and some vitamins. Other plants may contain compounds that can mitigate the negative effects of certain chemicals or drugs. For example, some studies suggest that milk thistle (*Silybum marianum*) may be effective at reducing liver damage caused by certain toxins and drugs, such as acetaminophen (Abenavoli *et al*., 2010; Atal *et al*., 1985; Shoba *et al*., 1998).

Synergic effect- Each plant contains a diverse array of bioactive substances that might interact simultaneously, which might produce a greater effect than the sum of their individual effect. However, there can also be antagonistic interactions between compounds, in which one compound reduces or negates the effect of another (Jia *et al*., 2009; Rather *et al*., 2013).

According to Newman and Craig, more than 30% of drugs used between 1981 and 2019 came from natural sources, with attention on compounds originating from plants. The existence of secondary compounds in plants is responsible for its therapeutic property and these secondary metabolites maybe present in the whole plant or in any part of the plant. Bioactive compounds reported from plants are usually a combination of secondary metabolites (Newman and Cragg, 2020). Scientific evidence of the therapeutic properties of a number of medicinal plants is limited, even though they have been used for centuries to treat ailments. The conventional employment of plants as medicine is not arbitrary, but is somewhat influenced by their taxonomic classification. This may be attributed to a range of factor, including unique evolutionary adaptations or environmental factors that favor the manufacture of bioactive compounds in certain species. It is worth noting that the relationship between species diversity and the number of medicinally useful species is not necessarily a linear one, and may vary depending on the specific ecosystem being studied (Moerman, 1996). The advantage of using plants as an alternative for synthetic medicine is its cost effectiveness and worldwide accessibility.

1.1.1. Advantages of Medicinal plants over synthetic drugs

Additional advantages of using medicinal plants include their relative safety, as they usually have lower toxicity than synthetic drugs, and the potential for fewer

side effects (Ekor, 2014). The utilization of medicinal plants also boasts a lengthy history, providing a wealth of traditional knowledge that can be valuable for guiding modern research and drug development. Moreover, the sustainable use of medicinal plants can support local economies and promote conservation of biodiversity (Sofowora *et al*., 2013). However, the use of medicinal plants also has limitations and challenges. Some plants may have toxic or harmful effects if used improperly, and their quality and potency can vary depending on factors such as growing conditions and harvesting practices (Van Wyk *et al*., 2020). Additionally, the regulatory framework for herbal medicines can be complex and inconsistent across different countries, leading to concerns about safety and efficacy (Ekor, 2014b; Anonymous, 2002). To overcome these challenges, conducting thorough scientific research is crucial for confirming the therapeutic attributes and establishing consistent protocols for their preparation and quality assurance. Collaboration between traditional healers, researchers, and healthcare professionals can also help to integrate the use of medicinal plants with mainstream medicine, which helps guarantee their secure and efficient utilization.

Alternative medicine is the utilization of plants for healing purpose and it has been used around the world in all cultures, especially in the Asian and European cultures. The presence of many plant species possessing medicinal properties has given rise to specialized fields such as medicinal herbalism. In numerous countries, this field of science has been instrumental in driving substantial progress in traditional medicine. This field of science has been instrumental in advancing folk medicine significantly in numerous nations. However, the use of each of these plants relies on where it is consumed and the people that use them (Mohammed, 2019; Rajan *et al*., 2020).

Sharma (2011) reported that a significant portion of the worldwide population depend on plants and their crude products for healthcare needs and over 30% of all plant species have been applied for medicinal purposes at different points in time. In developed countries, plant-based drugs make up around a quarter of the total drugs available, although in rapidly flourishing countries, this contribution can reach as

high as 80%. Consequently, the economic significance of medicinal plants holds far greater importance for developing countries in comparison to the developed countries.

1.1.2. Utilization of plants as medicine

The employment of plants as medicine is an indispensable part of human history. Since olden times, people have instinctively turned to nature in their quest to find remedies to cure ailments, seeking medicinal plants as a source of relief. This practice mirrors the innate behavior observed in animals. During those days, there was no sufficient information on the cause of the illness, or the utilization of plants that could cure the illness, everything was down to experience. However, over time, the rationale for using various medicinal plants to treat specific ailments began to emerge, resulting in a transition from empirical techniques to a more scientifically grounded knowledge. As a result, the usage of therapeutic plants shifted from an instinctual to an explanatory framework. A Sumerian clay slab from Nagpur, believed to be 5000 years old, provides the oldest recorded documentation of the use of medicinal plants for drug preparation (Kelly, 2009). Around 6000 years ago, the Egyptians had established a sophisticated and effective pharmacological system that relied on natural resources. The primary method of treatment recommended in their medical papyri was the use of drugs derived from a wide range of animal, mineral and plant substances. They prepared plant extracts that could be taken internally or applied topically, and they also administered them though fumigation or vapor inhalation (Nunn, 1996). Oakes and Gahlin (2003) emphasized that the Egyptians were pioneers in utilizing several drugs that modern research has subsequently proven to have medicinal effectiveness. According to the ancient Indian holy book known as the Vedas chronicles the use of natural remedies derived from the region's bountiful flora. India, in particular, has been a source of several spice plants that are still widely used today, such as nutmeg, pepper, clove, and many more (Tucakov, 1971). Information available from the Bible and Talmud, a sacred Jewish scripture, aromatic plants such as myrtle and incense were used during many events linked with healing practices (Dimitrova, 1999).

Theophrastus (371-287 BC) is credited with establishing the foundation of botanical science though his notable works, namely “De Historia Plantarum” (Plant History) and “De Causis Plantarum” (Plant Etiology). Within these books, he meticulously classified over 500 medicinal plants that were known during that period (Pelagic, 1970). Monkshood, cardamom, iris rhizome, fragrant hellebore, pomegranate, false hellebore, mint and cinnamon were among the therapeutic plants widely chronicled by Theophrastus. Notably, he emphasized the importance of gradually increasing the dose in order for humans to develop tolerance to the poisonous effects of these plants. Because of his extraordinary contributions to identifying and describing medicinal plants, he was dubbed "the father of botany" for his profound insights into these disciplines (Bazala, 1943).

When it comes to writing about plant drugs, Dioscorides, renowned as "the father of pharmacognosy," was a notable figure in ancient history. While accompanying Nero’s Army as a physician and phytochemist, he researched medicinal plants thoroughly. Dioscorides wrote the famous treatise "De Materia Medica" in 77 AD. This ancient treatise, which has been translated multiple times, has a wealth of information about medicinal plants that served as the foundation of the Materia Medica during the late Middle Ages and Renaissance (Thorwald, 1991). Traditional Chinese medicine and Ayurveda, which are two surviving forms of ancient medicine, have significantly contributed to the existing body of knowledge concerning medicinal plants (Patwardhan *et al*., 2005).

Throughout history, various cultures such as those in Africa, Ethiopia, China and India have relied on medicinal properties of plants. In Africa, the understanding surrounding plants with medicinal properties has been traditionally transmitted orally from one lineage to the next, resulting in a wealth of African traditional medicine that remains largely undocumented. In contrast, Chinese and Indian traditional medicine have benefited from extensive documentation in books, and are now readily accessible online. To create a comprehensive compilation of medicinal lants for disease prevention, it is crucial to gather original data from the custodians of traditional knowledge in order to bridge the existing gaps (Tan *et al*., 2010).

1.1.3. **Significance and role of plants as antioxidants**

Reactive oxygen species (ROS) or free radicals are exceedingly reactive entities generated by cells as part of their function in cell mediated immunity and the process of respiration (Chang *et al*., 2012). They can also originate from alternative sources such as automobile emissions, pesticides, radiation and so forth (Masoko and Eloff, 2006). In low to moderate concentrations ROS has a beneficial role in functions such as gene expression, defense against mechanism, cellular growth etc. Nevertheless, when the ROS level in the organism surpasses its capacity to supply sufficient antioxidant for protection, it gives rise to a condition known as oxidative stress. Oxidative stress has the potential to contribute to a wide range of diseases like arthritis, inflammation, cancer, coronary heart disease, lung damage and diabetes (Tochhawng *et al*., 2013; Valko *et al*., 2007).

Cells typically have antioxidant enzymes and molecules which effectively manage usual production of ROS. Although, these agents prove to be inadequate in restoring the redox balance during episodes of oxidative stress. In normal circumstances, cells have their defenses in the form of antioxidant enzymes, and molecules capable of managing the usual generation of ROS. Nonetheless, these mechanisms fall short when it comes to restoring the redox balance during episodes of oxidative stress. Hence, to reinstate the cellular redox balance, there may be a need for the external provision of antioxidants (Seifried, 2006). Similar to ROS, reactive nitrogen species, (RNS) are also generated as part of regular cellular metabolism. Nitric oxide (NO) is considered a free radical due to its possession of an unpaired electron, enabling it to make interactions with some specific proteins and other free radicals, including superoxide. Prolonged exposure to nitric oxide can develop certain conditions, including juvenile diabetes, arthritis, various forms of cancer and multiple sclerosis (Amaeze *et al*., 2011; Kumar *et al*., 2010). Nitric oxide scavengers decrease the generation of nitrite ions by engaging in competition with oxygen (Ebrahimzadeh, 2010).

Plants produce phytochemicals, or bioactive substances, primarily for self-defence. Over a thousand have been identified to date and are found in a wide range

of foods, like fruits, nuts, vegetables, herbs and whole grains. Some important families of phytochemicals are polyphenols, carotenoids, phytosterols, isoprenoids, dietary fibres and saponin and certain polysaccharides. These phytochemicals are known to possess a variety of effects, including antioxidant, anthelmintic, antidiarrheal, antibacterial, antiallergic, antispasmodic, and antiviral activities (Jaeger and Cuny, 2016; Sharma *et al*., 2018)

Antioxidants safeguard against oxidative stress by actively seeking out and neutralizing free radicals, inhibiting lipid peroxidation, and utilizing a range of other mechanisms for defence, which helps in impeding the advancement of diseases. Plant-derived natural antioxidants have gained significant popularity as a preferable and safer alternative to synthetic antioxidants in various applications (Mandal Sourav, 2011). Plant antioxidants not only protect cells from the detrimental impacts of free radicals but also decelerate the apoptotic process, thereby hindering additional harm (Chen, 2018). Boosting the body's natural antioxidant defences and integrating dietary antioxidants as supplements can reduce the risk of persistent illnesses and prevent the advancement of diseases. The pursuit of products having great antioxidants with minimum side effects is a vibrant area of research. Consequently, there is a strong desire to develop and employ natural-origin antioxidants that are more potent. Over the recent years, there has been research into the antioxidant and free radical neutralization attributes of natural antioxidants derived from certain plants.

Research into products having antioxidant properties with minimum negative reaction is an actively pursued area. Consequently, there is a keen interest in advancing and utilizing more potent antioxidants of natural origin. (Anagnostopoulou *et al*., 2006; Braughler, 1986; Stanner *et al*., 2004)

Over recent years, investigations on natural antioxidants derived from plants have been conducted to assess their capabilities in terms of antioxidant activity and radical scavenging potential. Although synthetic drugs have long been dominant in the field of drug discovery and manufacturing, bioactive plants still hold significant promise for offering novel and innovative therapeutic treatments. In the realm of

scientific inquiry, there is a strong current focus on antioxidant products that exhibit reduced side effects. Consequently, the pursuit of more effective natural antioxidants is highly desirable. Plants synthesize numerous antioxidant compounds, present in varying concentrations, and exhibiting a diverse array of chemical and physical properties. Antioxidants play a crucial role in neutralizing free radicals, thereby serving as a preventive measure against numerous diseases (Anagnostopoulou *et al*., 2006; Braughler, 1986; Hu *et al*., 2009; Patel *et al*., 2010; Sharma *et al*., 2013; Stanner *et al*., 2004).

1.1.4. Plants as antimicrobial agents

Human microbial infections represent a highly critical issue, with bacteria and fungi being the most prevalent pathogens responsible for these infections. Illnesses provoked by these microorganisms stand as the foremost global cause of mortality. Diseases caused by these microbes are the leading cause of death worldwide (Bhogavalli and Chenreddy, 2012). There has been a noticeable emergence of multi-resistant variants among clinically important pathogens in recent times, leading to a surge in strains that are resilient to antibiotics (Pillai, 2014). Plant secondary metabolites can be used as antimicrobial agents in pharmaceutical sciences where the present antibiotics fail to perform their duty due to multidrug resistance (Appendino *et al*., 2014; Seukep *et al*., 2020).

Plant extracts, when combined with conventional antibiotics, can enhance antimicrobial activity. Secondary metabolites from plants show promise as effective antimicrobial substance in the field of pharmaceutical sciences, particularly when faced with multidrug-resistant strains that challenge the efficacy of current antibiotics. Furthermore, the co-administration of plant extracts with conventional antibiotics can potentiate their antimicrobial effects (Dudhatra *et al*., 2012; Jeyanthi *et al*., 2013; Mawalagedera *et al*., 2019; Tatiraju *et al*., 2013). Clinical microbiologist s have a dual incentive to be intrigued by the subject of antimicrobial plant extracts. Firstly, there is a high likelihood that these phytochemicals will become part of the array of antimicrobial medications prescribed by medical professionals, with several already undergoing testing (Clark, 1996).

In developing countries, food poisoning is recognized as a leading contributor to illness and mortality. Majority of food borne illnesses are linked to bacterial contamination, particularly from gram-negative bacteria such as *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli*. Certain gram-positive bacteria, such as *Staphylococcus aureus*, have also been recognized as responsible agents for foodborne illnesses and food spoilage (Braga *et al*., 2005; Doughari *et al*., 2007; Khan *et al*., 2013; Solomakos *et al*., 2008).

1.2. Diuretics

Diuretics are drugs that pharmacologically alter the kidneys' fluid regulatory balance, facilitating the excretion of electrolytes and water. Consequently, they enhance urine production and volume. This category of drugs mainly achieves this by blocking the receptors that allow sodium, the most common extracellular cation, to be reabsorbed in the renal tubules. It thereby raises the osmolality in the renal tubules, which lowers the absorption of water (Donato *et al*., 2014; Wile, 2012).

The majority of diuretics lead to water loss as they enhance sodium and chloride ion elimination. Increasing urine volume causes blood volume to decrease, making diuretics useful for conditions such as hypertension and edema, especially when fluid retention is a concern. In a range of clinical conditions, including cirrhosis, nephrotic syndrome, renal failure, and heart failure, they are essential for maintaining the composition of body fluid. Accumulation of bodily fluids is due to the kidney’s incapability to release sodium followed by excretion of water. Sodium and water initially undergo filtration within the glomerular capsule of the nephron and are subsequently taken back into the bloodstream at various points along the nephron. Diverse categories of diuretics hinder sodium reabsorption at distinct locations though varied mechanisms, consequently leading to an increase in sodium excretion.

1.2.1. Importance of diuretics

Originally, diuretics were primarily used to adjust the volume of bodily fluids and ion levels. As a result, utilizing them either on their own or alongside particular

therapeutic regimens proves greatly advantageous in addressing hypertension, cardiovascular conditions, and, more broadly, in managing edema in individuals (Arumugham and Shahin, 2023). Lately, diuretics have found indications in conditions like type 2 diabetes, obesity and the management of metabolic irregularities. Chemically speaking, diuretics encompass a diverse range of molecules that operate in different ways (Scozzafava *et al*., 2013; Supuran, 2011). Diuresis is important for both edematous and non-edematous illnesses since it aids in the removal of extra water from the body, especially when fluid is excessively held in the third space and causes edema. Maintaining euvolemia and electrolyte balance is paramount for survival given that water constitutes for approximately 60% of the average adult body weight and is involved in various physiological functions. Any interruption in this equilibrium can have serious effects, emphasizing the need of fluid and electrolyte homeostasis (Roumelioti *et al*., 2018a).

Though a variety of physiological systems, the management of total body fluid balance ensures a careful balance between fluid intake and loss. These include the elimination of salt and water, vasopressin and natriuretic peptide hormone control, skin-related management, hemodynamic adaptations, and brain regulation of thirst. Notably, the renal excretion of urine is essential for maintaining fluid homeostasis since it not only gets rid of extra water but also metabolic waste products and extra electrolytes from the body (Popkin *et al*., 2010; Roumelioti *et al*., 2018b). Edematous conditions ultimately lead to heart failure (HF) wherein the heart's compromised pumping efficiency gives rise to various ailments such as:

1. Congestion, characterized by increased weight, difficulty breathing, and widespread swelling due to prolonged venous stasis and fluid leakage into the interstitial area, results from prolonged venous stasis (Miller, 2016a).

2. Renin-angiotensin-aldosterone system (RAAS) activation is caused by decreased renal blood flow (Miller, 2016b).

Pulmonary edema, frequently arising due to HF, is an indication for the administration of diuretics. Loop diuretics are the key component of diuretic therapy

used to treat symptomatic heart failure. Most prevalent among them is furosemide. However, Torsemide remains relatively underutilized despite its superior pharmacological properties. Transform-HF, a comparative study assessing the merits of these diuretics, is currently in progress. Both the New York Heart Association (NYHA) and the European Society of Cardiology endorse these recommendations (Mullens *et al*., 2019; Shah *et al*., 2017; Sica *et al*., 2017).

Diuretics in combination with salt restriction are also used to treat liver cirrhosis-induced ascites (Haberl *et al*., 2018). Spironolactone is the drug of choice for the first management of ascites in cirrhotic patients, primarily because of its antiandrogenic effects. Loop diuretics may be used as an adjuvant to spironolactone, or in combination with it to improve results (Garbuzenko and Arefyev, 2019; Moore and Aithal, 2006). Higher mortality rates are associated with elevated fluid levels in individuals with renal insufficiency or acute kidney injury. Initially, loop diuretics are prescribed to treat this problem, but renal replacement therapy is better in the long run (Khan *et al*., 2016; Sica, 2012). In Nephrotic Syndrome (NS), low albumin levels, proteinuria, and elevated lipid levels lead to edema, which requires diuretic treatment (Sica, 2012).

Thiazide-like diuretics are associated with reduced blood pressure (BP) on account of direct vasodilatory effects. Thiazides are still recommended in cases where hypertension and chronic kidney disease (CKD) coexist or when the glomerular filtration rate (GFR) falls below 30 mL/min. Additionally, potassium-sparing diuretics are useful in people suffering from hypertension with potassium (K^+) or magnesium (Mg^{2+}) losses (Blowey, 2016; McNally *et al*., 2019; Misha, 2016; Roush and Sica, 2016; Scheen, 2018). Diabetes insipidus, characterized by excessive urination, leads to the excretion of diluted urine accompanied by low sodium levels. Interestingly, thiazide diuretics can offer assistance by enhancing the excretion of sodium in the distal tubules, which in turn triggers compensatory sodium and water retention in the proximal tubule. This, however, compromises the kidneys' ability to achieve maximum dilution (Loffing, 2004).

In the realm of medical treatment for elevated intracranial pressure following injury to the brain caused by trauma and swelling within the cerebral region, osmotherapy takes centre stage. Hyperosmolar therapy involving mannitol swiftly alleviates heightened ICP in under an hour, although there is a potential for a rebound effect, which may initially lead to increased ICP. In cases of acute renal failure, mannitol also promotes diuretic effects, assisting in the removal of harmful metabolites and chemicals (Witherspoon and Ashby, 2017). As a secondary approach, loop diuretics in combination with salt tablets are applied to reduce urine concentration in cases of syndrome of inappropriate antidiuretic hormone secretion (SIADH) (Yasir and Mechanic, 2023). In this context, the primary treatment method involves limiting water intake. Bumetanide, a loop diuretic, has demonstrated effectiveness as a treatment for seizures. In addition to other disorders of the central nervous system such autism and schizophrenia, it is used to treat temporal lobe epilepsy (Witherspoon and Ashby, 2017).

The use of diuretics dates back to ancient times. In the 17th century, diuretics became more common in Europe as physicians started prescribing substances such as digitalis, squill, and mercury chloride to treat edema and heart failure. In the 20th century, the discovery of thiazide diuretics, such as chlorothiazide, revolutionized the management of heart failure and high blood pressure. The development of loop diuretics, such as furosemide, in the 1960s further expanded the range of conditions that could be treated with diuretics. These medications demonstrate notable efficacy in addressing edema linked to conditions such as heart failure, liver disease, and certain ailments. Mercurials were the most effective diuretics from 1919 to 1960, but their toxicity led to their discontinuation. Other diuretics used during this period included osmotic diuretic such as mannitol, urea, and sucrose, acidifying salts such as xanthene derivatives, ammonium chloride, and digoxin. In 1937, sulphanilamide was found to be a carbonic anhydrase inhibitor, when patients treated with it produced alkaline urine in addition to increase in sodium and urine excretion (Wile, 2012). From then on, the search for better and more effective diuretic with lesser side effects began which led to the discovery of thiazide, thiazide-like diuretics, loop diuretics, potassium sparing and osmotic diuretics. Other diuretics used during this

period included osmotic diuretics such as mannitol, urea, and sucrose, and acidifying salts like xanthene derivatives, ammonium chloride, and digoxin. There are several classes of diuretics. Each class of diuretics works by targeting different mechanisms involved in sodium and water reabsorption in the kidney. Each of these diuretics has a different mode of action as follows:

Thiazide diuretics: The mechanism of action of these diuretics is suppressing the Na^+/Cl^- cotransporter in the early part of the distal convoluted tubule, which leads to increased excretion of sodium, chloride, and water are commonly used to treat hypertension and edema associated with heart failure, kidney disease, and liver disease (Salvetti and Ghiadoni, 2006).

Loop diuretics: These diuretics function by blocking the sodium-potassium-chloride symporter in the ascending portion of Henle's loop, resulting in the excretion of sodium, chloride, and potassium ions in urine. These are used to treat edema associated with heart failure, kidney disease, and liver disease, as well as hypercalcemia and hyperkalemia (Ellison and Felker, 2017).

Potassium-sparing diuretics: Potassium-sparing diuretics operate by inhibiting the Na^+/K^+ ATPase pump in the final sections of the renal tubule and the duct system, which leads to elevated sodium excretion while minimizing potassium excretion. It is used to treat hypertension and edema, especially in patients with low potassium levels (Horisberger and Giebisch, 1987a).

Osmotic diuretics: These diuretics work by increasing the osmotic pressure of the blood, which draws water into the kidneys, resulting in increased urine production. It is used to decrease intracranial and eye pressure or IOP, as well as to promote urine production in acute renal failure (Dhawan, 2019).

Carbonic anhydrase inhibitors: These diuretics function by suppressing the enzyme carbonic anhydrase in the proximal tubules of the kidneys, which results in decreased reabsorption of bicarbonate ions and increased excretion of bicarbonate ions and other electrolytes. They are used to treat glaucoma, altitude sickness, and epilepsy (Karioti *et al*., 2016).

Diuretics are typically employed for the management of conditions like fluid retention, hypertension, heart failure and disorders affecting the kidneys and liver. However, their use must be carefully monitored, as they can cause electrolyte imbalances, dehydration, and other adverse effects. A number of side effects have been reported from the use of these synthetic drugs and therefore the pursuit for more novel diuretic involving plants have been going on in the scientific world.

1.2.2. Significance of natural diuretics

Synthetic diuretics usually have the tendency to project negative effects like metabolic alterations and electrolyte imbalance. Natural diuretics normally do not have adverse effects and moreover crude drugs are easily available as well as comparatively cheaper (Abdel-Aziz, 2016). Historically, traditional medicine relied on the use of plants possessing diuretic attributes to address a range of health issues. Diuretics have been around for a long time in different forms and the use of plants as diuretics have been reported by Pliny the Elder (23-79 AD) (Melillo, 1994). Joseph Plenick listed several plants in his treatise, out of which 115 plants had diuretic properties including licorice, sassafras, Chinese lantern, fennel and dandelion. The latter got its name from the French ‘Dent e lion’ on account of the shape of its leaves which is responsible for its diuretic property. In French it is commonly called ‘pissenlit’ which literally translates to ‘piss in bed’(Aliotta, 1994; Calvet, 1993; Funk, 1950).

1.3. Future perspective

Plants provide a rich assortment of resources for addressing or potentially even curing a broad spectrum of diseases. Multiple studies and preclinical trials have demonstrated the effectiveness of plants and their derivatives, such as phytochemicals, in managing various kidney-related ailments. Moreover, they have also demonstrated compounds with highly encouraging outcomes as diuretics. Additionally, plants are recognized for their potent antioxidant properties and promising efficacy against specific microorganisms. In the forthcoming years, the alignment of strategies aimed at preventing kidney ailments and advancing

therapeutic development may present promising prospects for natural compounds to tackle concerns associated with major kidney diseases with lesser risks. Further preclinical studies and clinical trials are undoubtedly necessary to fully elucidate the comprehensive range of activities and mode of action exhibited by the chosen natural compounds against kidney illnesses, either independently or in synergistic combination with other small molecules. These endeavours are crucial for substantiating the efficacy of these agents in combating kidney problems, thereby advancing our understanding and validating their utility in kidney disease treatment.

Therefore, considering the significance of natural plant-based products in treating several human disorders, the present study was focused towards characterizing the antioxidant, antimicrobial and diuretic potential of the selected plant in a comprehensive manner.

1.4. Objectives

The specific objectives of the present study are given below:

1. To collect and prepare *Hedyotis scandens* extracts.

2. To estimate phytochemical components and free radical scavenging properties of *H. scandens* extracts.

3. To assess antimicrobial activity of various extracts of *H. scandens.*

4. To evaluate diuretic effect of *H. scandens* extracts in Wistar rats (*Rattus norvegicus*).

CHAPTER-2

REVIEW OF LITERATURE

A multitude of research works have been conducted to explore the medicinal properties of various plants. In this chapter, an attempt has been made to review relevant literatures on the use and contents of plants as diuretics as well as various properties of the *Hedyotis* species focusing particularly on *Hedyotis scandens* Roxb.

2.1. Plants with diuretic property

According to Ntchapda (2016), phytochemical analysis of crude extract from *Vepris heterophylla* had shown the existence of various metabolites like saponins, tannins, anthraquinones etc, and it is traditionally used as a cure for heart disease and malaria. He also stated that the plant contained sterols and phenols, and further observed that the plant's extract increased urine and urine electrolyte output and may act as a loop diuretic. Additionally, the extract also triggers the suppression of reabsorption of water and electrolytes in the renal tubules, and this effect can be ascribed to the existence of previously mentioned phytochemicals within the plant which might work individually or might have a synergistic effect.

Hailu (2014), had reported that the crude extract of *Ajuga remota* Benth (Lamiaceae) exhibited diuretic properties and high dosage of the extract showed similar activity to that of furosemide; he further noted that the diuretic attribute of the plant extract might be linked to the existence of secondary metabolites in the plant including saponin which has already been proven to possess diuretic property.

Phytochemical analysis of *Withania aristata* Ait. (Solanaceae) was done by Herrera (2008). He highlighted the presence of phytosterols, withaminol, withanolides, and suggested that the plant's diuretic effect could be linked to these active compounds. Additionally, he proposed that the diuretic properties of the plant's aqueous extract may also be due to its high potassium content.

Najafian (2018) observed that *Plantago major* increases urine output though the diuretic actions of iridoid compounds. He confirmed that the plant possessed therapeutic applications for the treatment for constipation, cough, wounds, infections and inflammation and these effects are attributed to various constituents including flavonoids, terpenoids, lipids and iridoid glycosides. Notably, the plant also demonstrates diuretic effects and concurrently inhibits the function of angiotensin converting enzyme. The leaf extract of the plant contains compounds such as catalpol and aucubin, which has diuretic properties. The plant also contains iridoid glycosides which is known for its diuretic effect.

Kamboj and Saluja (2010) examined the diuretic effects of *Xanthium Strumarium* L. in albino rats. Two distinct doses: 250 mg/kg and 500 mg/kg of the petroleum ether extract were used which significantly increased diuresis, natriuresis, kaliuresis, and glomerular filtration rate. In comparison to standard saline solution, the extracts resulted in an increase in urine production and elevated excretion of sodium (Na^+), potassium (K^+), and chloride ions (Cl^-). Their conclusion pointed towards the likelihood that the diuretic activity of the extract might be attributed to the existence of certain compounds such as saponins, flavonoids and organic acids. It is also plausible that the increase in urine output could be from the action of secondary active metabolites. Alternatively, it may be linked to indirect alterations in certain physiological parameters preceding the filtration.

Suresh *et al* in 2010 employed the Lipschitz method to evaluate the diuretic properties of the bark of *Samanea saman* (Jacq) Merr in rats. In contrast to the control group, the plant's methanolic extract of this plant at a dose of 200 and 400 mg/kg resulted in elevated urine volume and greater excretion of electrolytes. Furosemide at 20 mg/kg of body weight was used for reference. Based on their discoveries, *Samanea saman* (Jacq) Merr, methanolic extract exhibited diuresis akin to that of the reference drug, particularly when administered at higher dosages. The plant extracts increased electrolyte output along with volume of urine.

According to Shenoy *et al* (2011)**,** *Morinda Citrifolia* Linn possesses diuretic property; two doses of the fruit juice (10 mg/kg and 5 mg/kg) were administered to

Wistar rats. Volume of urine and electrolyte content were examined at the end of 24h and data collected was analysed. Based on their findings, the methanol extract of *Morinda Citrifolia Linn* demonstrated a diuretic effect that was akin Furosemide, particularly when administered at higher concentrations. Notably, there was a statistically significant reduction in the excretion of sodium ions. Although potassium excretion decreased similarly, it did not achieve statistical significance. These findings suggest that the increase in urine formation due to Noni fruit juice may be related to its aquaretic action rather than a natriuretic effect.

Khan *et al* (2012) used Wistar rats to test the diuretic effects of *Holarrhena antidysenterica* along with its diverse fractions, including aqueous, n-butanol and n-hexane extracts; he observed that the crude aqueous ethanolic extract, when administered at 30 and 100 mg/kg, led to a dose-dependent increase in urine production. Moreover, it elevated the sodium (Na^+) and potassium (K^+) output, in addition to increase in urine volume, changes in pH value, and alterations in electrolyte levels, all of which pointed to diuretic effects. Nevertheless, none of the isolated fractions showed a diuretic effect that was on par with the initial crude extract. The hexane extract showed no diuretic activity, while the butanol extract displayed a minimal diuretic impact at 30 mg/kg. Conversely, the aqueous extract, significantly increased urine output, but only at a dosage of 100 mg/kg. He concluded that the diuretic effect is dispersed across all fractions in a way that is consistent with the solvent's rising polarity.

Diuretic properties of the crude ethanolic extract and diverse fractions from *Euphorbia thymifolia* Linn were validated by Kane *et al* (2009) in albino rats. These extracts were administered to the rats at doses of 200 and 400 mg/kg body weight. The extract exhibited a diuretic effect that depended on the dosage administered. Furthermore, the fractions derived from the extract enhanced this diuretic effect. The phytoconstituents present in *Euphorbia thymifolia* Linn are responsible for this it's diuretic effect. At the higher concentration of 400 mg/kg administered orally, the extract not only increased urine output but also led to elevated concentrations of electrolytes. The diethyl ether fraction demonstrated the highest effectiveness. The

findings suggests that *Euphorbia thymifolia* Linn, particularly its ethanolic extract and fractions, possess diuretic properties that could be due to the presence of certain phytochemicals. Notably, the diuretic impact was most pronounced at the higher concentration of 400 mg/kg, with the diethyl ether fraction displayed the most potent activity.

Sasmal *et al* (2007) examined the diuretic activity of water-soluble portions of ethanolic extracts obtained from different parts of *Euphorbia thymifolia* Linn, including barks, flower, leaves, and seed. They discovered that there was strong diuretic activity in the ethanolic extracts of various components, evident though increased urine volume and excretion of electrolytes. It's interesting to note that, especially at larger doses, the ethanolic extract of seeds and leaves obtained from the plant, exhibited a higher level of electrolyte excretion. The study's most significant finding is that these extracts are safe up to doses of 2g per kg of body weight.

Devi (2011) had assessed the diuretic effect of *Mangifera indica* bark extract. In order to test the ability of *Mangifera indica* to induce urination, 250 mg/kg of ethanol, water, and ethyl acetate extracts were given orally and the study has shown that the aqueous extract exhibited the highest Na^+/K^+ ratio as compared to the ethanol and ethyl acetate extracts.

Radhika *et al* (2010) demonstrated the diuretic potential of *Bixa Orellana's* petroleum ether, methanol, and water extracts was assessed using a standard method on Wistar rats. Using petroleum ether, methanol, and water as solvent, extractions were performed on the dried leaf powder using a Soxhlet. Subsequently, these extracts were examined for their diuretic effects in Wister rats. The methanolic extract showed notable diuretic effect as it enhance the overall urine production and elevated the sodium, potassium and chloride excretion.

Kane *et al* (2009) and Racz *et al* (1974) studied the effectiveness of *Taraxacum officinale* as a diuretic on mice. 2g per kg body weight of the aqueous extract of the plant was used which increased urine output exponentially and its effectiveness was found to be comparable to that of furosemide. Given that the plant

is abundant in potassium, certain researchers speculate about its ability to replenish potassium that might be depleted due to diuresis.

The assessment of diuretic potential of fractions and ethanolic extract of *Euphorbia Thymifolia* Linn was studied by Kane *et al* (2009) in rats. Furosemide at 10 mg/kg, was used for standard reference. Certain portions of the extract enhanced diuresis when compared to the reference drug. The positive outcomes can be ascribed to the existence of secondary metabolites in the ethanolic extract of *Euphorbia Thymifolia* Linn.

2.2. Phytochemicals from plants as diuretics

Synthetic medicines are often associated with well-known drawbacks like high cost, and also the potential for adverse health effects. Medicinal plants promise good alternative for these drugs due to their natural efficacy and lower toxicity with comparatively fewer side effects. They act as treatment for curing a wide range of illnesses (Abdel-Aziz *et al*., 2016). These medicinal plants yield chemical compounds with healing properties. Both single and multiple-herb formulations have been employed as diuretics, adding to a notable increase in herbal drug manufacturers. Consequently, herbal remedies have become the preferred choice for diuretic medications (Herrera *et al*., 2008). The medicinal significance of these plants stems from certain chemical compounds that show specific effects on the human body. Saponins, glycosides, flavonoids and alkaloid compounds are some of crucial bioactive compounds found in plants. Many of these native medicinal plants are employed for therapeutic applications (Ntchapda *et al*., 2016).

2.2.1. Alkaloids

Alkaloids constitute a group of secondary metabolites, normally present in all plant groups, at different concentration. Quinine, Ephedrine, Nicotine, Strychnine and morphine are some examples of alkaloids. Most alkaloids are known for their diuretic property. The leaves and roots of *Cissampelos pareira* are employed to treat ulcers and diarrhoea. An isoquinoline alkaloid called berberine is also derived from this plant.

The whole plant is used in managing urinary tract infection, rheumatism, inflammation of muscles etc. the major phytochemical constituent of the root of this plant includes berberine, which is reported to have diuretic activity. When tested in rats, berberine increased urine excretion, it also increased the K^+ and Na^+ excretion, which suggests saluretic effect (Sayana, 2014).

Hullatti *et al* (2011) had reported important compounds from *Cycas peltate* (Lam) Hook. f. and Thoms of the family Manispermaceae. The leaf of this plant was used as a treatment for herpes, coolant and as a diuretic. They found that the leaves of this plant contain bebeerine, hayatinin, hayatidin and cycleanine which are alkaloids. Five bisbenzylisoquinoline alkaloids were extracted from this plant's root. Experiments have proved that this plant possesses furosemide like diuretic activity

Acorus calamus is known to have antidiabetic, diuretic, antioxidant, anti-diarrheal and nephroprotective activities. The extract of this plant is known to have a dose dependent increasing effect on urine electrolyte, which is quite similar to that of furosemide. This study revealed that rapid and acute diuretic activity associated with quinone like alkaloid and other phytochemical like steroids, tannins, phenolic compounds and flavonoid (Ghelani *et al*., 2016).

Moringa stenopelata is used to cure malaria, infertility and diabetes. Several secondary metabolites like flavonoid, saponins, coumarin and alkaloids have been identified from this plant. This plant possesses an appreciable diuretic activity when compared to furosemide, as it also increases Na^+ and K^+ excretion. A combination of tannin, flavonoid and alkaloid is known to produce diuretic effect (Fekadu *et al*., 2017).

2.2.2. Flavonoids

Flavonoids are polyphenol compounds found in plants and they can be subdivided into categories such as flavones, flavonols and isoflavone. Flavonoids bind with the A1 receptor and thus exert diuretic activity. The latex of *Euphorbia granulate* is known to have anthelmintic and diuretic activity, it also finds application in the treatment of intestinal worm and is employed to cure snake bites

and counteract the effects of scorpion venom. It is also shown to inhibit Human Immunodeficiency Virus. The phytochemicals present in this plant work individually or in combination to produce diuretic effects. These phytochemicals act by increasing blood perfusion in specific areas, causing vasodilation, and inhibiting tubular secretion, all of which can be associated with an increased urinary excretion and potentially contribute to diuretic effects (Saleem, 2015).

Saffron, obtained from the dried stigma of *Crocus sativus*, is known to exhibit potent diuretic activity. Its diuretic activity is reported to be very prominent, which was almost similar to furosemide. Preliminary study has shown that diuretic activity is affected by flavonoids and other water-soluble compounds such as tannins, carbohydrates, saponin and alkaloids (Dadgarnejad, 2014).

Quercetin, classified as a flavonoid, is derived from the polyphenol category of plants. It is also found in *Hibiscus sabdariffa* L, which is utilized as a diaphoretic and diuretic agent. An analysis of the plant's phytochemical composition verified the existence of numerous bioactive compounds. These active compounds are able to act as ACE inhibitor, which in turn acts as potassium sparing diuretics (Alarcon-Alonso, 2012).

Smilex canariensis wild (Smilacaceae) also called Zarzaparilla, which is employed as a diuretic, antimicrobial, antiviral, antioxidant, antiglyceamic and anti-inflammatory. This species contains many polar compounds like flavonoids and saponin. The polar compounds found in plants are known to aid in diuresis by increasing the glomerular filtration rate and renal function (Nishanthini *et al*., 2014).

Desmostachya bipinata, of the family Poaceae, is a known aphrodisiac, analgesic, antipyretic, diuretic, anti-asthma etc. Studies have shown that this plant contains many phytochemicals like terpenoids and flavonoids which could potentially underlie its diuretic effects (Golla, 2014).

2.2.3. Saponin

Saponin is a glycoside which is commonly found in plants. It has a foamy texture and hence can be utilized as a mild cleanser. When it comes to the staining process for intracellular histochemistry staining, it allows the antibody to enter intracellular proteins, therefore it can function as a treatment for elevated blood glucose level, high cholesterol, as a diuretic, antioxidant, anti- inflammatory and might also help in weight loss.

2.3. Phytochemicals from *Hedyotis* species

Wang *et al* (2013) had documented the discovery of five novel glycosides from *Hedyotis scandens* and ten previously identified compounds, encompassing a cyanogenic glycoside. Additionally, various *Hedyotis* species, such as *Hedyotis hedyotidea*, *Hedyotis leschenaultiana*, and *Hedyotis diffusa* Willd, have been noted for their significant content of phenols, flavonoids, iridoids, triterpenes, anthraquinones, and sterols (Sornalakshmi, 2016).

Lajis and Ahmad (2006), reported the presence of several compounds from *Hedyotis auricularia- β-Carboline alkaloid*: auricularine. According to Ahmad *et al* (2005), *Hedyotis chysotricha* contains phytochemicals including- *β-Carboline alkaloid*: chysotricine (Ahmad *et al*., 2005). Similarly, Phuong *et al* (1999) also reported several secondary metabolites from this plant including *β-Carboline alkaloid:* chysotricine. *Iridoid glucosides*: asperuloside; scandoside methyl ester *Iridoids:* hedycoryside A–C.

Hedyotis capitellata- β-Carboline alkaloids: capitelline; cyclocapitelline; isocyclocapitelline;hedyocapitelline; hedyocapitine, *Anthraquinones*: capitellataquin one A–D; rubiadin; anthagallol; 2-methyl ether;alizarin-1-methyl eter; digiferruginol; lucidin-3-*O*-β-glucoside (Lajis and Ahmad, 2006).

In a study conducted by Huu and Phung (2014), it was found that *Hedyotis crassifolia* contains: *Triterpenes*: ursolic acid; 3β-hydroxyurs-11-ene-23(13)-lactone;

3α,13β-dihydroxyurs-11-ene-28-oic acid; oleanolic acid; 3-β-D-glucopyranosyl-β-sitosterol and3β,6β-dihydroxyolean-12-ene-28-oic acid.

Xu *et al* (2010), found that *Hedyotis diffusa* contained: *Iridoid glycosides*: dunnisinoside; *E*-6-*O*-*p*-methoxycinnamoyl scandoside methyl ester; *Z*-6-*O*-*p*-methoxycinnamoyl scandoside methyl ester; *E*-6-*O*-*p*-feruloyl scandoside methyl ester; *E*-6-*O*-*p*-coumaroyl scandoside methyl ester; *Z*-6-*O*-*p*-coumaroyl scandoside methyl ester, *Iridoid glucosides:* diffusosides A–B.

Hedyotis intricate was also found to possess *Triterpene*: lupeol; oleanolic acid, Iridoid-Asperuloside (Dominguez, 1992).

As per Peng *et al* (1998), *Hedyotis hedyotidea* was affluent in secondary metabolites like hedyotoside; asperulosidic acid; asperuloside; deacetyl asperuloside and *Iridoids*: deacetyl lasperulosidic acid ethyl ester.

In research conducted by Lajis and Ahmad (2006) and Hamzah (1996), *Hedyotis herbacea* was reported to contain pyranoside; quercetin 3-*O*-galactoside, rutin; kaempferol 3-*O*-glucoside; kaempferol 3-*O*-arabinopyranoside; kaempferol-3-*O*-arabino and *Flavonoids*: kaempferol 3-*O*-rutinoside.

According to Konishi *et al* (1998), *Hedyotis nudicaulis* contains phytochemicals such as nudicaucins A–C and guaiacin which are triterpene glycosides.

Duy (2014) reported the presence of several compounds from *Hedyotis pinifolia* including 1,6-dihydroxy-2- methylanthaquinone; Anthaquinones:1,6-dihydroxy-7-methoxy-2-methylanthaquinone; 1,3,6-trihydroxy-2-methylanthaquinone and 3,6-dihydroxy-2-methylanthaquinon .

According to Zhao *et al* (2005), *Hedyotis tenelliflora* was found to contain, *Iridoids:* teneoside B.

Based on the investigation led by Lajis and Ahmad (2006), it was found that *Hedyotis verticillate* had *Flavonoids*: kaempferitrin, rutine; isohametin 3-*O*-rutinoside; vomifoliol 9-*O*-β-D-glucopyranoside and *Stereoid*: phytol.

Hedyotis vestita was also reported to contain *Iridoid:* 6α-methoxygenyposide; *Phenolic compound:* sodium (1*S*,4a*R*,5*R*,7a*R*)-7-hydroxymethyl-5-methoxy-1-β-D-glucopyranosyloxy-1,4α,5,7α-tetrahydrocyclopenta[c]pyran-4-carboxylate (Hang, 2014).

2.4. Antioxidant property of some *Hedyotis* species

Ahmad *et al* (2005), conducted antioxidant assays and free radical scavenging property of some *Hedyotis* species. In the FTC assay conducted on *H. capitellata* leaves and stems demonstrated inhibitions of 98.2% and 97.8%, while in the TBA assay, they exhibited inhibitions of 97.5% and 95.3%. *H. verticillata* stems and leaves displayed inhibitions of 98.0%, 96.5%, 97.6%, and 94.2% in the FTC and TBA assays, respectively. *H. dichotoma's* aerial parts and roots showed inhibitions of 98.0%, 95.7%, 97.1%, and 92.4% in the FTC and TBA assays, respectively. *H. pinifolia* (aerial parts) exhibited inhibition percentages of 97.1% and 93.9%. *H. corymbosa* (aerial parts) displayed inhibition percentages of 96.7% and 89.9%. *H. nudicaulis* aerial parts had inhibition percentages of 95.2% and 88.8%. In this study, the IC_{50} values for methanolic extracts from various *Hedyotis* species against the DPPH radical were determined as follows: *H. dichotoma* (aerial parts) exhibited an IC_{50} of 64, *H. verticillata* (leaves) had an IC_{50} of 100, *H. pinifolia* (aerial parts) showed an IC_{50} of 90, and *H. herbacea* (aerial parts) displayed an IC_{50} of 32. Additionally, the study assessed the percentage of nitric oxide inhibition by different *Hedyotis* species at varying concentrations (25µg, 50µg, and 100µg), yielding the following results: *H. corymbosa* - 9.1%, 9.10%, 8.6%; *H. dichotoma* - 12.4%, 12.9%, 122.3%; *H. herbaceae* - 3.6%, 9.5%, 11.2%; *H. nudacaulis* - 41.4%, 22.4%, 18.1%; *H. pinifolia* - 19.1%, 6.7%, 0%; *H. capitellata* (stems) - 40.6%, 22.4%, 0.5%; *H. verticillata* (leaves) - 38.9%, 18.8%, and 7.7%.

2.5. Antibacterial effect of some *Hedyotis* species

As per the findings by Ahmad *et al* (2005), the inhibition zones observed for various parts of *Hedyotis capitellata* and *Hedyotis dichotoma* against different microorganisms are as follows:

H. capitellata: The stems exhibited inhibition zones of 9mm, 6.5mm, 8mm, and 10mm against methicillin-resistant *Staphylococcus aureus*, both *Bacillus subtilis* mutant and wild type strain and *Pseudomonas aeruginosa* respectively. The roots, on the other hand, displayed inhibition zones measuring 10mm, 7mm, 9mm, and 11mm against the same organisms. *H. capitellata* leaves demonstrated an inhibition zone of 8mm against *Bacillus subtilis* mutant strain, *Pseudomonas aeruginosa* and *Staphylococcus aureus* which was resistant to methicillin.

H. dichotoma: Roots of *H. dichotoma* exhibited significant inhibition zones, measuring 17mm, 15mm, 19mm, and 20mm against the tested microorganisms. *H. capitellata* (stems) were found to have an inhibition zone of 9,6.5,8 and 10mm for mutant and wild strain of *Bacillus subtilis*, *Staphyloccocus aureus* (methicillin-resistant) and *Pseudomonas aeruginosa*. *H. capitellata* (roots) on the other hand inhibition zone of 10,7, 9 and 11mm for the organisms. *H. capitellata* (leaves) had an inhibition zone of 8mm against *Bacillus subtilis* mutant strain, methicillin- resistant *Staphyloccocus aureus* and *Pseudomonas aeruginosa*. *H. dichotoma* (roots) on the other hand, had a zone of 17,15,19 and 20mm against the tested organisms.

Sikarwar *et al* (2019), investigated the antibacterial properties of *Hedyotis hedyotidea* against two bacteria, *Staphylococcus aureus* and *Bacillus pumilus*. In their findings, they observed that when the *Hedyotis hedyotidea* extract was tested against *Bacillus pumilus*, it displayed varying levels of inhibition depending on the concentration used. Specifically, at 60mg/ml, it produced an inhibition zone measuring 1.92cm, while at 80mg/ml, the inhibition zone increased to 2.12cm. Penicillin which was used as standard, resulted in an inhibition zone of 5.34cm. Whereas, for *Staphylococcus aureus*, penicillin exhibited a significant inhibitory effect with an average diameter of 6.86cm. In contrast, the plant extract demonstrated

varying degrees of inhibition at different concentrations. At 40mg/ml, the average inhibition zone had a diameter of 1.74cm, while at 60mg/ml, it increased to 2.46cm. Further, at 80mg/ml, the inhibition zone expanded to an average diameter of 3.11cm, and at 100mg/ml, it decreased to 1.56cm. Sikarwar *et al* (2019) found that *Hedyotis hedyotidea* exhibited antibacterial effects against both *Staphylococcus aureus* and *Bacillus pumilus*, with the degree of inhibition varying with different extract concentrations, while penicillin, used as a control, had consistent and generally greater effect.

2.6. *Hedyotis scandens*

Hedyotis scandens remains relatively unexplored in comparison to other plants belonging to this family. Our objective is to shed light on the potential medicinal benefits of *Hedyotis scandens* and contribute to the broader understanding of its therapeutic properties within the context of medicinal plant research. *Hedyotis scandens* has long been utilized in Mizoram as traditional medicine to cure various ailments such as stomach troubles, eye problems, oliguria and certain kidney diseases like kidney swelling and kidney stone. However, there is a notable scarcity of comprehensive scientific research elucidating the properties of this plant. Consequently, the present study has been proposed to adequately address these concerns.

Hedyotis scandens Roxb. (Rubiaceae) is a highly valued ethnomedicinal plant known for its diverse array of properties. It belongs to the genus *Hedyotis*, also known as ‘star violet’ (Anonymous, 2015). Many species belonging to this genus are well known medicinal plants. *Hedyotis scandens* is a widely distributed, cosmopolitan plant and usually thrives well in disturbed sites. Shrubs or herbs, perennial, that can climb, creep or twine reaching several meters, stems flattened or rounded with smooth surfaces. Previous studies have shown that *H. scandens* contain secondary metabolites, which may contribute to its potential medicinal properties. Wang *et al* (2013) had identified five new important phenolic glycosides from *H. scandens*. The ethanolic extract of *H. scandens* have also shown protective activity

against the bacteria *Sarcina lutea, K. pneumoniae, P. vulgaris, E. coli* and *S. aureus* in a concentration dependent manner (Subba and Basnet, 2014).

Although scientific information on this plant is limited, many species belonging to the same genus have reported many scientific activities, for eg. *Hedyotis diffusa* Willd is known to have scavenging property, anticancer activity and diuretic effect. *Hedyotis corymbosa* is reported to have hepatoprotective, antibacterial, antifungal and free radical scavenging property (Marisetti, 2019; Sadasivan *et al*., 2006). The medicinal plant *Hedyotis scandens* is utilized for its therapeutic properties by numerous tribal communities across India. It is locally known as Kelhnamtur or Laikingtuibur in Mizoram.

2.6.1 Ethno-medicinal uses of *Hedyotis scandens*

Hedyotis scandens is a recognized therapeutic plant with applications in addressing a variety of ailments, although the specific illnesses it addresses vary depending on the region of use. Jain (1994) reported that people in Jaipalguri (West Bengal) administer root paste of *H. scandens* to address issues related to diarrhoea and stomach pain. Additionally, it is also employed in the management of eye conditions and postpartum complications (Chopra, 1986). The plant's root is ground and applied to alleviate sprains, and also administered for indigestion whereas a paste prepared from the plant is provided for peptic ulcer treatment (Manandhar, 1991; Singh and Borthakur, 2011). In West Sikkim, the plant's roots serve as a vermifuge, and the extract obtained from crushed roots is employed to treat jaundice (Bennett, 1985).

Naga communities utilize the crushed leaves of *H. scandens* to remedy wart-like ailments (Rao and Jamir, 1982; Bennet, 1985). In Assam, the whole plant is employed to cure heartburn, treat gastric ulcers and facilitate the healing of wounds. Meanwhile, in Bangladesh and Meghalaya it is employed to treat mouth sores, abscesses and as a remedy for malaria (Biswas *et al*., 2010; Bhuyan and Laskar, 2021). Naga communities utilize the crushed leaves of *H. scandens* to remedy wart-like ailments, while in West Sikkim, the plant's roots serve as a vermifuge, and the

extract obtained from crushed roots is employed to treat jaundice (Rao and Jamir, 1982; Bennet 1985). Similarly, the ethnic community in Jorhat also use a decoction of the leaves in the cure for urinary and kidney problems (Sengupta, 2017). In Meghalaya, including the Khasi Hills, the entire plant is harnessed for managing conditions like cold and cough, fractured bones, muscles sprain, eye disorder, rheumatism and kidney stone (Singh and Borthakur, 2011; Kayang, 2003). The Adi tribe of Arunachal Pradesh make a decoction of the roots and use it for the treatment of gallstones (Kagyung, 2009).

In Mizoram, a decoction made from the leaves of the plant is taken orally for kidney problems including kidney stone and swelling, oliguria, eye trouble (Lalfakzuala *et al.*, 2007; Bose *et al*. 2015; Rozika, 2005). Oral consumption of a decoction made from the root, leaves, or rhizome is employed to remedy fever, stomach pain, kidney stones and dysuria. Additionally, the plant is utilized to treat rheumatism, jaundice and as a cure for tapeworm infections (Laldinsanga, 2019). A decoction made from the leaves is used to treat diabetes and provides pain relief (Laldingliani *et al*., 2022).

CHAPTER-3

METHODOLOGY

3.1. Collection and preparation of *Hedyotis scandens* extracts.

The plant *Hedyotis scandens* Roxb. was collected from Tanhril, Mizoram (23º44'20.13" N and 92º40'35.78" E) during June-July, 2019. The plant was authenticated and confirmed by Dr. Khomdram Sandhyarani Devi, Department of Botany**,** Mizoram University, Aizawl (Accession no- MZU000246) and deposited in the Mizoram University Herbarium, Department of Botany, Mizoram University.

3.1.1. Details of *Hedyotis scandens*

Hedyotis scandens Inflorescence

Fig 1: Habit and inflorescence of *Hedyotis scandens* Roxb.

Kingdom- Plantae

Phylum- Tracheophyta

Class- Magnoliopsida

Order- Gentianales

Family- Rubiaceae

Genus- *Hedyotis*

Species- *scandens*

3.1.2. Botanical description

Hedyotis scandens Roxb. is a perennial herb or shrubs, lianescent up to several meters, stems are either compressed and cylindrical or have angular shape, smooth, furrowed or covered with fine hairs in linear patterns. Leaves have a short stem, with petioles measuring up to 5mm in length, smooth; leaf blade is usually papery to somewhat leathery, light-coloured abaxially, elongated and lance shaped, elliptic, narrowly elliptic, or elliptic-oblong, stipules are usually united or joined to the base of the petiole. Inflorescence clusters at the tip of the stem and upper leaf axils arranged in pattern ranging from simple from branched clusters. Flowers are on stalks, distylous and have a smooth calyx. Corolla is either white or light yellow and it has a shape that ranges from tubular funnel shaped to funnel shaped to funnelform, smooth [or downy in India], capsule fruit, nearly spherical, oblong, ovoid, opens along the top and sometimes split at the septa, seeds are numerous, black in colour and have distinct angle. The flowering and fruiting time period of this plant is from March-December.

3.1.3. Preparation of extract

After washing and air drying the leaves of *H. scandens*, it was ground into a coarse powder. The sample was then subjected to sequential extraction in a soxhlet extractor using petroleum ether, chloroform, methanol and distilled water in an

ascending order of polarity, for a minimum of 72h till the solvent turns colourless while maintaining a temperature slightly under the boiling point of the respective solvents. The extracted samples were than dried with a rotary evaporator and kept at -20°C until use. The extracted petroleum ether, chloroform, methanol and aqueous extract of *H. scandens* is written as:

HSPE- *Hedyotis scandens* petroleum ether extract,

HSCE- *Hedyotis scandens* chloroform extract,

HSME- *Hedyotis scandens* methanol extract and

HSAE- *Hedyotis scandens* aqueous extract.

3.2. Estimation of phytochemical components and free radicals scavenging properties of extracts of *H. scandens*

3.2.1. Qualitative Phytochemical Analysis:

Qualitative phytochemical analysis of the extracts was performed by standard procedures given by Trease and Evans (1989), Sofowara (1993), Kokate (1994), Gokhale (2008).

a. Test for Saponin

2ml of the extracted plant material were combined with the same amount of distilled water in a graduated cylinder and mixed for 15 min. Saponins are present when a 1cm layer of foam forms.

b. Test for Flavonoids

Zinc-hydrochloride test- The existence of flavonoids in the plant extract was established by the appearance of red colour on the addition of hydrochloric acid combined with zinc dust.

c. Test for Alkaloids

Dragendroff's and Mayer's test was used to identify the existence of alkaloids in the plant extracts. A small quantity of the reagent was blended with 1ml of the plant extract. The detection of alkaloids was indicated with the production of reddish-brown precipitate after the addition of Dragendroff's reagent and yellow or white precipitate following the use of Mayer's reagent.

d. Test for glycosides:

1. **Keller-Killiani's test**- A blend of glacial acetic acid and 2% ferric chloride was combined to 10ml of the extracts, after which 1ml concentrated sulphuric acid was introduced. The presence of cardiac steroidal glycosides is affirmed by the appearance of a brown ring between the layers.

2. Legal's test:

2ml of pyridine and sodium nitroprusside were added to the extracts, after which 2ml of NaOH was added. The detection of glycosides was signalled by the pink colour indicated the presence of glycoside.

e. Test for carbohydrate

1. Molish's test- An ml of the plant extract was blended with a small quantity of Molish's reagent. The formation of a blue to purple ring serves as confirmation of the presence of carbohydrates.

2. Benedict's test- The confirmation of the presence of carbohydrates occurred when 1ml of plant extract was combined with a small quantity of Benedict's solution, leading to the development of a brick-red precipitate.

3. Fehling's test- 2ml each of extracts was combined with 2ml of both A and B Fehling's solutions. The mixture then was brought to a boil for 10 min. The detection of carbohydrates was indicated by the creation of red precipitate.

f. Test for tannin

The presence of tannin in the plant extract was demonstrated by the production of a greyish precipitate on addition of a few drops of ferric chloride solution to it.

g. Test for phytosterols

1. Leibermann-Burchard Test- 1ml each of the plant extracts were mixed with some drops of acetic anhydride, and then boiled. Concentrated sulfuric acid was then added after cooling to produce a bluish-green solution. This confirmed the presence of phytosterols.

2. Salkwoski reaction- Some drops of concentrated sulfuric acid were added to the plant extract and subsequently allowed to sit for a while. Appearance of brown ring indicated the presence of phytosterols.

3.2.2. Quantitative Phytochemical Screening

a. Total Phenol Content

Total phenol content of the plant extracts was estimated by using Folin - Ciocalteu assay (Singleton and Rossi 1985; Singleton *et al*. 1999). 1ml of gallic acid solution of various concentration ranging from 10 µg/ml to 100 µg/ml was mixed with 5ml Folin - Ciocalteu reagent that had been diluted tenfold. After a 3 min interval, 4ml of 0.7M sodium carbonate solution was introduced, and the resultant solution was left to sit at room temperature for an hour. Absorbance was subsequently recorded at 765nm using UV- visible spectrophotometer. The identical procedure was performed for HSPE, HSCE, HSME and HSAE. The absorbance measurements of gallic acid at various concentrations were recorded and used to generate a calibration curve. Using the calibration curve, the total phenolic content was determined and expressed as mg of Gallic acid equivalent (GAE) per gram of the dried extract.

b. Total Flavonoid Content

Total flavonoid content of the plant extracts was determined by Aluminium chloride method (Zhishen *et al*. 1999) with quercetin as standard. 1ml of each extract was mixed with 2ml of distilled water, followed by 3ml of a 5% sodium nitrite ($NaNO_2$) solution. After a 5 min wait, 0.3ml of a 10% Aluminium chloride solution was added to the mixture, which followed by incubating at room temperature for 6 min. After incubation, 1ml of 1M sodium hydroxide was added to the solution, and the final volume was adjusted to 10ml with distilled water. Using a UV-Vis spectrophotometer, the absorbance of the sample was measured at 510nm. The experiment was repeated the times for accuracy, and the results were presented in units of Quercetin equivalent (QE) per gram of dry weight.

3.3. Antioxidant assays

The confirmation of the antioxidant activity of the various extracts was carried out through a phosphomolybdenum test, ferric reducing test and various *in-vitro* assays. These included tests for free radical scavenging potential employing DPPH and $ABTS^+$, as well as superoxide and nitric oxide assays.

3.3.1. DPPH scavenging activity

The extracts' ability to neutralize DPPH radicals was conducted using the procedure described by Leong and Shui (2002) with minor adjustments. The extent of DPPH (2, 2- diphenyl-1-picrylhydrazyl) discoloration by the extracts reveals their scavenging potential. Plant extracts and butylated hydroxytoluene (reference drug) was prepared at a range of concentrations spanning from 10 - 100 µg/ml. Subsequently, 1ml of 0.1mM solution of DPPH in methanol was combined with 3ml of different concentration of the extract and incubated at 37 ± 1 °C for 30 min in the dark. During this incubation period, change in colour was monitored visually. A control sample was also maintained. A wavelength of 517nm was used to measure the absorbance. Percentage of absorbance was determined by comparing the absorbance values of the extracts to those of the control sample.

3.3.2. ABTS scavenging activity

ABTS assay was conducted following the protocol suggested by Re *et al* (1999). HSPE, HSCE, HSME and HSAE along with butylated hydroxytoluene (standard) were prepared at concentrations from 10 - 100 µg/ml. ABTS stock solution was prepared by mixing equal volumes of 7mM ABTS and 2.45mM potassium per sulphate. This solution was then kept in dark at room temperature for 12h, which ultimately formed a solution containing $ABTS^{+}$ radicals. Before starting the experiment, a fresh working solution was prepared by diluting it with 50% methanol to achieve an absorbance of 0.70 (±0.02) at 745nm. The scavenging activity was evaluated by adding 500µl of different concentration of various extracts of *Hedyotis scandens* (ranging from 10 to 100µg/ml solubilized in distilled water) with 1ml of the previously prepared working solution. The decrease in absorbance was measured up to 3min after mixing of the solutions. Variation in absorbance of the solutions was observed for 3min at 745nm. The experiment was done in triplicates.

3.3.3. Superoxide ($O_2^{\bullet-}$) scavenging activity

The superoxide anion scavenging ($O_2^{\bullet-}$) potential of *H. scandens* extract was determined by the method put forward by Hyland *et al* (1983) with minor changes. NBT stock solution was prepared in DMSO at a concentration of 1mg/ml. 0.2ml of this solution was mixed with 0.6ml of different *H. scandens* extracts which were prepared in concentrations ranging from 10 to 100µg/ml. In a total volume of 2.8ml, 2ml of alkaline DMSO (comprising 1ml of DMSO containing 5mM NaOH dissolved in 0.1ml of distilled water). The control sample contained alkaline DMSO instead of pure DMSO and the blank sample contained of pure DMSO instead of alkaline DMSO. The result was quantified in terms of ascorbic acid equivalence, which served as the standard reference. The absorbance was assessed at a wavelength of 560nm.

3.3.4. Nitric oxide (NO) scavenging activity

Nitric oxide scavenging capability was estimated using standard protocol (Marcocci *et al*., 1994). The extracts and ascorbic acid (standard) were prepared at different concentrations (10, 20, 40, 60, 80, 100, 150, 200 µg/ml). 2ml of different concentration of the extract was then mixed with 2ml of 10mM sodium nitroprusside and kept aside for 2h. After that, 0.5ml of Greiss reagent was added to the mixture. The measurement of absorbance was conducted at 550nm with a UV-Vis Spectrophotometer.

3.3.5. Total Antioxidant Activity (Phosphomolybdenum Assay)

Total antioxidant activity (TAA) of the plant extracts was determined using the phosphomolybdate estimation method used by Prieto *et al* (1999). A standard curve for ascorbic acid was generated by preparing it in various concentrations (ranging from 10 to 100 µg/ml) and measuring the absorbance at 695nm using a UV-Vis spectrophotometer. The TAA of the plant extracts was however calculated from a stock solution with concentration of 1mg/ml. Following this, the extracts' TAA was calculated and written as milligram of ascorbic acid equivalent per gram of dried extract.

3.3.6. Ferric reducing capacity

Oyaizu's method was used to assess the reducing capacity of the extracts, with ascorbic acid acting as the reference (Oyaizu, 1986). To prepare the solution, 1ml each of HSPE, HSCE, HSME, HSAE and ascorbic acid at varying concentrations such as 10 µg/ml-100 µg/ml were mixed with 2.5ml of phosphate buffer (with a pH of 6.6) and 2.5 ml of 1% potassium ferricyanide. This solution was then incubated at 50° C for 20 min. Following this incubation, 2.5ml of 10% tri-chloroacetic acid was introduced. Subsequently, the samples underwent centrifugation at 3000 rpm for 10 min. The supernatant (if formed) was collected and combined with 2.5ml of distilled water. This mixture was then subjected to vortexing with 0.5ml of a freshly prepared 0.1% ferric chloride solution. Absorbance was

recorded at a wavelength of 700nm. A rise in absorbance corresponds to enhancement in reducing power.

3.4. Assessment of antimicrobial activity of various extracts of *H. scandens.*

3.4.1. Microorganisms used

Antibacterial activity of the extracts was tested against two-gram positive bacteria *Bacillus subtilis* (ATCC- 11774), *Micrococcus luteus* (ATCC- 10240), and thee-gram negative bacteria *Escherichia coli* (ATCC-10536), *Pseudomonas aeruginosa* (ATCC-10145), *Klebsiella pneumoniae* (ATCC-10031), *Salmonella typhi* (ATCC 51812). These microorganisms were acquired from the Institute of Microbial Technology (IMTECH), Chandigarh, Punjab, India. The bacteria were sub-cultured in nutrient broth before the experiment began, and then incubated at 37°C for around 20-24h.

3.4.2. Disc Diffusion Method

Antimicrobial efficacy of the plant extract was tested against the microorganisms by using the Kirby-Bauer disc diffusion method (Bauer *et al*., 1996). Two concentrations of the plant extract 10 mg/ml and 20 mg/ml were loaded on to a sterile paper disc of 6mm in diameter. A standard of 100µg ceftriaxone was maintained for comparison. The control experiment was retained involving solely the bacterial culture. 100µl of standardized bacterial culture (0.5 McFarland) was introduced on the solidified Mueller Hinton Agar media, followed by the placement of discs containing the plant extract on the surface of the solidified media. After that, the plates were incubated for 12h at 37°C. The size of the zone of growth restricted region was measured after incubation, observed, and compared to that of Ceftriaxone, a broad-spectrum antibiotic used as reference drug.

3.4.3. Minimum Inhibitory Concentration

The determination of MIC for the plant extracts involved the utilization of resazurin as an indicator of cell viability (Elshikh *et al*., 2016; McNicholl *et al*.,

2007). The experiment was conducted in a sterile 1.2ml 96-well. A stock solution was formulated by adding 10g of resazurin in a litre of sterile water, which was then diluted to a ratio of 1: 10 in sterile water when needed. Following the addition of 0.5ml of sterilised Mueller Hinton Broth, the plant extracts were added to the wells of the sterile plate in varying amounts. Subsequently, 0.1ml of the bacterial inoculum (at 5×10^8 cfu/ml) was added to each well, thoroughly mixed, and then the well plates were incubated for 12h at 37°C. After the incubation period 10µl of resazurin was introduced into the solution and once again incubated for an additional 1-2h. Change in colour was observed visually. Colour change in the wells was monitored visually. Wells exhibiting pink colour after the incubation period indicated bacterial growth whereas plates displaying blue indicated no growth.

3.5. Evaluation of diuretic effect of *H. scandens* extract in Wistar rats (*Rattus norvegicus*)

3.5.1. Animals

Adult Wistar rats weighing 180-185 grams were obtained from authorized vendors. Each rat was housed in individual propylene cages and kept in a controlled environment maintaining a temperature of 22±2ºC with a 12h light/dark cycle, and continuous access to water and food until the day before the study. The protocol approved by the Mizoram University Institutional Animal Ethical Committee (Approval No. MZU/IAEC/2021-22/13) was adhered to during all experimental procedures for this investigation.

3.5.2. Acute Toxicity Study

Test for acute toxicity was performed in compliance with the guidelines outlined by the Organization of Economic Cooperation and Development (423 recommendation). Four groups of six animals each were created at random(n=6). Subsequently, the animals received oral administration of HSME and HSAE dissolved in distilled water at doses of 1000, 3000 and 5000 mg/kg. For control, the animals were administered distilled water. All treatments were administered orally. Following administration, the animals were checked for the first four hours for any

signs of acute toxicity. Subsequently, the animals were checked for symptoms of delayed toxicity once every day for 14 days at an 8h interval. The rats were euthanized on the 14th day and serum and organs were taken for subsequent analysis.

3.5.3. Grouping and dosing of animals

Animals were randomised into the following groups (n=6) and all treatment were given orally using an oral gavage. Each rat received an oral load of 2.5ml of 0.9% NaCl/100g of body weight to ensure uniform water and salt load (Wielbelhaus and Weinstock, 1965).

Control group: Animals were administered with water (2.5 ml/kg b.wt).

Furosemide group: Animals were administered with furosemide (10 mg/kg b.wt) serve as positive control.

H. scandens group: Animals were administered with *H. scandens* extract at different doses based on the result of acute toxicity study.

Furosemide and *H. scandens* group: Animals were administered with a combination of furosemide (10mg/kg b. wt) and the different doses of the *H. scandens* extracts.

3.5.4. Diuretic activity

The assessment of diuretic activity was performed using the method reported by Lipschitz *et al*, (1943) with slight modification (Lipschitz *et al*., 1943). Six Wistar rats, in random, were assigned to each treatment group. Prior to commencing the experiment, the rats experienced an 18h fasting period during which they were deprived of both food and water. Additionally, before the experiment began, the animals were relieved of urine by gently compressing their pelvic area and pulling their tails. Volume of the doses was made similar in all the animals. The same amount of saline as mentioned above was given to the control group in addition to normal water. Furosemide (10mg/kg), was given as the standard reference. All the treatment was given orally using a gavage. Animals were kept at room temperature immediately after

treatment and housed individually in metabolic cages designed specifically to segregate urine and feces. Urine volume was recorded after the 5^{th} and 24^{th} hour post treatment. At the end of the experiment, animals were sacrificed and serum and organs collected for further analysis.

3.5.5. Urinary pH and Urine electrolyte

Urinary pH of all groups was measured with a calibrated digital pH meter (Model: Labwan benchtop pH meter) (Asif *et al*., 2014a). The concentrations of K^+, Na^+ and Cl^- in the urine were measured using an ion selective electrode analysis (9180 Electrolyte analyzer, Roche). $Cl^-/Na^+ + K^+$ and Na^+/K^+ were estimated to determine natriuretic and carbonic anhydrase inhibitory activity, respectively (Somova *et al*., 2003).

3.5.6. Biochemical assay

Blood samples were collected into tubes and then subjected to centrifugation at 12000 rpm for a duration of 15 min. The serum from each sample was taken and kept at -80°C for the ensuing biochemical test. Biochemical assay viz. SGOT, SGPT, Bilirubin, Urea, Blood Urea Nitrogen (BUN), Creatinine, were performed using the Coral Clinical Systems kit as per the kit's instructions.

3.5.7. Glomerular filtration rate

Glomerular filtration rate was estimated using creatinine clearance, based on serum and urine creatinine levels with values expressed in ml/min (Bazzano *et al*., 2015; Pellicer-Valero *et al*., 2022).

3.5.8. Histology of the kidney

Histological studies were carried out according to the previously described protocol (Bancroft and Gamble, 2008**).** Kidneys of the rats administered with the plant extract were taken out and immersed in Bouin's solution for a 24h period. After being immersed in Bouin's solution, the tissues were dehydrated through a sequence of increasing concentrations of ethanol (70%, 90% and 100%), cleaned with xylene

and incorporated in paraffin wax. Using a Leica rotary microtome, namely the type RM2125 RTS, a ‘tissue block’ was created and subsequently cut into 7mm thick slices. The slides were coated with poly-L-lysine to ensure adhesion, and the tissue sections were carefully dispersed in a 43 °C water bath. The slides were deparaffinized using xylene, and then rehydrated using a graded ethanol series for 10 min at each concentration. Haematoxylin and eosin stains were used to stain the slides, and subsequently, the slides were dried using varying concentrations of alcohol. The slides were then mounted with DPX after being cleared with xylene to complete the process and then meticulously scrutinised and captured on camera with a Nikon E200 microscope.

3.6. Compound identification using GC-MS

GCMS-QP2010 Ultra was used to identify phytochemicals present in HSPE, HSCE, HSME, and HSAE, with the column oven set to 120°C, the injection temperature set to 200°C, and the column flow rate set to 1.21 ml/min. The ion source was held at a temperature of 220°C. About 500µg of each extract, each with a concentration of 1mg/ml, were blended in a separating funnel and thoroughly agitated with a 1:4 mixture of water and ethyl acetate. The upper layer was gathered, reduced to 1ml though a rotary evaporator, and then subjected to additional processing with 50µl of N, O-Bis (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (BSTFA+TMCS), followed by the addition of 10µl of pyridine. The mixture was subjected to heating at a temperature of 60ºC for 30 min. Following this, the solutions were moved to GC vials, dehydrated using nitrogen gas, and then solubilized in methanol prior to GC-MS analysis. The compounds discovered were identified by matching them to recognised compounds in the National Institute of Standards and Technology (NIST) database.

CHAPTER 4

RESULTS

The results and interpretations of the present study has been presented in this chapter as follows:

4.1. Qualitative phytochemical analysis

The preliminary phytochemical screening of *H. scandens* extracts revealed the presence of important phytochemical constituents such as, carbohydrates, flavonoid, phytosterols, alkaloids, saponin, glycosides and tannins. However, protein was found to be absent in all four extracts. Glycoside, tannin, saponin and alkaloids were found to be absent in HSPE and HSCE whereas HSME and HSAE did not show the presence of phytosterol and glycoside (Table 1).

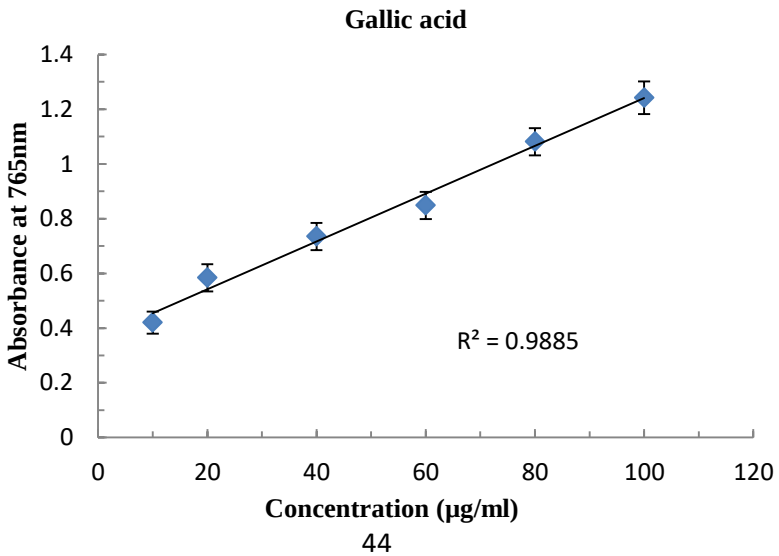
Table1. Phytochemical screening of *H. scandens* extract

Sl. No	Compounds	Phytochemical test	Present/Absent			
			HSPE	HSCE	HSME	HSAE
1	Alkaloids	Mayer's test	Absent	Absent	Present	Present
		Dragendroff's test	Absent	Absent	Present	Present
		Wagner's test	Absent	Absent	Present	Present
		Hager's test	Absent	Absent	Present	Present
2	Carbohydrates	Molisch's test	Absent	Absent	Present	Present
		Fehling's test	Present	Present	Present	Present
		Benedict's test	Present	Absent	Present	Absent
3	Phytosterol	Leibermann-Burchard's test	Present	Present	Absent	Absent
		Salkwoski reaction	Present	Absent	Absent	Absent
4	Glycosides	Legal's test	Absent	Absent	Absent	Absent
		Keller Killiani's test	Absent	Absent	Absent	Absent

5	Tannin	$FeCl_3$	Absent	Absent	Present	Present
		$K_2Cr_3O_7$	Absent	Absent	Absent	Absent
		Lead acetate test	Absent	Absent	Absent	Absent
6	Saponins	Foam of froth test	Absent	Absent	Absent	Present
7	Proteins and amino acid	Xanthoproteic test	Absent	Absent	Absent	Absent
		Hydrolysis test	Absent	Absent	Absent	Absent

4.2. Quantitative phytochemical analysis

A standard graph was used to analyze and quantify the Total Phenol Content (TPC) and Total Flavonoid Content (TFC) of the *H. Scandens* extracts **(Fig 2 and 3).** Gallic acid served as the standard for determining TPC, while Quercetin was employed as the standard for TFC calculations. The quantification of phytochemicals of different extracts of *H. scandens* revealed that HSME exhibited the highest phenolic content, measuring at 27.2 ± 0.6 mg of Gallic Acid Equivalent (GAE) per gram. This was followed by HSAE with a TPC of 23.6 ± 0.7 mg GAE/g. HSPE displayed a TPC of 14.5 ± 0.6 mg GAE/g, while HSCE had the lowest TPC at 13.7 ± 0.5 mg GAE/g. The highest TFC content of *H. scandens* was shown by HSME, which had a value of 29.9±0.3 QE mg/g, followed by HSAE which had 27.4 ± 0.3 QE mg/g of flavonoid. HSPE possessed a TFC of 25.1 ± 0.2 QE mg/g and HSCE on the other hand had the lowest flavonoid content at and 24.6 ± 0.2.



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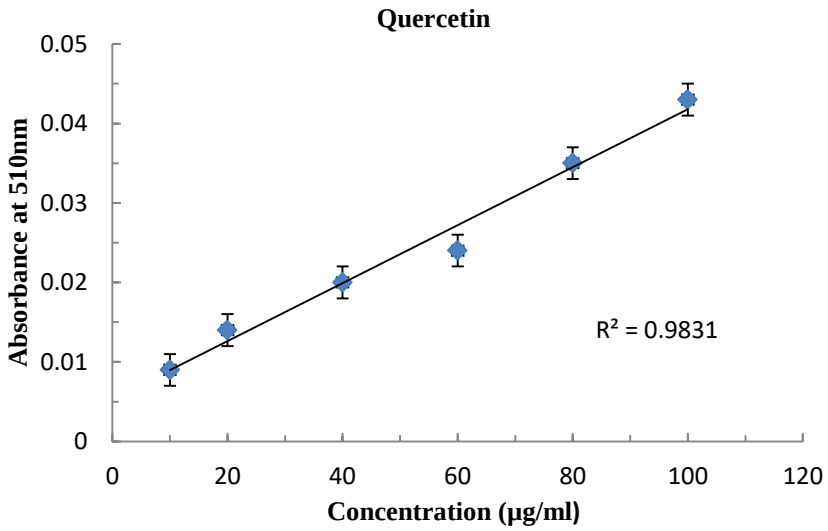


3.

Fig 2 and 3: Standard graph of gallic acid for Total Phenol Assay and Standard curve of quercetin for total flavonoid assay. Dotted line represents the linear graph (Values are in mean ± SEM, n = 3).

4.3. Antioxidant activities

To evaluate the antioxidant properties of various extracts, a range of *in-vitro* assays, including the phosphomolybdenum assay (Total Antioxidant Activity), Ferric reducing capacity, DPPH, $ABTS^{+}$, Superoxide scavenging assay, Nitric oxide was employed and IC_{50} of the extracts in comparison to the standard drug as given below in Table 2 and capacity of the different extracts in scavenging the radicals is depicted in Figures 4 to 9.

Table 2. IC_{50} values and Total Antioxidant Activity (TAA) for various extracts of *H. scandens* against different free radicals.

	DPPH (µg/g)	$ABTS^{+}$(µg/g)	O^{2-} (µg/g)	NO (µg/g)	TAA (mg/g)
HSPE	108.6± 0.6	126.6 ± 0.2	69.5 ±1	_	32.1 ± 0.3 AE.
HSCE	118.2 ± 0.5	74 ± 0.1	_	_	38.8 ± 0.2 AE
HSME	24.5±0.5	50.03 ± 0.1	32.6±0.7	83.6±0.2	98.03±0.1 AE
HSAE	74.1 ± 1	53.7 ± 0.3	60.3±0.6	173.2±0.3	51.1 ± 1 AE
BHT	13.9± 0.5	8.4 ± 0.2	_	_	_
ASC	_	_	26.6±0.3	64.2±0.1	_

4.3.1. DPPH radical scavenging activity

The DPPH scavenging activity of the different extracts of *H. scandens* increased proportionally with higher concentration of the extract (10-100 µg/ml) as shown in Figure 4. The scavenging activity was effective in the order: HSME (24.5±0.5 µg/ml)> HSAE (74.1 ± 1 µg/ml)> HSPE (108.6 ± 0.6 µg/ml)> HSCE (118.2 ± 0.5 µg/ml) with respect to BHT (13.9± 0.5µg/ml) as given in Table 2.

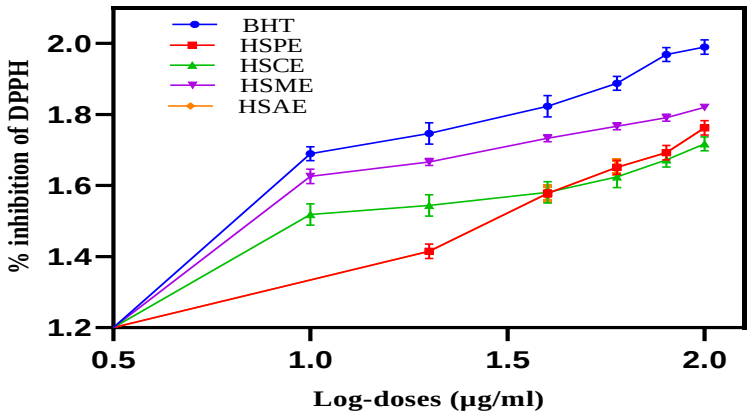


Fig 4: Plots of log-doses of *H. scandens* extracts against DPPH radical. Values are expressed as Mean ± SEM, n= 3

4.3.2. ABTS^{+} scavenging activity

As seen in Figure 5, the ability of *H. scandens* extracts to scavenge ABTS^{+} cations increased as concentration increased (10-100 g/ml). The order of effectiveness for the scavenging activity was as follows: HSME (50.03 ± 0.1µg/ml)> HSAE (53.7 ± 0.3 µg/ml)> HSPE (126.6 ± 0.23 µg/ml)> HSCE (74 ± 0.1 µg/ml), whereas the standard BHT had an IC_{50} value of 8.4 ± 0.2 µg/ml.

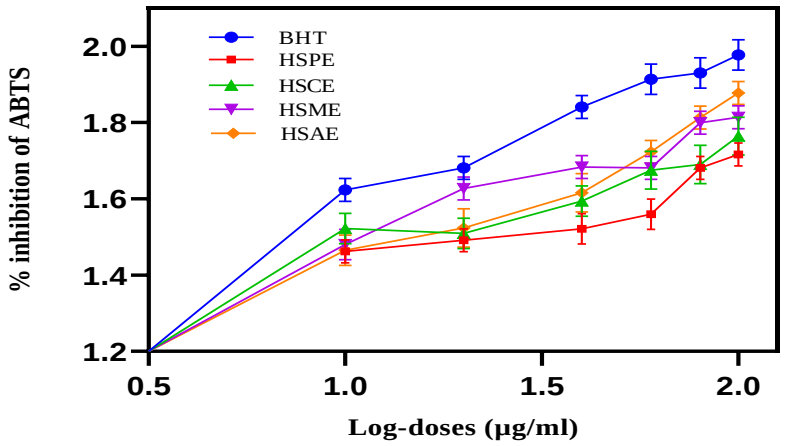


Fig 5: Plots of log-doses of *H. scandens* extracts against ABTS radical. Values are expressed as Mean ± SEM, n= 3

4.3.3. Superoxide scavenging activity ($O^{2\bullet-}$)

HSPE, HSME and HSAE showed scavenging potential against superoxide ion. However, HSCE could not scavenge the radical even at the highest tested concentration. The superoxide scavenging activity of the extracts displayed an incremental increase in activity with rising concentrations (10-100 µg/ml), as illustrated in Figure 6. The following was the order of the scavenging activity's effectiveness: HSME (32.6 ±0.7 µg/ml)> HSAE (60.24±0.6 µg/ml)> HSPE (69.5±1 µg/ml), standard ascorbic acid on the other hand had an IC_{50} of 26.6±0.3 µg/ml.

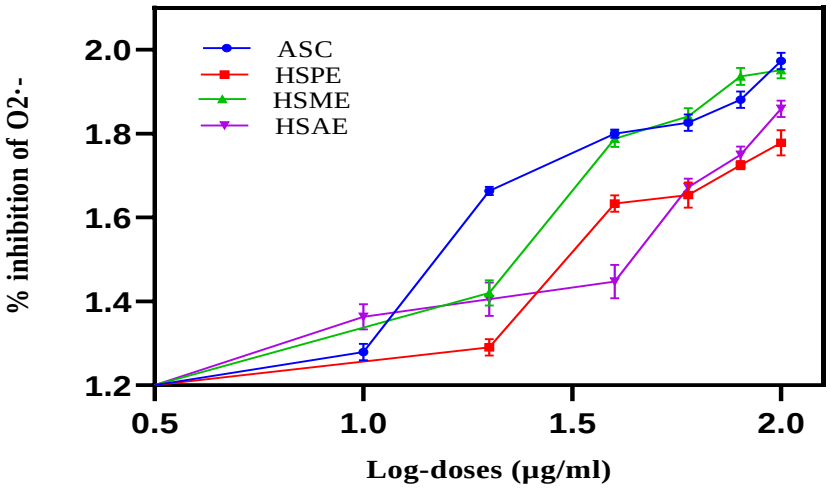


Fig 6: Plots of log-doses of *H. scandens* extracts against O_2^- radical. Values are expressed as Mean ± SEM, n= 3

4.3.4. Nitric oxide scavenging activity (NO)

HSPE and HSCE were unable to effectively scavenge nitric oxide at the tested concentration range of 10-200µg. In contrast, HSME and HSAE exhibited scavenging activity that increased as their concentration increased as illustrated in Figure 7 and Table 2. HSME had higher scavenging potential at IC_{50} of 83.6±0.2 µg/ml, while HSAE had an IC_{50} of 173.2 ± 0.3 µg/ml**.** Ascorbic acid was used for standard reference and it had an IC_{50} value of 64.2±0.1 µg/ml.

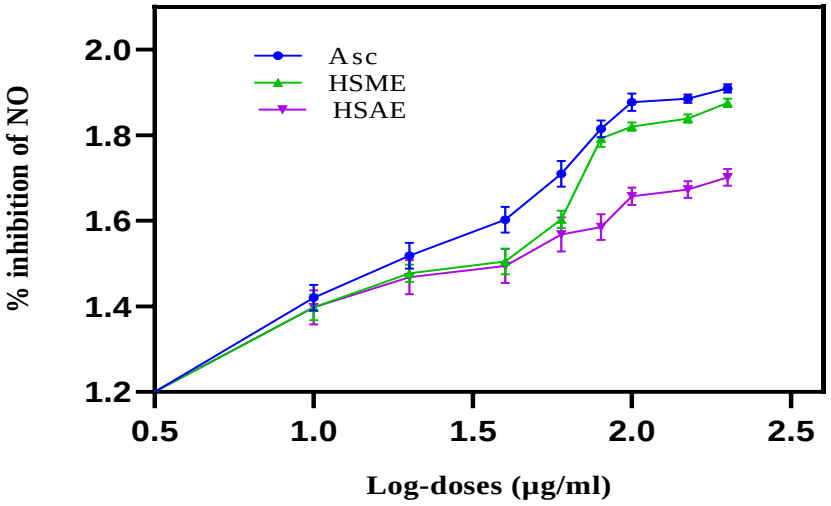


Fig 7: Plots of log-doses of *H. scandens* extracts against Nitric oxide radical. Values are expressed as Mean ± SEM, n= 3

4.3.5. Ferric reducing capacity

Ferric reducing capacity of *H. scandens* was determined in a concentration dependent manner, which proved that the extracts were capable of reducing potassium ferricyanide with rise in concentration ranging from 10µg/ml to 100µg/ml. HSME had the highest reducing potential followed by HSAE, HSCE and HSPE. However, the standard ascorbic acid showed the highest reducing potential. The reducing potential of the extracts and ascorbic acid is given in Figure 8.

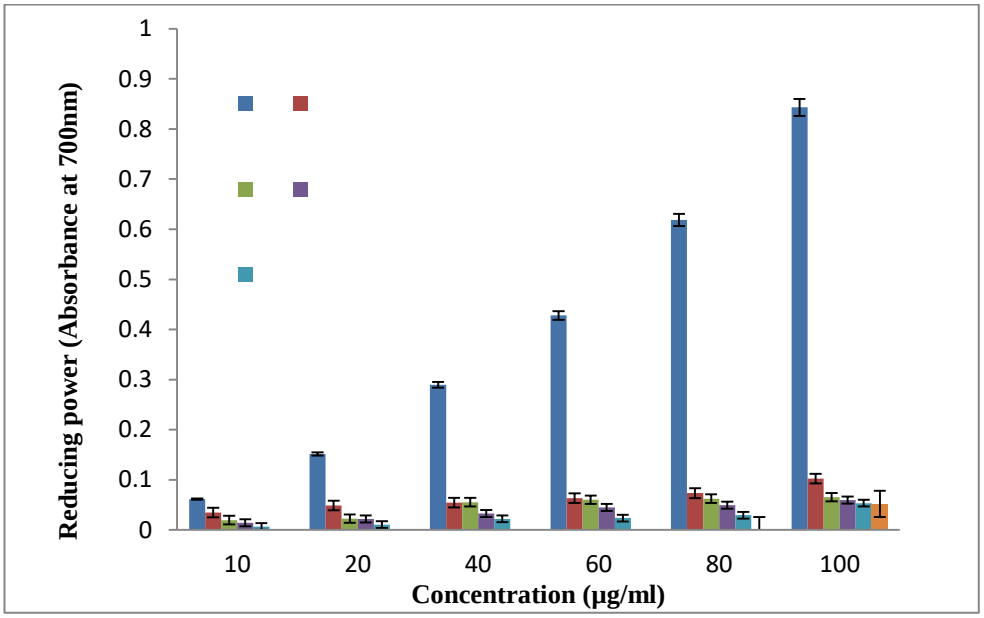


Fig 8: Reducing power of *H. scandens* extracts and Ascorbic acid at different concentrations. Values are expressed as Mean ± SEM, n= 3.

4.3.6. Total Antioxidant Activity (Phosphomolybdenum Assay)

Phosphomolybdenum assay was used to calculate the total antioxidant potential of different extracts of *H. scandens*. The TAA of each extract was calculated from a standard graph given below (Figure 9) and the value was given in terms of ascorbic acid equivalent (AE) per gram of dried extract which was used as standard reference. The values, ranked from highest to lowest, are as follows: HSME 98.03±0.1 AE mg/g, HSAE 51.1 ± 1 AE mg/g, HSCE 38.8 ± 0.2 AE mg/g and HSPE 32.1 ± 0.3 AE mg/g.

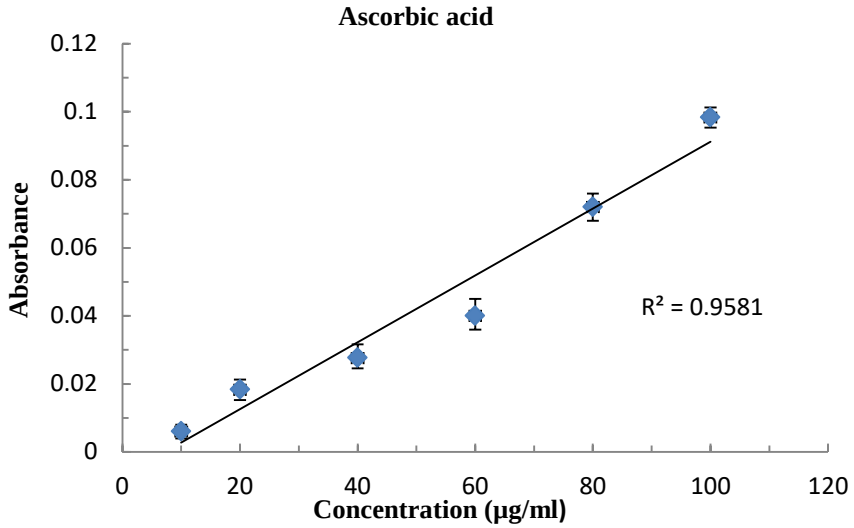


Fig 9: Standard curve of Ascorbic acid for Total Antioxidant Assay. The percentage of inhibition is plotted against concentration of sample. Values are expressed as Mean ± SEM, n= 3.

4.4. Antibacterial activity of *H. scandens* extract

HSPE, HSCE, HSME and HSAE were tested against all the aforementioned microorganisms. However, it was observed that HSPE and HSCE did not exhibit any activity against all the tested microorganisms even at concentration as high as 100mg, therefore it was concluded that both the extract did not possess antibacterial property against the tested organisms. HSME and HSAE on the other hand showed positive effect against all the organism tested. HSME however showed pronounced inhibition activity compared to HSAE.

HSME had highest activity against *Micrococcus luteus* and *Salmonella typhi* followed by *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus subtilis* and lastly, *Pseudomonas aeruginosa*. HSAE on the other hand showed highest inhibition against *Klebsiella pneumonia* followed by *Pseudomonas aeruginosa*, *Bacillus*

subtilis, *Micrococcus luteus*, *Salmonella typhi* and *Escherichia coli*. The measured zone of inhibition against the different microorganism in manner of highest to lowest activity is given in a table below:

Table 3. Inhibition zone of HSME and HSAE in mm against different bacteria as compared to Ceftriaxone

HSME	10mg	20mg	Ceftriaxone (10µg/ml)	HSAE	10mg	20mg	Ceftriaxone (10µg/ml)
	Zone of inhibition in mm				Zone of inhibition in mm		
M. luteus	11.3 ± 0.3^{b}	15.3 ± 0.3^{b}	24 ± 0.01^{a}	***K. pneumoniae***	9.3 ± 0.3^{b}	11.3 ± 0.3^{b}	25 ± 0.03^{a}
S. typhi	11.6 ± 0.6^{b}	15.6 ± 0.6^{b}	25 ± 0.03^{a}	***P. aeruginosa***	9 ± 0.5^{b}	10.6 ± 0.3^{c}	25 ± 0.03^{a}
K. pneumoniae	9.3 ± 0.6^{c}	14 ± 0.5^{c}	25 ± 0.03^{a}	***B. subtilis***	8.6 ± 0.3^{c}	10.3 ± 0.3^{c}	25 ± 0.03^{a}
E. coli	8.3 ± 0.3^{d}	10.6 ± 0.3^{d}	22 ± 0.3^{a}	***M. luteus***	7.6 ± 0.3^{d}	9 ± 0.5^{d}	24 ± 0.01^{a}
B. subtilus	8 ± 0.3^{d}	10 ± 0.3^{d}	25 ± 0.03^{a}	***S. typhi***	7.6 ± 0.3^{c}	9.3 ± 0.3^{d}	25 ± 0.03^{a}
P. aeruginosa	7 ± 0.5^{c}	10 ± 0.8^{c}	25 ± 0.03^{a}	***E. coli***	7 ± 0.5^{d}	9 ± 0.3^{d}	22 ± 0.3^{a}

Values are expressed as Mean ± SEM, n= 3. Different letters indicate significant variation at $p < 0.05$

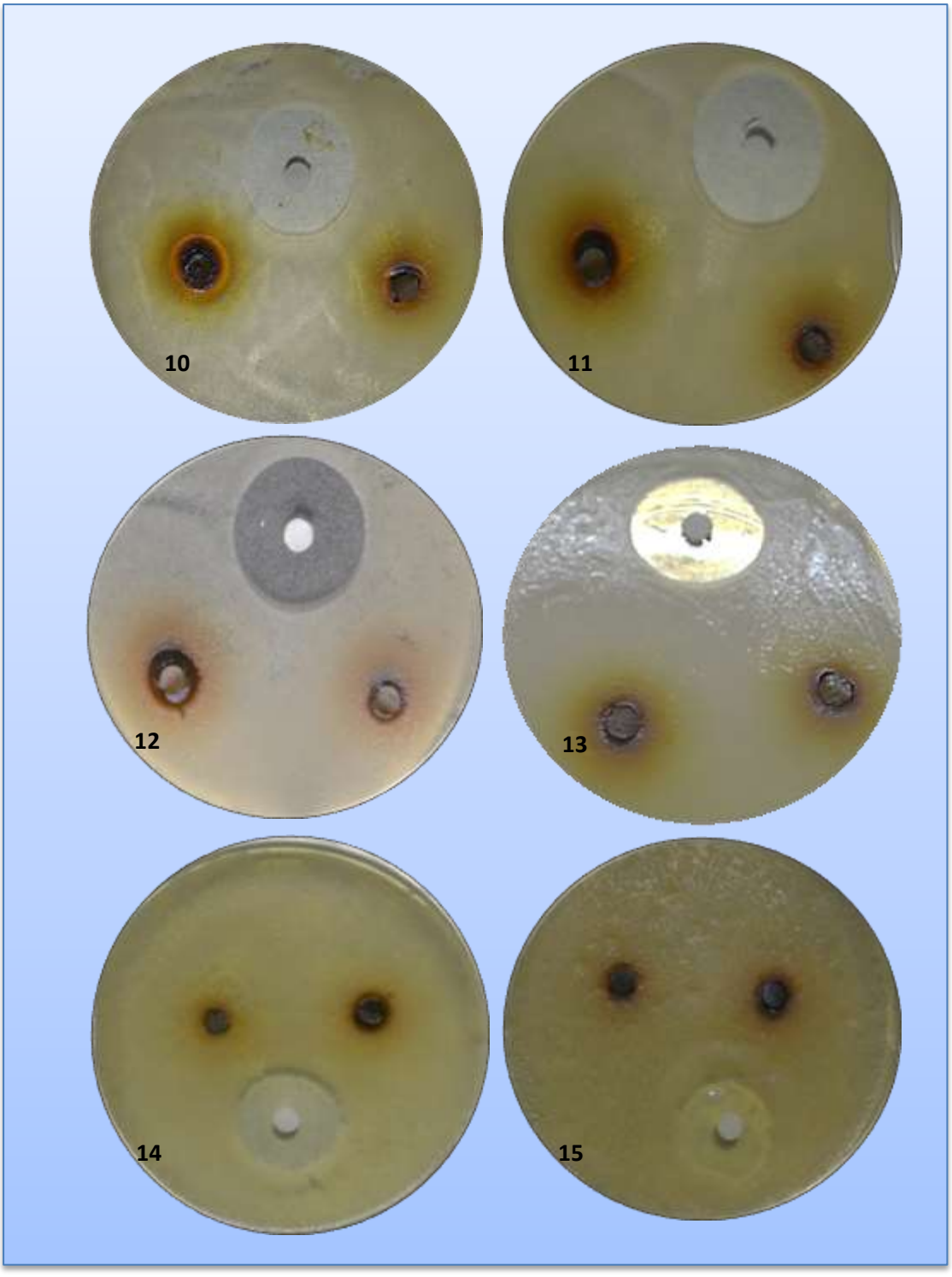


Fig. 10 to 15: Antibacterial activity of HSME against *Micrococcus luteus*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus subtilus*, *Pseudomonas aeruginosa*

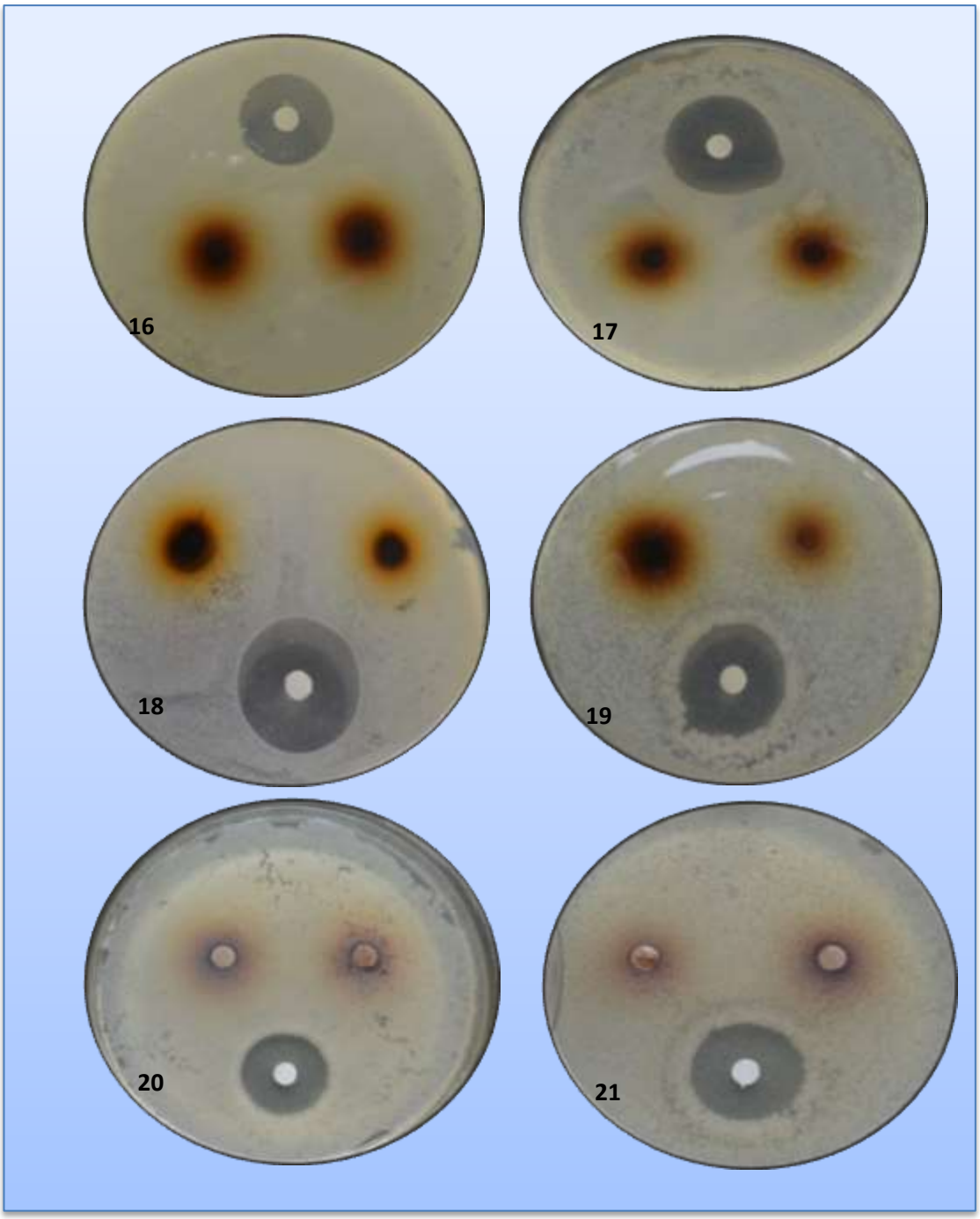


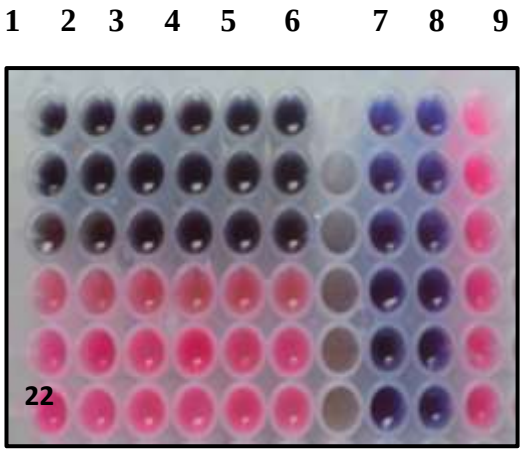
Fig 16 to 21: Antibacterial activity of HSAE against *Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus subtilus, Micrococcus luteus, Salmonella typhi, Escherichia coli.*

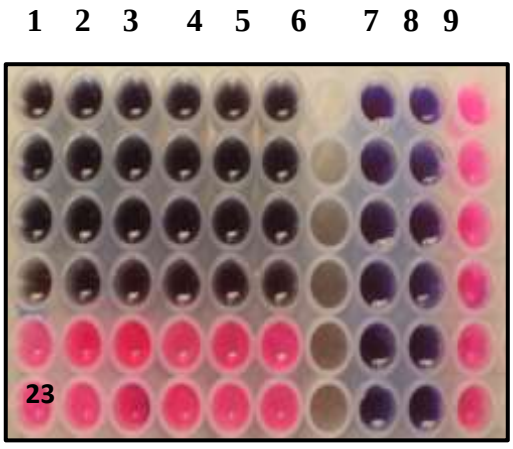
4.4.1. **Minimum Inhibitory Concentration (MIC)**

The MIC of HSME and HSAE were tested on concentrations below the lowest tested concentration that is 10mg. The MIC was determined by identifying the lowest concentration of the extract at which colour change occurred on addition of resazurin. Colour change in the wells was monitored visually. Wells exhibiting pink colour after the incubation period indicated bacterial growth whereas plates displaying blue indicated no growth. The results are given below:

Table 4: Minimum Inhibitory Concentration of HSME and HSAE against different bacteria

	HSME (mg/ml)	**HSAE (mg/ml)**
Micrococcus luteus	6	10
Salmonella typhi	4	10
Klebsiella pneumoniae	8	8
Escherichia coli	10	10
Bacillus subtilus	10	10
Pseudomonas aeruginosa	10	10





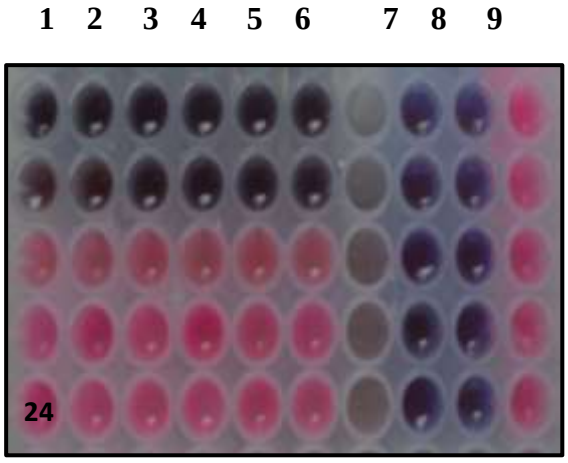


Fig 22, 23 and 24. Determination of MIC for HSME against *Micrococcus luteus, Salmonella typhi, Klebsiella pneumoniae*.

Various concentrations were organized from top to bottom in descending order, from the highest to the lowest. The last row showing change of colour was considered as the MIC. After the period of incubation, resazurin dye was added. Column 9, a negative control shows a change of resazurin natural color (*blue/purple*) to the reduced form (*red-colourless*). The highest concentration incorporated into the plate is 10 mg/ml and the lowest concentration tested was 0.05 mg/ml. Column 7, 8 are positive controls which showed no change of color which indicated that the standard Ceftriaxone showed positive inhibition against all tested bacteria.

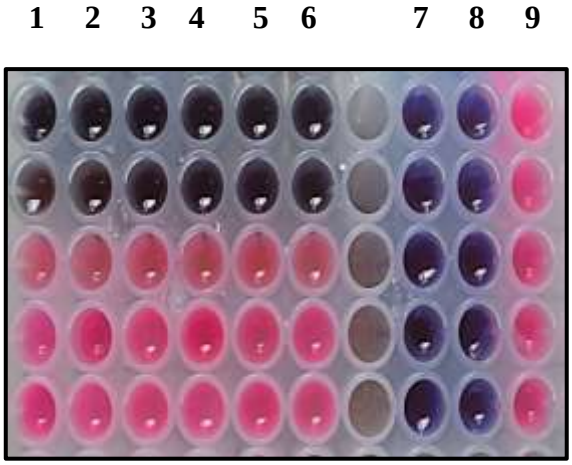


Fig 25. Determination of MIC for HSAE against *Klebsiella pneumoniae*.

Various concentrations were organized from top to bottom in descending order, from the highest to the lowest. The last row showing change of color was considered as the MIC. After the period of incubation, resazurin dye was added. Column 9, a negative control shows a change of resazurin natural color (*blue/purple*) to the reduced form (*red-colorless*). The highest concentration incorporated into the plate is 10 mg/ml and the lowest concentration tested was 0.05 mg/ml. Column 7, 8 are positive controls which showed no change of color which indicated that the standard Ceftriaxone showed positive inhibition against all tested bacteria.

Based on results obtained from the experiments mentioned earlier, along with an extensive review of the literature and initial investigations, it was decided to choose only two out of the four extracts, namely HSME and HSAE, for further *in vivo* studies. These studies were focused specifically on evaluating their diuretic effects.

4.5. Acute Toxicity Study

Table 5. Effect of HSME and HSAE on clinical parameters of acute toxicity

Clinical sign of toxicity	1000 mg/kg b. wt	2000 mg/kg b. wt	5000 mg/kg b. wt
Mortality	-	-	-
Diarrhea	-	-	-
Tremors	-	-	-
Body weight	+	+	+
Impaired limb function	-	-	-
Loss of righting reflex	-	-	-
Excessive salivation	-	-	-

- indicates no effect, + indicates positive effect

Throughout the entire 14-day observation period, in accordance with OECD guideline 423, all the animals involved in the acute toxicity study consistently survived without displaying any noticeable neurological or behavioural abnormalities. Also, none of the parameters for acute toxicity listed in the above table showed positivity, except for slight increase in body weight, which was still within the normal range. This comprehensive result provides compelling evidence that both HSME and HSAE does not fall within the scope of classification under the 'Global Harmonization System,' as indicated by Jadhav *et al* in 2010. Considering the absence of detectable toxicity at the administered doses, the need to determine the exact LD_{50} value was deemed unnecessary.

4.5.1. Selection of doses

Based on the acute toxicity study result, three doses of extract were selected, a low dose of 25 mg/kg, a middle dose of 500 mg/kg and a high dose of 1000 mg/kg. Similarly, for the *H. scandens* + furosemide group, furosemide at a dose of 10 mg/kg was combined with the various doses of HSME and HSAE (250 mg/kg, 500 mg/kg, and 1000 mg/kg b. wt). For control group, the animals were administered water (2.5 ml/kg b. wt) whereas for the positive control group, animals were given furosemide at 10mg/kg b.wt. Animals were randomised into the following groups (n=6) and all treatment were given orally using an oral gavage. Each rat received an oral load of 2.5ml of 0.9% NaCl/100g of body weight to ensure uniform water and salt load.

4.5.2. Effect of HSME and HSAE on urinary pH

HSME and HSAE did not show any adverse effect on urinary pH and exhibited minimal alteration. The control rats had a urinary pH of 7.08 ± 0.5. In contrast, the rats treated with standard furosemide had a urinary pH of 7.5 ± 0.08. Rats treated with the extract resulted in a dose dependent increase of urinary pH and HSME 1000 mg/kg+F, 10 mg/kg showed the highest value of 7.5± 0.09 followed by 1000 mg/kg at 7.4± 0.6 respectively. Furthermore, the rats treated with HSAE 1000 mg/kg+F, 10 mg/kg had a pH of 7.4± 0.9 HSAE followed by HSAE1000 mg/kg at 7.3± 0.07 (Table 6).

Table 6. Effect of HSME and HSAE on urinary pH

	5h	24h		5h	24h
Control	7.08± 0.5		**Control**	7.08± 0.5	
Furosemide,10 mg/kg	7.5± 0.08		**Furosemide, 10mg/kg**	7.5± 0.08	
HSME, 250mg/kg	7.1± 0.4	7.3± 0.5	**HSAE, 250mg/kg**	7.04± 0.08	7.1± 0.5
HSME, 500mg/kg	7.2± 0.2		**HSAE, 500mg/kg**	7.2± 0.05	7.2± 0.5
HSME, 1000mg/kg	7.4± 0.6		**HSAE, 1000mg/kg**	7.3± 0.07	7.4± 0.5
HSME 250mg/kg+F, 10 mg/kg	7.1± 1		**HSAE 250mg/kg+F, 10mg/kg**	7.2± 0.3	
HSME 500mg/kg+F, 10 mg/kg	7.4± 0.05		**HSAE 500mg/kg+F, 10mg/kg**	7.31± 0.5	
HSME 1000mg/kg+F, 10 mg/kg	7.5± 0.09		**HSAE 1000mg/kg+F, 10 mg/kg**	7.4± 0.9	

Data are expressed as mean ± SEM, $n = 6$

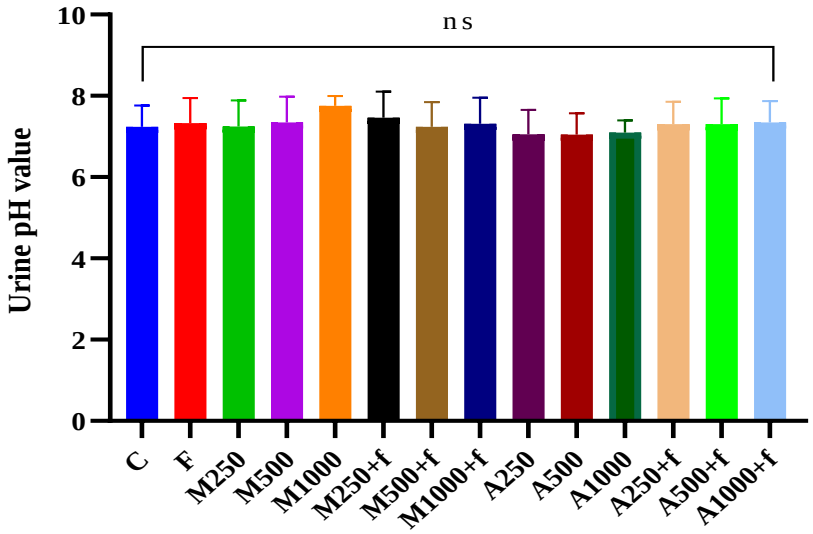


Fig 26: pH of various groups of HSME and HSAE. Values are expressed as Mean ± SEM, n= 6. Statistical analysis was conducted using a one-way ANOVA at p < 0.05

4.5.3. Effect of *H. scandens* on urinary volume

HSME and HSAE increased urine output in a dose dependent manner, when compared to the control group (Tables 7 and 8). The extracts at all administered doses exhibited pronounced diuresis at the 5th hour, and there was an absence of pronounced diuresis at the 24th hour, which was consistent with the reference drug, except for HSAE at 250 mg/kg and HSAE at 250, 500, and 1000 mg/kg, which displayed diuresis even at the 24th hour. Interestingly, at a dosage of 1000 mg/kg, HSME demonstrated the highest diuretic effect at 5 hours, while HSAE exhibited its peak diuretic action at 24 hours. When both extracts were combined with furosemide (HSME+F and HSAE+F), there was an enhancement in diuretic activity compared to the administration of the extracts alone. However, it's worth noting that furosemide displayed the highest urine output and reached its peak diuretic effect within 5 hours.

Across all the tested dosages, HSME and HSME+F consistently showed higher urine output than HSAE. Remarkably, HSME and HSME+F demonstrated much higher urine production than the control group, even at the lowest dose of 250

mg/kg. However, furosemide exhibited the highest diuretic activity value of 1, closely followed by HSME 1000 mg/kg+F at 0.9. At the dosage of 500 mg/kg+F, HSME had a diuretic activity of 0.58 and comparable diuretic activity of 0.43 was shown by HSME at 250 mg/kg+F and HSAE at 500 mg/kg. On the hand, the control group showed the lowest activity.

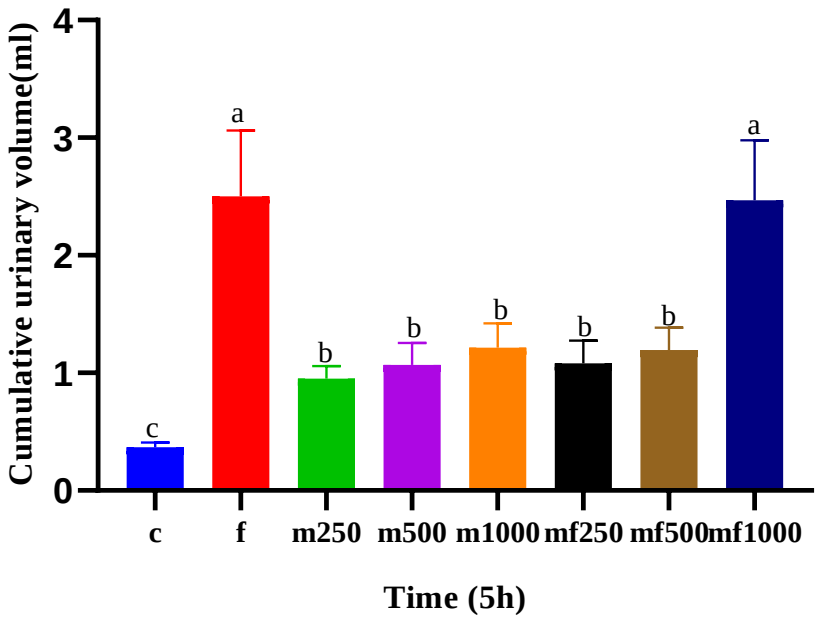


Fig 27. Urine volume of HSME at 5h. Values are expressed as Mean ± SEM, n= 6. Different letters indicate significant variation at p < 0.05. Statistical analysis was conducted between the groups using a one-way analysis of variance (ANOVA), followed by the DMRT test.

Table 7. The effects of *Hedyotis scandens* methanol extract (HSME) and its combinations with furosemide (HSME+F) on urine volume at two time points: 5 hours (5h) and 24 hours (24h).

	Urine volume		**Diuretic action**		**Diuretic activity**	
	5h	24h	5h	24h	5h	24h
Control	2.2±0.4		0.15			
Furosemide, 10 mg/kg	15±0.2*		7.1		1	
HSME, 250 mg/kg	5.7±0.1	5.2±0.3	2.6	2.4	0.38	0.34
HSME, 500 mg/kg	6.4±0.2		3		0.2	
HSME, 1000 mg/kg	7.3±0.2		3.32		0.48	
HSME 250 mg/kg+F, 10 mg/kg	6.5±0.2		2.9		0.43	
HSME 500 mg/kg+F, 10 mg/kg	7.18±0.2		3.26		0.47	
HSME 1000 mg/kg+F, 10 mg/kg	14±0.7**		6.36		0.9	

The data presented in the table represent the mean values along with the standard error of the mean (SEM) for a sample size of 6. Statistical analysis was conducted using a one-way analysis of variance (ANOVA), followed by the Dunett's test for multiple comparisons. Significance levels are indicated as follows: *$p < 0.05$: Indicates statistical significance at the 0.05 level, suggesting that the compared groups are significantly different from the control group. **$p < 0.001$: Indicates high statistical significance at the 0.001 level, suggesting that the compared groups are significantly different from the control group with a higher level of confidence.

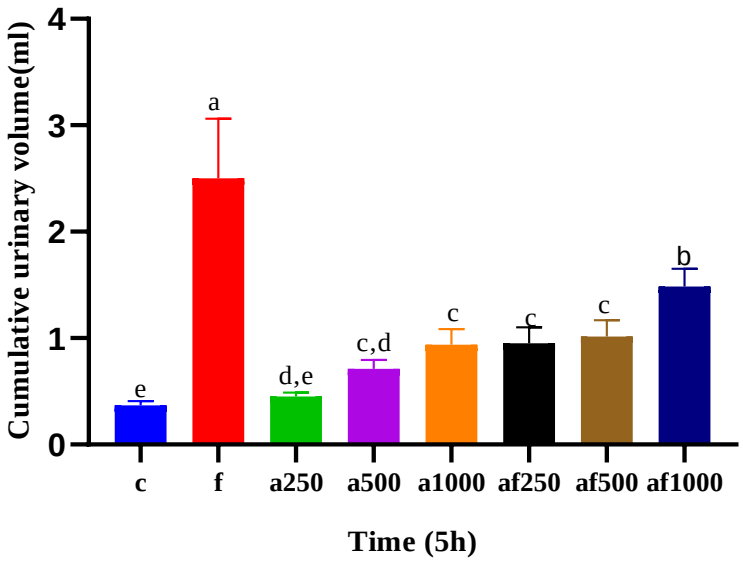


Fig 28: Urine volume of HSAE at 5h. Values are expressed as Mean ± SEM, n= 6. Different letters indicate significant variation at p < 0.05. Statistical analysis was conducted between the groups using a one-way analysis of variance (ANOVA), followed by the DMRT test.

Table 8. The effects of various treatments of *Hedyotis scandens* aqueous extract (HSAE) and its combinations with furosemide (HSAE+F) and on urine volume, diuretic action, and diuretic activity at both 5-hours (5 h) and 24-hours (24 h) time points.

	Urine volume		**Diuretic action**		**Diuretic activity**	
	5h	24h	5h	24h	5h	24h
Control	2.2±0.4		0.15			
Furosemide, 10 mg/kg	15±0.2*		1		1	
HSAE, 250 mg/kg	2.7±0.5	5.45±0.2	1.23	2.5	0.18	0.36
HSAE, 500 mg/kg	4.32±1	6.5±0.2	1.97	2.9	0.28	0.43

HSAE, 1000 mg/kg	5.63±1	7.5± 0.2	2.56	3.4	0.37	0.5
HSAE 250 mg/kg+F, 10 mg/kg	5.7±0.9		2.5		0.38	
HSAE 500 mg/kg+F, 10 mg/kg	6.1±1.01		3		0.41	
HSAE 1000 mg/kg+F, 10 mg/kg	8.9±1.4*		4.04		0.59	

The data presented in the table represent the mean values along with the standard error of the mean (SEM) for a sample size of 6. Statistical analysis was conducted using a one-way analysis of variance (ANOVA), followed by the Dunett's test for multiple comparisons. Significance levels are indicated as follows: *p < 0.05: Indicates statistical significance at the 0.05 level, suggesting that the compared groups are significantly different from the control group.

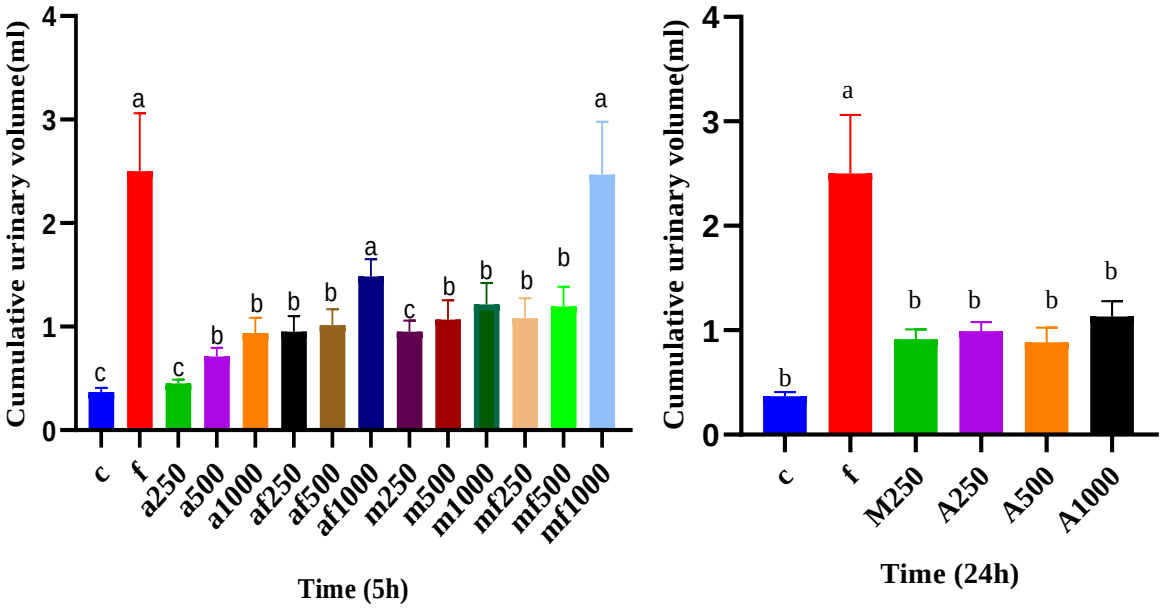


Fig 29 and 30: Combined cumulative assessment of urine volume for HSME and HSAE at 5h and 24h. Statistical analysis was conducted using a one-way analysis of variance (ANOVA), followed by the DMRT test. Different letters indicate significant variation at p < 0.05

4.5.4. Effect of *H. scandens* on urinary electrolyte output

Tables 9 and 10 provide a breakdown of the urinary electrolyte content of each of the groups. Once again, it was found that furosemide showcased the highest urinary electrolyte content, while the control group had the lowest urinary electrolyte level. Intriguingly, HSME+F at 1000 mg/kg exhibited a sodium (Na) output of 109 ± 2, closely resembling the Na output of furosemide at 115 ± 2. Additionally, HSME+F at 500 mg/kg displayed a Na output of 95.1 ± 3, somewhat comparable to HSME at 1000 mg/kg (91.6 ± 2). This experiment revealed that when combined with furosemide, both HSME and HSAE exhibited the highest Na output.

Notably, HSME at dosages of 500 and 1000 mg/kg demonstrated a higher potassium (K) output of 49.6 ± 2 and 50.5 ± 3, respectively, compared to furosemide, which had a K output of 49.3 ± 0.8. HSME 500 mg/kg exhibited slightly higher chloride (Cl) output (162 ± 3) than HSME 1000 mg/kg+ F (158.6 ± 3). HSAE 1000 mg/kg+ F, 10 mg/kg displayed a high Na output of 75.6 ± 4. HSAE 500 mg/kg+ F exhibited a K output similar to furosemide at 48.1 ± 5, while the K output of HSAE 1000 mg/kg+ F was higher than furosemide at 50 ± 2, akin to the K output of HSME at 500 mg/kg. Cl output of HSAE alone did not significantly differ from the control group. However, when combined with furosemide, the Cl output significantly exceeded that of the control group.

Table 9. The impact of HSME and HSME+Furosemide 10mg/kg on urinary electrolyte concentrations at the 5-hours mark.

	Na^+(mmol/L)	K^+(mmol/L)	Cl^- (mmol/L)	Saluretic index			Na^+/K^+	$Cl^-/Na^+ + K^+$
				Na^+	K^+	Cl^-		
Control	46.5± 3	34.3±2	77.5±1.4	1	1	1	1.4	0.95
Furosemide, 10 mg/kg	115±1.8*	49.3±0.8*	170.5±1*	2.5	1.43	2.2	2.3	1.03
HSME, 250 mg/kg	78.2±2*	41±2.8	135.6±5*	1.68	1.19	1.74	1.9	1.13
HSME, 500 mg/kg	90.8±3*	49.6±2.3	162±3*	1.95	1.44	2.09	1.83	1.15
HSME, 1000 mg/kg	91.6±2*	50.5±2.5	163±8*	1.9	1.47	2.1	1.81	1.14
HSME 250 mg/kg+F, 10 mg/kg	76.3±5*	42.5±5.1	141.8±6*	1.64	1.23	1.83	1.7	1.19
HSME 500 mg/kg+F, 10 mg/kg	95.1±3*	46.3±2.4	152.8±1*	2.04	1.34	1.97	2.1	1.1
HSME 1000 mg/kg+F, 10 mg/kg	109±2*	48.6±2.9	158.6±3*	2.34	1.41	2.04	2.2	1

The data presented in the table represent the mean values along with the standard error of the mean (SEM) for a sample size of 6. Statistical analysis was conducted using a one-way analysis of variance (ANOVA), followed by the

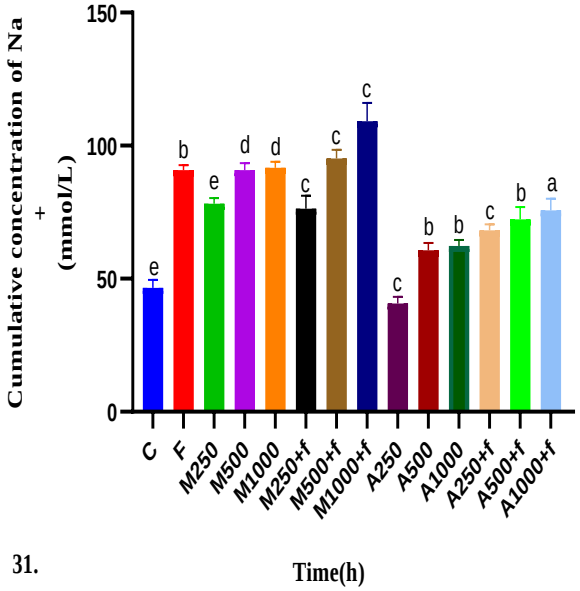
Dunett's test for multiple comparisons. Significance levels are indicated as follows: *p < 0.05: Indicates statistical significance at the 0.05 level, suggesting that the compared groups are significantly different from the control group.

Table 10. The influence of HSAE and HSAE+F on urinary electrolyte concentrations at the 5-hour interval. The parameters under study include Sodium (Na^+), Potassium (K^+), Chloride (Cl^-), Saluretic index, Na^+/K^+ ratio, and $Cl^-/Na^+ + K^+$ ratio.

	Na^+(mmol /L)	**K^+(mmol/L)**	**Cl^- (mmol/L)**	**Saluretic index**			**Na^+/K^+**	**$Cl^-/Na^+ + K^+$**
				Na^+	K^+	Cl^-		
Control	46.5± 3	34.3±2	77.5±1.4	1	1	1	1.4	0.95
Furosemide, 10 mg/kg	15±1.8*	49.3±0.8*	170.5±1*	2.5	1.43	2.2	2.3	1.03
HSAE, 250 mg/kg	40.6±3	35.8±2	83±2.9**	0.87	1.04	1.01	1.13	0.9
HSAE, 500 mg/kg	60.6±3*	41.6±4	162±3*	1.3	1.21	1.1	1.45	0.81
HSAE, 1000 mg/kg	62.3±2*	42.5±2	78±2.5**	1.33	1.24	1	1.46	0.74
HSAE 250 mg/kg+F, 10 mg/kg	68.1±3*	46.8±3*	118.5±2**	1.46	1.36	1.52	1.45	1.03
HSAE 500 mg/kg+F, 10 mg/kg	72.3±5**	48.1±5*	127.5±3**	1.55	1.4	1.64	1.5	1.05
HSAE 1000 mg/kg+F, 10 mg/kg	75.6±4**	50±2*	138.2±8**	1.62	1.45	1.78	1.51	1.1

The data presented in table 10 represents the mean values along with the standard error of the mean (SEM) for a sample size of 6. Statistical analysis was conducted using a one-way analysis of variance (ANOVA), followed by

Dunnett's test for multiple comparisons. Significance levels are indicated as follows: *p < 0.05: Indicates statistical significance at the 0.05 level, suggesting that the compared groups are significantly different from the control group. **p < 0.001 indicates high statistical significance at the 0.001 level, suggesting that the compared groups are significantly different from the control group with a higher level of confidence.



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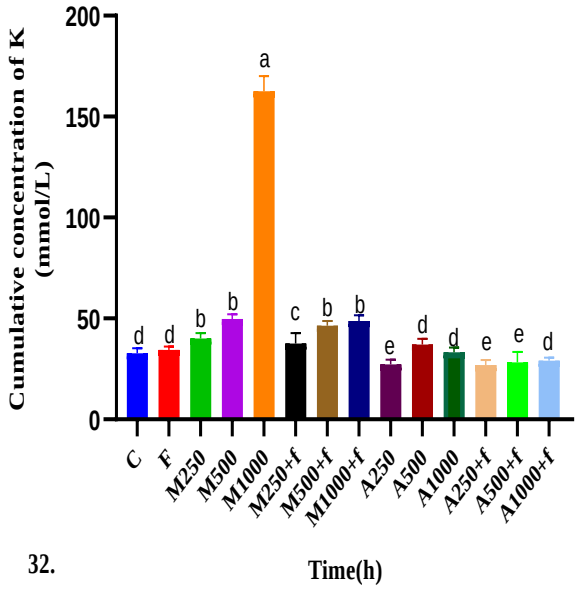

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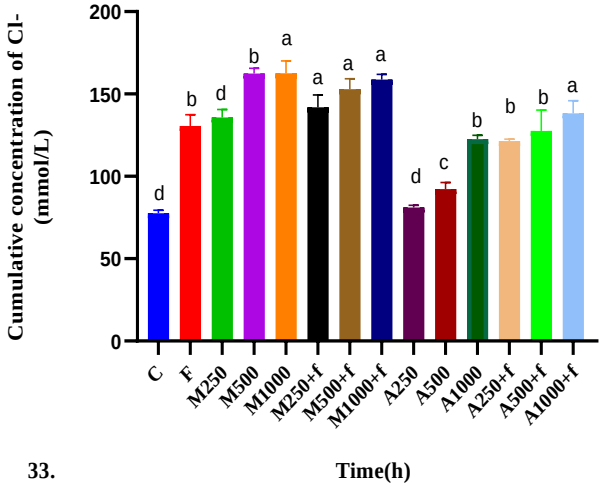


33.

Fig 31, 32 and 33: Effect of HSAE and HSME on urine electrolyte at 5h. Values are expressed as Mean ± SEM, n= 3. Different letters indicate significant variation at 0.05 level.

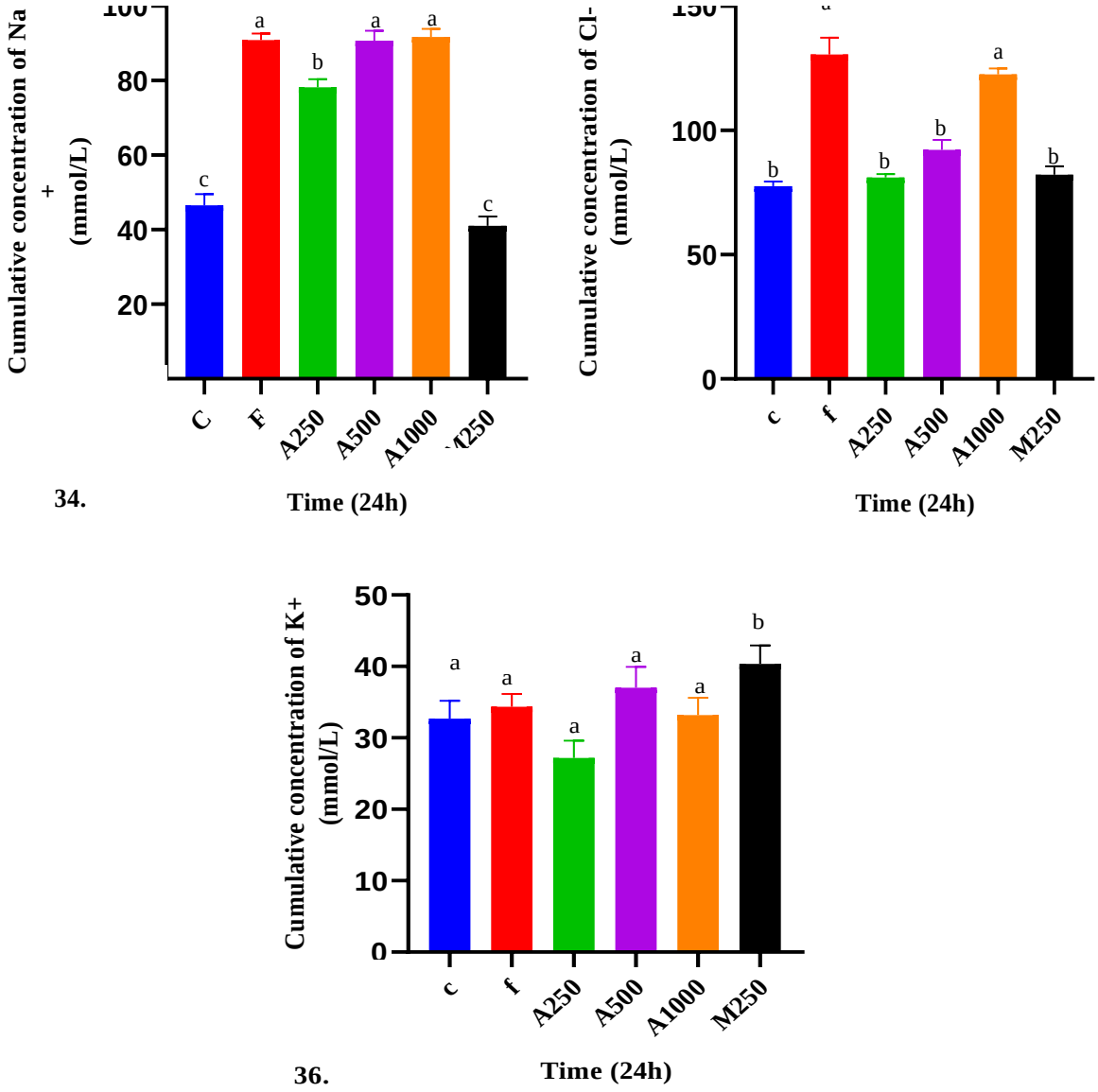


Fig 34, 35 and 36. Effect of HSAE and HSME on urine electrolyte at 24h. Values are expressed as Mean ± SEM, n= 3. Different letters indicate significant variation at 0.05 level.

4.5.5. Effect of *H. scandens* on biochemical parameters

The results of the biochemical analysis of serum indicated that the administered extracts, across various doses, did not result in significant increases in tested parameters when compared to the control group. Nevertheless, doses of HSME

250 mg/kg+F and HSAE 250 mg/kg+F, 10 mg/kg, exhibited a minor increase in urea concentration within the normal range. Furthermore, HSAE 500 mg/kg+F, 10 mg/kg, resulted in a slight elevation in creatinine levels, though still within the normal range.

Table 11: Results of biochemical analyses conducted on serum samples, revealing the impact of different treatments on various parameters. The parameters assessed include Serum Glutamic Oxaloacetic Transaminase (SGOT/U/L), Serum Glutamic Pyruvic Transaminase (SGPT/U/L), Urea (mg/dl), Blood Urea Nitrogen (BUN, mg/dl), and Creatinine (g/l).

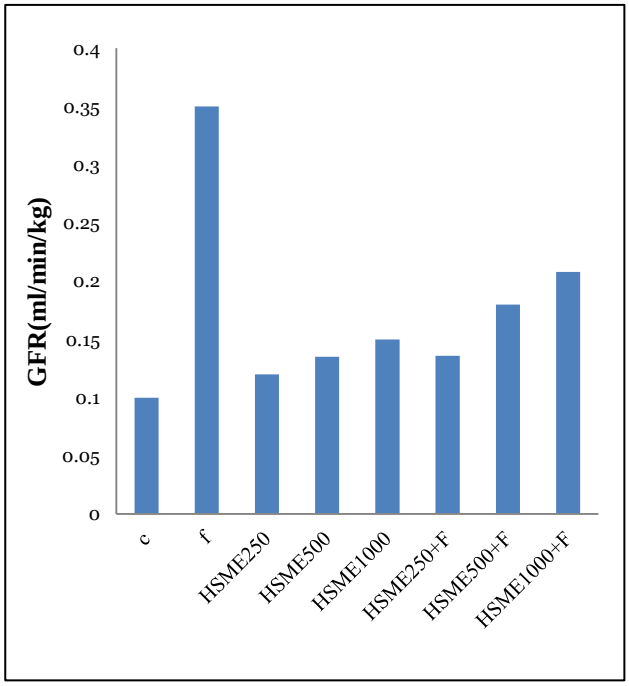
	SGOT(U/L)	**SGPT(U/L)**	**UREA (mg/dl)**	**BUN (mg/dl)**	**CREATININE (g/l)**
Control	44±0.7	28.5±2	36.6±2	17.8±2	0.85±0.4
Furosemide, 10 mg/kg	28.5±0.4	10*±0.9	13.34±1	11.1±0.5	0.6±0.2
HSME, 250 mg/kg	32.61±1	7.8**±3	22.8±1	11.5±1	0.66±0.4
HSME, 500 mg/kg	32.6±2	6.1**±0.8	25.7± 2	12.1±0.15	0.37±0.4
HSME, 1000 mg/kg	27.4±0.9	8.6**±0.2	11.09±0.6	6±0.7	0.76±0.1
HSME 250 mg/kg+F, 10 mg/kg	33.9±1	7.1**±2	38.6±3	14±0.3	0.26±0.5
HSME 500 mg/kg+F, 10 mg/kg	34.4±2	10*±3	29.7±2	16.83*±0.8	0.5±0.1
HSME 1000	31.65±2	14**±0.7	6.7±1	3.22±0.28	0.45**±0.2

mg/kg+F, 10 mg/kg					
HSAE, 250 mg/kg	24.8*±2	25.02±1	25±0.1	3.2±0.7	0.7±1
HSME, 500 mg/kg	29.1±0.8	25.96±2	12.1±0.1	4±1	0.5±1
HSAE, 1000 mg/kg	29.6±2	8.1**±0.9	11.13±2	4.55±0.3	0.4±0.5
HSAE 250 mg/kg+F, 10 mg/kg	24.7*±0.5	13.5*±0.7	36±2	5.43±0.3	0.28*±2
HSAE 500 mg/kg+F, 10 mg/kg	29.2±2	14*±6	12.6±1	5.91±0.6	0.86±0.9
HSAE 1000 mg/kg+F, 10 mg/kg	19.06**±2	6.6**±2	23±1	12.7±0.1	0.52±1.2

The data presented in table 11 represents the mean values along with the standard error of the mean (SEM) for a sample size of 6. Statistical analysis was conducted using a one-way analysis of variance (ANOVA), followed by the Dunnett's test for multiple comparisons. Significance levels are indicated as follows: *p < 0.05: Indicates statistical significance at the 0.05 level, suggesting that the compared groups are significantly different from the control group. **p < 0.001: Indicates high statistical significance at the 0.001 level, suggesting that the compared groups are significantly different from the control group with a higher level of confidence.

4.5.6. Effect of HSME And HSAE On Glomerular filtration rate and Histopathology

Renal function was evaluated using the creatinine clearance to determine the Glomerular Filtration Rate (GFR). Comparison of the GFR of treated animals at various doses to control and standard group is given in Figures: 38, 39 and 40. The results revealed significant changes in GFR dynamics. In comparison to the control group, GFR increased noticeably in both HSME and HSAE, indicating a possible benefit to renal filtration processes. Notable, among the groups examined, the well-known diuretic furosemide showed the highest GFR value, indicating a significant impact on renal filtration. The second highest GFR enhancer after furosemide was HSAE given at a dosage of 1000 mg/kg, demonstrating its ability to support effective renal function. In conclusion, these findings collectively underscore the potential renal benefits of HSME and HSAE, further solidifying their promising role in promoting renal health.



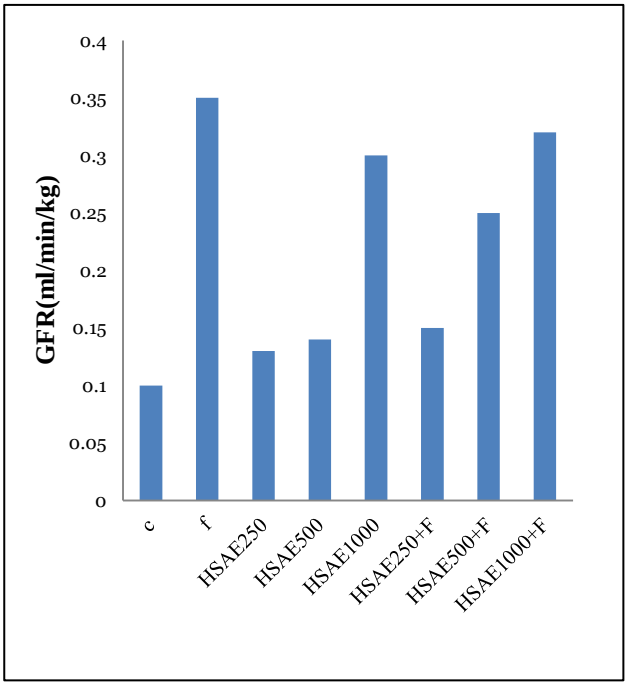


Fig 37 and 38. Effect of HSME on glomerular filtration rate at 5h (ml/min).

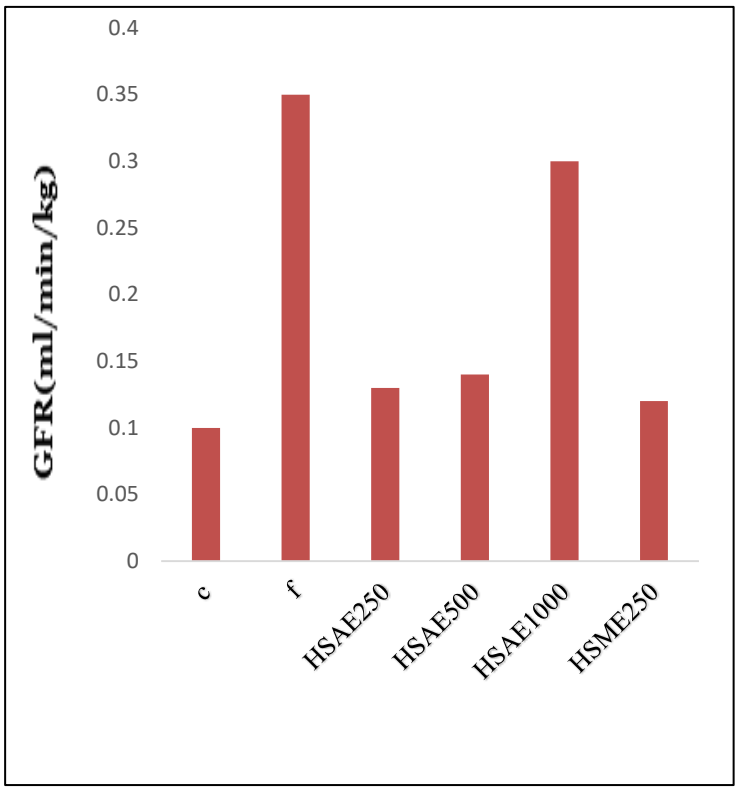


Fig 39. Effect of HSAE and HSME on glomerular filtration rate at 24h

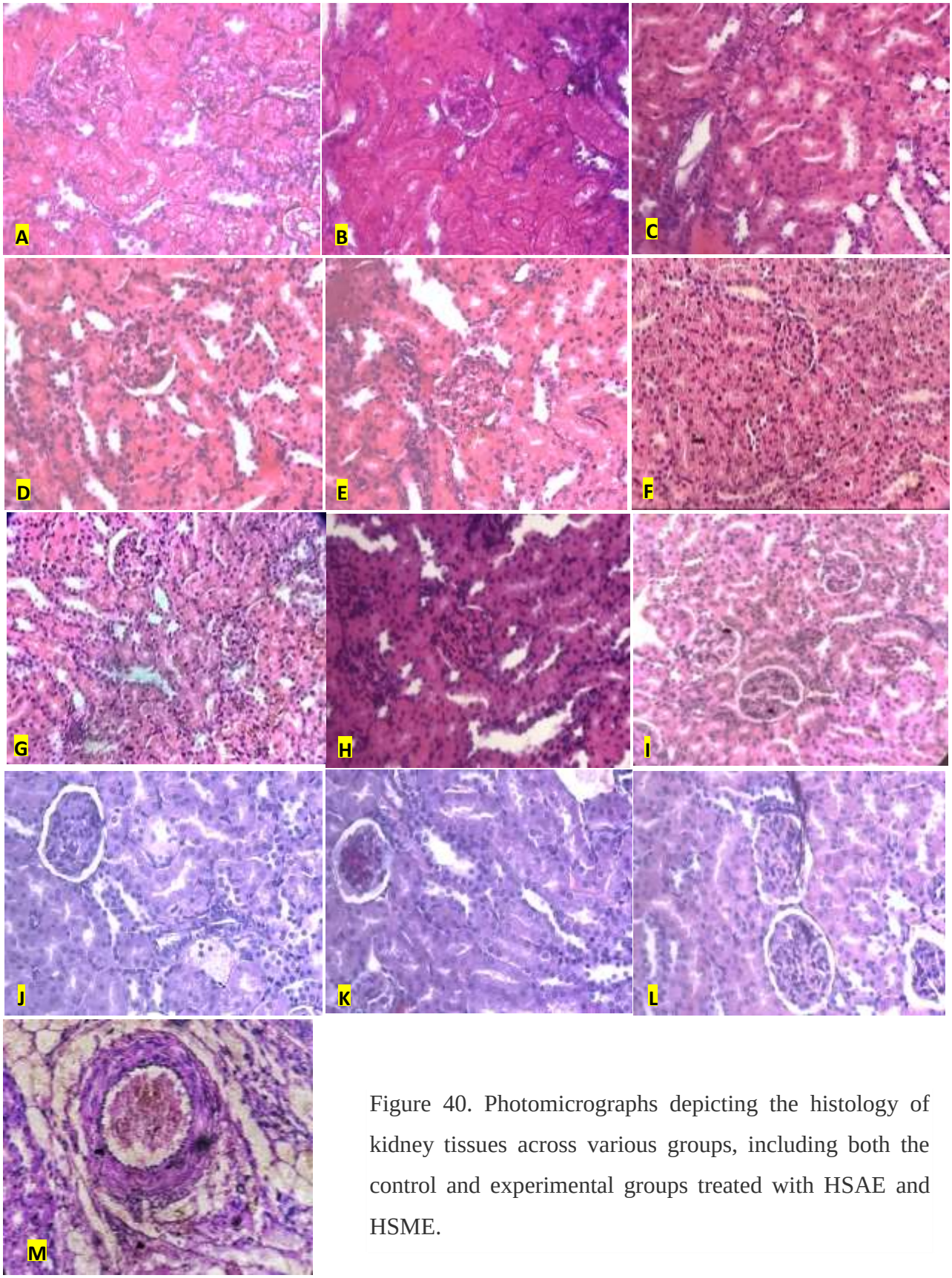


Figure 40. Photomicrographs depicting the histology of kidney tissues across various groups, including both the control and experimental groups treated with HSAE and HSME.

The control group (A) showcases the normal histological architecture of the kidney. Moving on to the experimental groups, (B) through (G) correspond to the HSME and HSAE treated groups at doses of 250 mg/kg, 500 mg/kg, and 1000 mg/kg, respectively. Similarly, (H) through (M) correspond to the HSME+F and HSAE+F treated groups at the same dosage levels. No observable alterations are evident in the histological features of the kidney tissues in these experimental groups, reaffirming the preservation of normal kidney structure throughout the study.

In conclusion, the findings suggest that HSME and HSAE exhibit dose-dependent diuretic effects, with HSME demonstrating notable diuretic action and diuretic activity across various dosages. The combined administration of extracts with furosemide enhances diuretic activity. Moreover, HSME and HSME+F showcased greater urinary output than HSAE and HSAE+F. The experiment also highlighted the distinctive effects of these extracts on urinary electrolyte content and indicated no significant adverse impact on serum biochemical parameters, reinforcing their potential as diuretic agents within safe levels of administration.

4.6. Identification of compounds using GC-MS analysis

In GC-MS study, *H. scandens* petroleum ether extracts revealed the presence of 18 compounds, while the chloroform extract exhibited 21 peaks, the methanol extract displayed 11 peaks, and the aqueous extract exhibited 31 peaks in total that were identified by peak retention time and peak area (%) to that of the known compounds described by the National Institute of Standards and Technology (NIST) library. Overall, the compounds with known biological activity identified in HSPE, HSCE, HSME and HSAE are given in Tables 12,13,14 and 15.

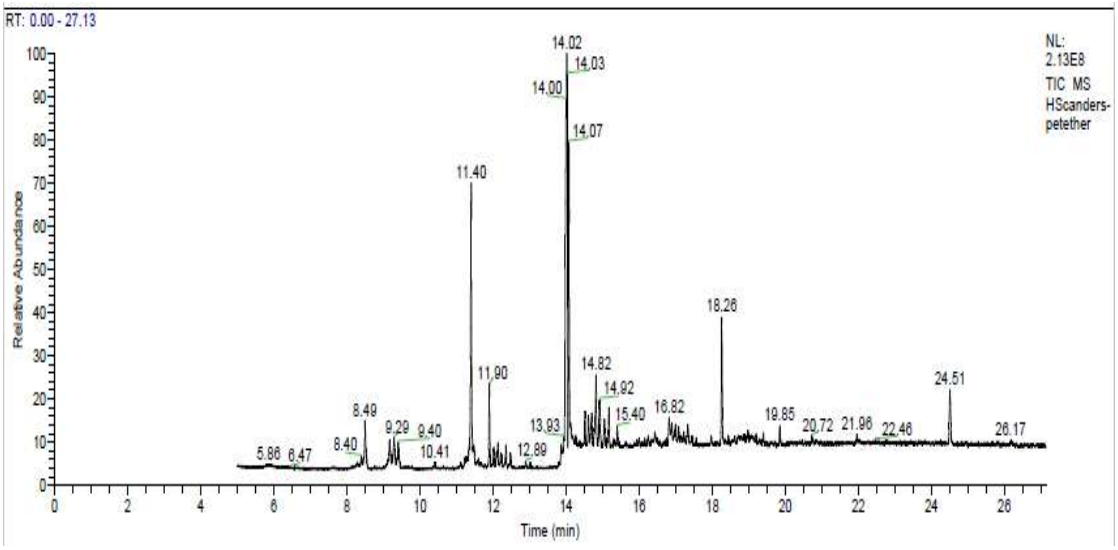


Fig 41. GC-MS chromatogram of HSPE

Table 12: Phytochemicals identified from HSPE using Gas Chromatography-Mass Spectrometry

Peak	RT	Area	Name	Activity	Reference
1	5.86	7343119	2,6 Dimethyl-6-trifluoroacetoxyoctane	No activity reported	
2	7.61		Hydroxylamine	Antibacterial	(Miret-Casals *et al*., 2018)
3	8.40	63103029	Benzene,1,3-bis(1,1-dimethylethyl)	Antibacterial	(Al-Youssef HM, 2015)
4	9.29		3-Eicosene	Cytotoxic, antioxidant, antifungal	(Banakar, 2018; Marimuthu, 2020)
5	9.40		2-Butyl-1-Octanol	Antimicrobial	(Muthulakshmi, 2012)
6	9.59		2,6-Dimethylheptadecane	No activity reported	
7	11.90	71940816	2,4-Di-tert-butylphenol	Antioxidant	(Wolf and Kaul, 2000)
8	11.40	294513241	E-11,13-	No activity	

			Tetradecadien-1-ol	reported	
9	11.90	71940816	Phenol, 2,4-bis(1,1-dimethylethyl)	Anticancer, antimicrobial, anti-inflammatory, antibacterial, antioxidant, antifungal	(Kuppuswamy1 *et al*., 2013 Rajeswari,2011;Rama nd Rao, 2015; Pereira *et al*., 2009)
10	12.13	395784	Trichloroacetic acid	Cytotoxic, Antioxidant	(Luo *et al*., 2014)
11	13.86		2-Propenoic acid, tridecyl ester	No activity reported	
12	14.02	721760358	Dodecyl acrylate	Antibacterial, cytotoxic	(Manilal, 2009)
13	14.82	84481509	2-Octyldecan-1-ol	No activity reported	
14	15.40		1-Propyl 12-methyl-tridecanoate	No activity reported	
15	16.82	30838139	Hexadecanoic acid	Antioxidant, nematicide, pesticide, alpha reductase inhibitor, anti-androgenic.	(Kumari, 2019)
16	16.99		11-Methyldodecanol	No activity reported	
17	18.26	111069102	Phytol	Antimicrobial, anti-inflammatory, anticancer, diuretic, antiashmatics	(Nishanthini, 2014)
18	18.98	111069102	2-Methyl-Z-4-tetradecene	No activity reported	
19	19.85	23042627	2-Propenoic acid, pentadecyl ester	No activity reported	
20	16.82	30838139	Hexanedioic acid, mono (2-ethyl	Antioxidant	

			hexyl) ester		
21	21.96	9850988	9-(2,2-Dimethylpropanoil hydrazono)3	No activity reported	
22	22.46		Dodecanoic acid	No activity reported	
23	24.51	69100047	Squalene	Skin protection, adjunctive to cancer therapy, antoxidant, antitumor, diuretic	(Karthikeyan, 2016) (Nishanthini, 2014)
24	26.97	13535447	1-Amininononadecane, N- trifluoroacetyl	Antimalarial, anticonvulsant	(Jaddoa, 2016)

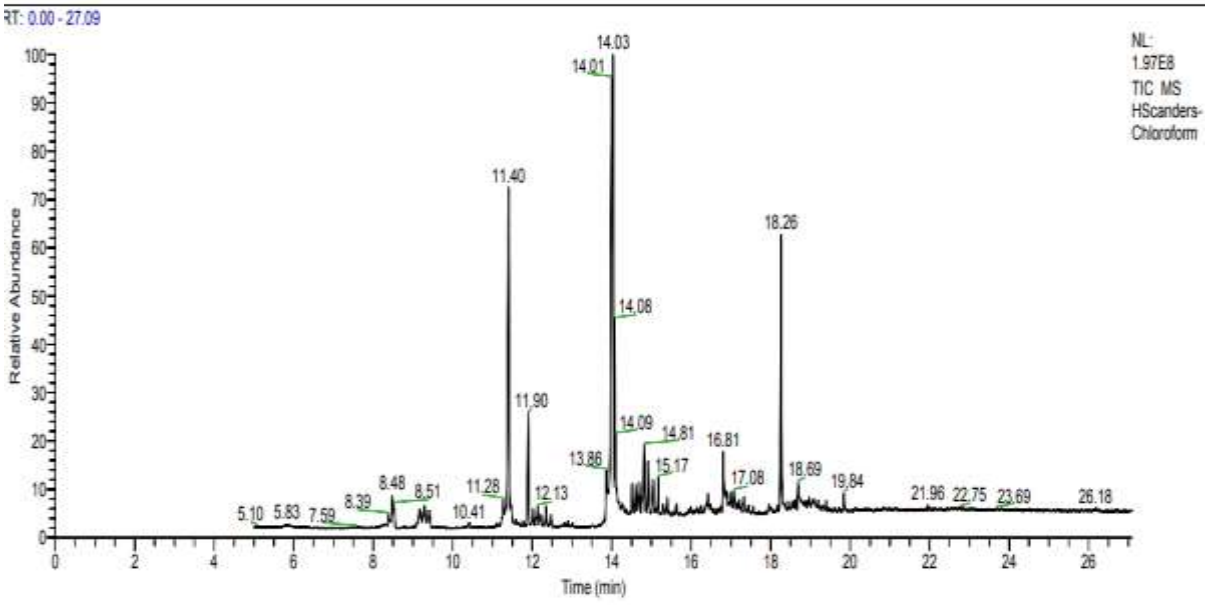


Fig 42. GC-MS chromatogram of HSCE

Table 13: Phytochemicals identified from HSCE using Gas Chromatography-Mass Spectrometry

Peak	RT	Area	Name	Activity	Reference
1	5.8				
2	7.59		Docosyltrichlorosilane	No activity reported	
3	8.48	49963684	Benzene,1,3-bis(1,1-dimethyethyl)	Antibacterial	(Al-Youssef, 2015)
4	9.29		2-Butyl-1-octanol	Antimicrobial	(Muthulakshmi, 2012)
5	9.40		E-14-Hexadecenal	No activity	
6	11.28		Hexadecen-1-ol	No activity	
7	11.40	314553992	1-Undecanol		
8	11.90	75071212	2,4-Di-tert-butylphenol	Antioxidant	(Wolf and Kaul, 2000)
9	12.13		Trichloroacetic acid	Antimicrobial	(Luo *et al*., 2014)
11	13.86		Dodecyl acrylate	Antibacterial, cytotoxic	(Manilal, 2009)
12	14.03	758916859	2-propenoic acid, pentadecyl ester	No activity reported	
13	14		Propanoic acid, decyl ester	No activity reported	
14	14.03	758916859	2-Octyldecan-1-ol	No activity	
15	14		2,3,5,6-Detetetrahydrocyclohexanone	No activity	
16	14.81	67134893	2-Methylhexadecan-1-ol	anticancer, anti-inflammatory and antimicrobial, antioxidant activities	(Ouyang, 2012)

17	14.92		Hexadecanoic acid	Antioxidant, nematicide, pesticide, alpha reductase inhibitor, anti-androgenic.	(Kumari, 2019)
18	16.42	60645082	2-Methylhexadecan-1-ol	anticancer, anti-inflammatory and antimicrobial, antioxidant activities	(Ouyang, 2012)
19	18.26	215107129	Phytol	Antimicrobial, anti-inflammatory, an ticancer, diuretic, antiashmatics	(Nishanthini, 2014)
20	18.69	46282368	Octadecanoic acid	Antibacterial	(Mohy and El-Ahwany, 2016a)
21	19.84		3-chloropropionic acid, heptadecyl ester	No activity reported	
22	20.84		3-Trifluoroacetoxypentadecane	Antinephrotic, antioxidant	(Jasna, 2020)
23	21.96	7226324	9-(2,2-Dimethylpropanoilhydrazono)-3	No avtivity	
24	22.75		i-Propyl 9-tetradecenoate	No activity	
25	23.69		Cyclopropanetetradecanoic acid	No activity	
26	24.19	4224632	Androstane-11	Antimicrobial, anti-inflammatory	(Susheela, 2018)

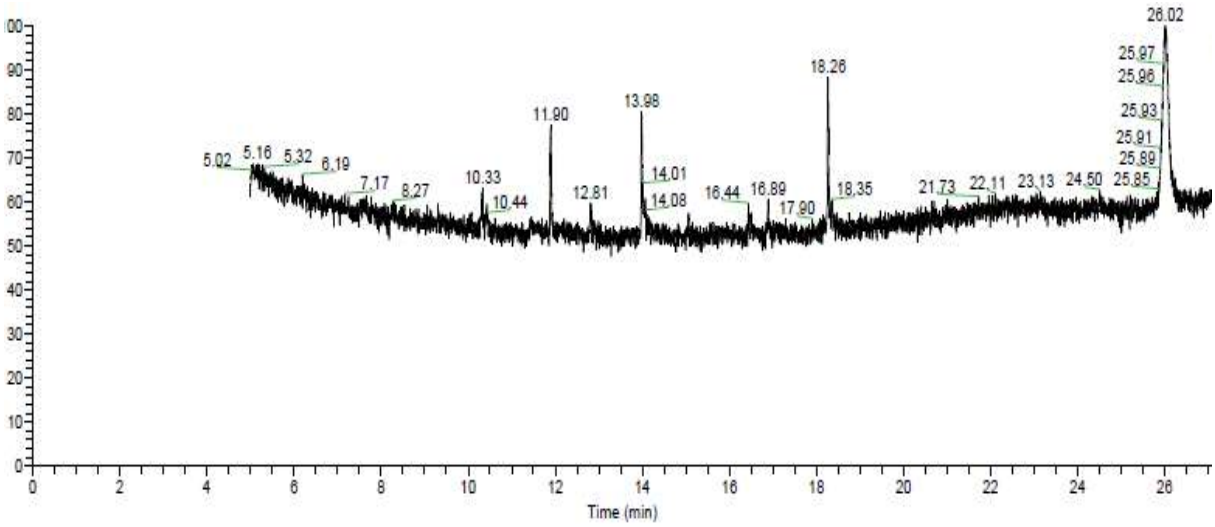


Fig 43. GC-MS chromatogram of HSME

Table14: Phytochemicals identified from HSME using Gas Chromatography-Mass Spectrometry

Peak	RT	Area	Name	Activity	Reference
1	10.33	9724679	4 Trifluoroacetoxytetra decane	Antimicrobial	(Sarada *et al.*, 2011.)
2	10.43		1,6:3,4- Dianhydro-2-deoxy-a-d-lyxo-hexopyranose	Antimicrobial	(Sushma and Raveesha, 2020)
3	11.90	17546587	Phenol, 2,5-bis(1,1-dimethylethyl)	Anticancer, antimicrobial, anti-inflammatory, antibacterial, antioxidant, antifungal	(Kuppuswamy1 *et al*., 2013; Rajeswari,2011; Rao, 2015; Pereira *et al*., 2009)
4	12.01	576560	E-14-Hexadecenal	Antibacterial	(Ram and Rao, 2015)

5	13.98	20031683	Dodecyl acrylate	No activity reported	
6	14.06		1-Ethyldodecyl acrylate	No activity reported	
7	16.89	10474501	Pthalic acid, butyl nonyl ester	No activity reported	
8	18.26	25262966	Phytol	Diuretic, to treat rheumatoid arthitis antioxidant, anti-inflammatory, anticancer	(Ogunlesi *et al*., 2009; Rajeswari *et al*., 2013; Malipeddi and Das, 2014; Sermakkani & Thangapandian, (2012); Nagalakshmi *et al*., 2015)
9	18.35	6748762	Cyclopentaneundecanoic acid, methyl ester	Antimicrobial	(Maha Lakshmi *et al*., 2018)
10	26	4017022	Pregnan-18-oicacid	No activity reported	
11	26.23	71237434	3 [(Trimethylsilyl)oxy] androstane-11,17-dione 17-(O-benzyloxime)	Antimicrobial, anti-inflammatory	(Susheela *et al*., 2018)

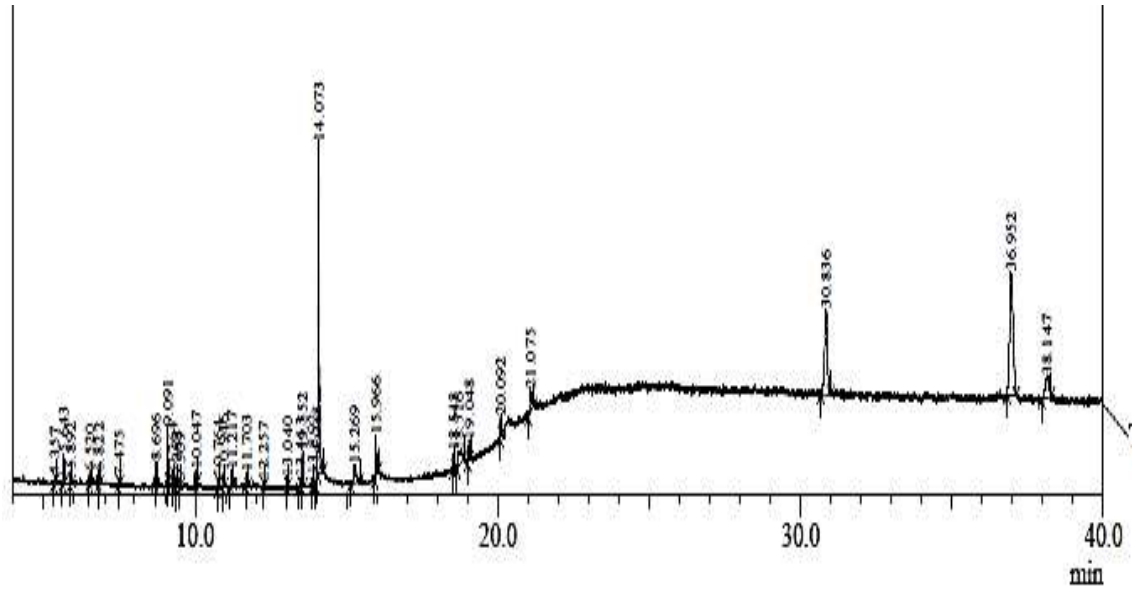


Fig 44. GC-MS chromatogram of HSAE

Table 15: Phytochemical constituents identified from HSAE using Gas Chromatography-Mass Spectrometry

Peak	R. T	Area	Name	Activity	Reference
1	5.357	12383	3,4-Diaminobenzonitrile	No activity reported	
2	5.643	58414	L-Proline, N-(3-fluorobenzoyl)-, isohexyl ester	No activity reported	
3	5.892	28242	Docosane	Antibacterial, antioxidant, lytotoxic	(Godwin *et al*., 2015)
4	6.822	23245	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl) ethyl] pentyl ester	Antimicrobial, insecticide	(Janakiraman *et al*., 2012)
5	7.475	104	9,10,11-	Anti-infective,	(Ali *et al*., 2022)

		59	Trioxabicyclo [6.2.1] undecane	antimyopathies, antineoplastic,antirickettsial	
6	8.696	308 08	Decane, 1-iodo-	No activity reported	
7	9.091	815 53	Phenol,2,4-Bis (1,1-dimethylethyl)-	Anticancer, antimicrobial, antiinflammatory, antibacterial, antioxidant, antifungal, antimalarial	(Mathivanan and Suseem 2015)
8	9.264	256 58	Decane, 1 - iodo-	No activity reported	
9	9.388	121 41	C13-E2	No activity reported	
10	9.509	669 7	3-Hexanone, 2,2-dimethyl-	No activity reported	
11	10.04 7	271 20	Ethyl 1,2,3,4,5,6,7,8-octahydro-8-oxo-1 Naphthalenecarboxylate ethyl	No activity reported	
12	10.76 4	112 70	3- Methoxy-2-undecanone	No activity reported	
13	10.94 6	237 35	Cheloviolin	No activity reported	
14	11.21 7	539 87	1- Iodotetradecane	No activity reported	
15	11.70 3	264 40	3,5-Dimethyl-4-octanone	No activity reported	
16	12.25 7	763 6	Docos-13-Enoic acid	No activity reported	
17	13.04 0	162 95	Phthalic acid, 4-bromophenyl heptyl ester	Potential antioxidant	(Karolina *et al*., 2019)
18	13.55 2	559 82	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	Antioxidant	(Merlin, 2009), (Conforti *et al*., 2009),(Riquet *et al*., 2016)

19	14.018	21873	Ethyl-6- beta-D-Ribofuranosylimidaz0	No activity reported	
20	14.073	758922	n-Hexadecanoic acid	Prostaglandin inhibitor, antioxidant	(Mohy and El-Ahwany, 2016b)
21	15.269	86812	1-Octadecene	Used for nano crystal synthesis	(Dhaene *et al*., 2019)
23	15.966	119891	Octadecanoic acid	Antibacterial	(Mohy and El-Ahwany, 2016b)
24	18.548	35885	1H- Indole-3-Ethanamine	No activity reported	
25	18.716	103182	1H-Indene, 1-Hexadecyl-2,3-Dihydro-	No activity reported	
26	19.048	49995	1,2-Benzenedicarboxylic acid	Cytotoxic activity	(Krishnan *et al*., 2014)
27	20.092	31033	5-ethoxy-5-oxonorvaline	No activity reported	
28	21.075	97121	13-Docosenamide, (Z)-	Human and plant metabolite	(PubChem Compound Summary for CID 5365369, 2023)
29	30.836	530481	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	Selective antioxidant and antimicrobial activity	(Malhotra, 2008)
30	36.952	973257	Tris(2,4-di-tert-butylphenyl) phosphate	Antioxidant	(Wolf and Kaul, 2010)
31	38.147	180266	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydrox	Antifungal, antioxidant	(Akpuaka et al., 2013)

The GC-MS analysis of HSPE revealed the existence of 24 bioactive compounds. These compounds along with their biological activities are detailed in Table 12. HSCE on the other hand revealed the presence of 26 compounds listed in Table 13, along with their reported biological activity. Table 14 lists the 11 bioactive compounds identified from HSME along with their biological activity. On the other hand, HSAE documented the highest bioactive compounds, with a total of 31 compounds as listed in Table 15.

CHAPTER 5

DISCUSSION AND CONCLUSION

Hedyotis scandens shows remarkable therapeutic property despite it being a relatively less explored species when compared to other species of its genus. Our study confirmed the presence of important phytochemical constituents in *H. scandens*. HSME and HSAE unveiled the presence of compounds like alkaloids, carbohydrate, tannin and saponin. On the other hand, HSPE and HSCE revealed the presence of solely carbohydrate and phytosterol with the absence of other compounds. *Hedyotis* species has been known to contain numerous important bioactive compounds including anthraquinones from which alizarin was isolated (Martins and Nunez, 2015). In accordance to this study, a variety of phytochemical like flavonoid and alkaloids were discovered from different *Hedyotis* species, including five new phenolic glycosides from *H. scandens* (Wang *et al*., 2013; He *et al*., 2013). Prior phytochemical investigations also verified the presence of abundant flavonoids and triterpenoids from *H. scandens* (Jabbar *et al*., 1996; Deng *et al*., 2012). *H. corymbose* (Linn.) and *H. diffusa* Willd has been known to contain a bioactive phytochemical iridoid glycoside (Shan *et al*., 2017). However, the present study does not confirm the presence of this particular substance in *H. scandens*. Our study validated the significant presence of phenol and flavonoid in HSPE, HSCE, HSME and HSAE.

Polyphenols are found in many different types of plants and are essential components of both human and animal diets. Polyphenols, found in plants, plays a pivotal role in the diets of both humans and animals, contributing significantly to a wide range of health benefits (Bravo, 1998; Chung *et al*., 1998; Crozier *et al*., 1997). Fruit and vegetable consumption is associated with great health advantages which are partially attributed to the flavonoids included in these foods (Lampila *et al*., 2009). Due to their antioxidant qualities, flavonoids and phenols have been shown to prevent the formation, growth, and spread of tumours as well as reduce the risk of coronary heart disease (Kittakoop *et al*., 2014; Harley *et al*., 1994).

Antioxidants combat free radicals, protecting individuals from a range of illnesses. This is accomplished by either scavenging reactive oxygen species or strengthening the antioxidant defence systems (Umamaheswari and Chatterjee, 2008). *H. scandens* extracts exhibited significant amount of antioxidant when the phosmolybdate test was conducted to test the total antioxidant activity. The presence of flavonoids and polyphenols in the plant is responsible for the reduction of Mo(V) to Mo(V) in phosphomolybdate test, which is used to test the total antioxidant property of plants, and this property can be attributed to the presence of phenols and flavonoids in the plant (Sharififar *et al*., 2009; Khan *et al*., 2012).

Both HSME and HSAE demonstrated effective antibacterial qualities against the tested species. All the bacteria employed in the present study are opportunistic pathogens and have been documented as causative agents of a number of illnesses. Consistent with the present finding, another investigation found that ethanolic extract of *H. scandens* had potent antibacterial effect against *K. pneumonia* which was similar with the current observation. It can be assumed that secondary metabolites generated from plants might be associated with antibacterial activities and these substances can safeguard plants from fungal, viral and bacterial infections. Although the presence of antibacterial chemicals in both HSPE and HSCE was confirmed by GC-MS testing, no action against the tested pathogens was detected. However, these substances may prove to be highly effective when evaluated against other organisms.

The diuretic property of *Hedyotis scandens* is apparent when compared to a control group over a 5-hour period and in certain doses, within a 24-hour timeframe. This observation aligns with the traditional utilization of this plant as a diuretic in Mizoram. Comparable findings were documented in studies assessing other plants known for their diuretic property such as *Ficus glumosa*, *Herniaria glabra L*and *Retama raetam* (Hakim *et al*., 2021; Maghani *et al*., 2005; Ntchapda *et al*., 2015). Consequently, it can be concluded that HSME, which caused a swift rise in urine output within 5h contains higher diuretic efficacy in comparison to the aqueous extract which exhibited peak diuresis within 24h. The diuretic action value functions as an indicator of a substance's diuretic potential. When the value is below 0.72, it is

classified as having no diuretic effect, while between 1.00 to 1.50, is considered to be small, suggesting a mild diuretic effect. On the other hand, when the value falls within the 0.72 to 1 range, it is considered to be moderate, indicating a moderate diuretic effect, A value exceeding 1.50 signifies a potent diuretic effect (Asif *et al*., 2014b). Thus, in the current investigation, all of the HSME and HSAE dosages demonstrated a diuretic action surpassing 1.50, signifying a vigorous diuretic effect, with the exception of HSAE 250mg/kg at 5h which showed a diuretic action of 1.23, which falls in the moderate range. When paired with furosemide, HSAE and HSME demonstrated increased diuretic activity, diuretic action and diuresis as opposed to when the extracts were administered independently. Thus, one can attribute the diuretic property of *H. scandens* to either the singular effect or the synergistic effect of the compounds it contains (Pezzani *et al*., 2019).

According to Feng *et al* saluretic activity is determined by using the value of chloride ion and sodium (Feng *et al*., 2013). HSME at 500 and 1000 mg/kg doses, as well as HSME 500+F and HME 1000+F, showed a significant rise in sodium (Na^+) and chloride (Cl^-) levels as compared to the control group. However, when compared to the activity of HSME, HSAE showed a dose-dependent increase in sodium (Na^+) and chloride (Cl^-) production, albeit to a smaller degree. These findings provide support for the reasonable theory that the diuretic effect of *H. scandens* extracts is more likely to be saluretic than aquaretic, a type of diuretic that is typically associated with most phytodiuretic drugs.

Additionally, it was observed that the administration of *H. scandens* treatment was comparable to that of furosemide, which is a well- known loop diuretic, recognized for its saluretic property. HSME and HSAE led to qualitative increase in both water and electrolyte output, which is an important characteristic of a diuretic agent. The increase in diuresis caused by HSME and HSAE might partially be explained by the rise in natriuresis (Na^+/K^+) caused by the administration of the extracts.

Carbonic anhydrase inhibition is unlikely to occur at ratios between 0.8 and 1.0. It becomes more likely that mild to strong inhibition will be felt when the ratios drop (Vogel, 2007).The assessment of urinary carbonic anhydrase inhibition of the extracts showed no discernible increase in inhibition. This suggests that the inhibition of carbonic anhydrase is not the primary mechanism by which HSME and HSAE exert their diuretic action (Solis and Gutierrez, 2002). Both the extracts appear to facilitate the excretion of sodium ions more prominent than potassium ions, as indicated by the significant rise in the ratio of sodium-to-potassium ion excretion. This characteristic is extremely advantageous in an efficient diuretic since it diminishes the risk of hyperkalemia, a typical electrolyte imbalance frequently linked with conventional diuretics (Horisberger and Giebisch, 1987b; Sarafidis *et al*., 2010). This effect may be due to the synergistic mechanism of the [HCO_3^-/Cl^-], [HCO_3^+/H^+] and the [Na^+/H^+] antiporter, leading to diuresis (Dubois, 1959) This is a characteristic of high ceiling diuretic. Furosemide acts by inhibiting electrolyte re-absorption in the thick ascending loop of Henle (Shinkawa *et al*., 1993).

Importantly, positive results from the analysis of various biochemical markers were obtained. Deviations from these biochemical proportions can lead to an organ's normal function to be disrupted (Afolayan and Yakubu, 2009). Notably, the investigation into diverse biochemical parameters resulted in favourable findings. The oral administration of HSME and HSAE at all the doses showed no negative effects on the treated animal’s liver and kidney function. This proves that *H. scandens* extract is a secure and well-tolerated choice for diuretic therapy.

GC-MS test performed on HSPE, HSCE, HSME and HSAE exposed the presence of numerous important bioactive compounds with significant functions, all of which have been documented in tables:12-15. The antioxidant and antibacterial property of the extracts can be attributed to the existence of these bioactive compounds, the antioxidant and antibacterial activity of which has already been documented. GS-MS analysis of *H. scandens* identified the presence of compounds like 3-Eicosene, 2,6-Dimethylheptadecane, 2,4-Di-tert-butylphenol, Phenol, 2,4-bis(1,1-dimethylethyl), Trichloroacetic acid, Hexanedioic acid, mono (2-ethyl hexyl)

ester, 2-Methylhexadecan-1-ol, 3-Trifluoroacetoxypentadecane, Phthalic acid, 4-bromophenyl heptyl ester which has been reported to have antioxidant property. Similarly, the extracts reported the presence of compounds such as phytol and squalene, whose diuretic property has also been reported.

Previous studies have provided proof of the diuretic property of saponin, the presence of which in *H. scandens* has been confirmed in this study (Amuthan *et al*., 2012; Martín-Herrera *et al*., 2007) Furthermore, its diuretic potential can also be attributed to its antioxidant capacity, comparable findings have been reported in other plants with diuretic properties, suggesting a potential link between the antioxidant property and diuretic activity among different plant species. However, further in-depth investigation is necessary to evaluate this claim.

Conclusion

Medicinal plants are superior to chemical products in a number of ways because compounds derived from plants are less harmful, better tolerated and less toxic to human cells. The already available therapies that require the use of diuretics are known to have side effects that are talked about in this study like increased blood sugar level, electrolyte imbalance, low blood potassium, impaired kidney function etc which can have detrimental effects on a person's health. Hence, an alternative approach is required to create a diuretic that is safe, potent and is also capable of fighting all these complications. Phytochemicals derived from significant ethnobotanical plants have garnered scientific interest owing to their potential as diuretic agents.

The present study showed the phytochemical content, antioxidant, antibacterial and diuretic effect of *Hedyotis scandens*. Qualitative phytochemical analysis of the plant extract confirmed the presence of key phytochemicals like alkaloid, saponin, carbohydrate, tannins and phytosterols. GC-MS analysis of the extracts also confirmed the presence of numerous important bioactive compounds that have been reported to possess significant and vital biological activities. Quantitative phytochemical test of the extracts also verified the presence of substantial amount of phenol and flavonoid. The antioxidant activity of the different extracts of *H. scandens* was validated though the phosphomolybdenum assay and ferric reducing capabilities along with several in-vitro tests, including DPPH and $ABTS^{+}$ assays, as well as superoxide and nitric oxide scavenging assays. The results indicated that the extracts demonstrated considerably significant amount of antioxidant potential.

The assessment of *H. scandens* extracts for diuretic activity confirmed its promising efficacy in this regard. The result of the present investigation has shown that the methanolic extract exhibited a superior diuretic effect compared to the aqueous extract. Furthermore, a noteworthy diuretic impact was seen when furosemide and the extracts were combined at all tested levels. However, furosemide implemented the highest diuresis. Both the extract at all doses, even the lowest dose

showed higher diuresis than the control group. This study proves that *H. scandens* may be useful as a safe and natural diuretic with fewer side effects in comparison to synthetic diuretics. In conclusion, this study offers the initial scientific evidence supporting the diuretic properties of *H. scandens*, as claimed by the traditional users in Mizoram. However, a more detailed study is required to confirm the mechanisms of action and active ingredients responsible for the diuretic property of *Hedyotis scandens.*

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BIO-DATA

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HSLC	2009	Board of Secondary Education, Assam	English, Alt.Eng, Hindi, Mathematics, Science, Social Sciences, Adv-History	65	First
HSSLC	2011	Assam Higher Secondary Education Council	English, Physics, Chemistry, Biology	61	First
B.Sc.	2015	Gauhati University	Botany, Zoology, Chemistry, English	62	First
M.Sc. (Botany)	2017	Mizoram University	Botany	76	First

List of Publications:

1. Elizabeth Vanlalruati Ngamlai, R.B. Pradhan, P.C Lalbiaknii, Vanlalhruaii Ralte*, F. Lalnunmawia, PC Vanlalhluna. S.K. Mehta. **Diuretic Activity Evaluation and Chemical Composition Analysis of *Hedyotis scandens* Extract from Mizoram, India, in Rat Models.** ***Journal of Ethnopharmacology*** 319 (17079).

2. Elizabeth Vanlalruati Ngamlai, P.C. Lalbiaknii, R. Vanlalpeka, Vanlalhruaii Ralte& F. Lalnunmawia*(2022) **Phytochemical and pharmacognostic study of *Hedyotis scandens* Roxb. from Mizoram, Northeast India.** ***Research Journal of Pharmacy and Technology*** 15(12): 5483-9.

3. Elizabeth Vanlalruati Ngamlai[1], PB Lalthanpuii[3], P.C. Lalbiaknii[1], Vanlalhruaii Ralte[2]*, & F Lalnunmawia[1] (2022) **Antioxidant property and free radical scavenging activity of *Hedyotis scandens* (Roxb). Rubiaceae.** ***Current Trends in Biotechnology and Pharmacy*** 16(1): 46–55.

4. Elizabeth Vanlalruati Ngamlai[1], P.C. Lalbiaknii[1], F. Lalnunmawia[1], K. Lalchhandama[3] & Vanlalhruaii Ralte[2]* (2021) *In vitro* antioxidant activity and phytochemical screening of *Hedyotis scandens* Roxb. (Rubiaceae). ***Science and Technology Journal (UGC care list)*** 10(2): 116-122.

5. P. C. Lalbiaknii, Lalrinmuana, **Elizabeth Vanlalruati Ngamlai**, Vanlalhruaii Ralte, P. C. Vanlalnunpuia and F. Lalnunmawia * *In-silico* validation and pharmacological activity of potent anti-viral and anti-inflammatory ethno medicinal plants used by traditional herbalists within Thorangtlang Wildlife Sanctuary, Mizoram, North-east India. ***International Journal of Pharmaceutical Sciences and Research*** 14(5): 2385-2400.

6. Lalbiaknii, P. C.; Lalnunmawia, F.; Ralte, Vanlalhruaii; Vanlalnunpuia, P. C.; **Ngamlai, Elizabeth Vanlalruati**; Pachuau, Joney Lalnunpuii. Morphological assessment and partial genome sequencing inferred from matK and rbcL genes of the plant *Tacca chantrieri*. ***Journal of Threatened Taxa*** 14(8): 21487-21750

7. Lalawmpuia, H. Lalruatsanga, F. Lalnunmawia, Lalbiakdika and **Elizabeth Vanlalruati Ngamlai**; Oil Palm Plantation: Carbon Sequestration potential and effective Carbon Management within Serchhip, Mizoram, India. ***Ecology, Environment and Conservation Paper. EM International.*** 29(1): 96-101

8. R. Vanlalpeka, **Elizabeth Vanlalruati Ngamlai**, P.C. Vanlalhluna, Vanlalhruaii Ralte, S.K. Mehta. Phytochemical profiling and pharmacognostic activity of

Lycopodiella cernua (L.) Pic. Serm. A lesser-known plant from Mizoram, North East India. ***Research Journal of Pharmacy and Technology***. 17(5)

Seminar and Workshop attended:

1. Presented "**Taxonomic study of Lamiaceae Family in Mizoram University Campus**" at National Conference on Emerging Trends in Environmental Research on 31st Oct to 2nd Nov, 2019 which was organized by Department of Chemistry, Pachhunga University College, Aizawl, Mizoram, India.

2. Presented "**Phytochemical screening & Antibacterial activity of Aqueous leaf extract of Hedyotis scandens Roxb. (Rubiaceae)**" at International Seminar on Recent Advances in Science and Technology (ISRAST) International Conference on Recent Advances in Animal Sciences (ICRASS) on 16th to 18th November, 2020 which was organized by NEAST, Mizoram University, Aizawl, Mizoram, India.

3. Presented "***In vitro* antioxidant activity and preliminary phytochemical screening of Aqueous leaf extract of Hedyotis scandens Roxb. (Rubiaceae)**" at Mizoram Science Congress on 3rd & 4th December, 2020 which was organized by Mizoram Science, Technology & Innovation Council (MISTIC) and Directorate of Science & Technology, Planning Department, Govt of Mizoram.

4. Presented "**Antibacterial and free radical scavenging activity of *Hedyotis scandens* Roxb. extracts**" at National Seminar on Plant Taxonomy and Traditional Knowledge in the Himalayas and North-east India & Annual Conference of East Himalayan Society for Spermatophyte Taxonomy (EHHST) on 21st to 22nd, February, 2022 which was organized by Department of Botany, Rajiv Gandhi University, East Himalayan Society for Spermatophyte Taxonomy and Botanical Survey of India.

5. Presented "**Assessment of free radical scavenging property, antimicrobial activity and diuretic effect of *Hedyotis scandens* Roxb. (Rubiaceae)"** at Pachhunga University College, Research Conclave- 2023 on 24th and 25th January, 2023, organized by Research and Development Cell, P.U.C

6. Participated in the Two-day National Workshop on Biodiversity Loss and Climate Change organized by the Department of Environmental Science, Pachhunga University College and Rajiv Gandhi National Institute of Youth development Programme (YLSDP) in Higher Educational Institutions from 9th to 10th February 2021.

7. Participated in the International Conference on Recent Advances in Animal Sciences-2019 (ICRAAS 2019) held at Pachhunga University College, Aizawl, Mizoram, India from November 6 to 8, 2019.

8. Participated in the 2 Days National Workshop on Plant Diversity of NE India with special reference to Mizoram organized by Department of Botany & Department of Life Sciences, Pachhunga University College, Mizoram University in collaboration with Govt. Zirtiri College from 23rd to 24th May, 2023 at Pachhunga University College, Aizawl, Mizoram.

9. Participated in the workshop on Molecular Phylogeny and Molecular Docking organized by UGC STRIDE Program (Component-1) held from 19th to 27th January.

PARTICULARS OF THE CANDIDATE

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DEGREE:	Doctor of Philosophy (Ph.D.)
DEPARTMENT:	Department of Botany
TITLE OF THESIS:	Assessment of free radical scavenging property, antimicrobial activity and diuretic effect of *Hedyotis scandens* Roxb. (Rubiaceae)
DATE OF ADMISSION:	3rd September 2018
APPROVAL OF RESEARCH PROPOSAL	
1. DRC:	2th May 2019
2. BOS:	3rd MAY 2019
3. SCHOOL BOARD:	17th March 2019
4. MZU REGN. NO.	1506806
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ABSTRACT

ASSESSMENT OF FREE RADICAL SCAVENGING PROPERTY, ANTIMICROBIAL ACTIVITY AND DIURETIC EFFECT OF *HEDYOTIS SCANDENS* ROXB. (RUBIACEAE)

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DEPARTMENT OF BOTANY
SCHOOL OF LIFE SCIENCES
APRIL, 2024

ASSESSMENT OF FREE RADICAL SCAVENGING PROPERTY, ANTIMICROBIAL ACTIVITY AND DIURETIC EFFECT OF *HEDYOTIS SCANDENS* ROXB. (RUBIACEAE)

BY

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Submitted

In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Botany of Mizoram University, Aizawl.

Hedyotis scandens Roxb. (Family: Rubiaceae) is a widely distributed, liana up to several meters and usually thrives well in disturbed areas. It is an important medicinal plant utilized in many communities for the treatment of stomach pain, ulcers, kidney problem, oliguria, to alleviate sprains, anthelmintic, wound healing etc. In Mizoram, it is commonly recognized as Laikingtuibur/Kelhnamtur and is used in treating oliguria and kidney problem like swollen kidney or kidney stone. However, scientific evidence for its therapeutic properties is quite poor and no scientific validation is available of its antioxidant capacity and diuretic property and very little about its antimicrobial potential and phytochemicals are known as per our knowledge.

Diuretics play a crucial role in addressing various medical conditions such as hypertension and edema. Across numerous communities, plants have served as diuretic agents, leveraging their abundant phytochemical composition. In certain instances, plant-based remedies have gained preference over synthetic drugs due to their affordability and ready availability. The ethnopharmacological exploration of plant diuretics not only preserves cultural traditions but also contribute to the potential discovery of novel therapeutic agents. In this study, we aim to study the qualitative and quantitative phytochemical constituents, free radical scavenging activity, antimicrobial property and diuretic activity of *Hedyotis scandens*.

H. scandens was collected from Tanhril and authenticated by Dr. Khomdram Sandhyarani Devi, Department of Botany**,** Mizoram University, Aizawl (Accession no- MZU000246) and deposited in the Mizoram University Herbarium, Department of Botany. The leaves of the plant were cleaned under running tap water, dried and then ground for extraction. The ground plant material was then extracted sequentially, using petroleum ether, chloroform, methanol and distilled water. The petroleum ether extract will be referred to as HSPE, chloroform extract as HSCE, methanol extract as HSME and aqueous extract as HSAE. Preliminary phytochemical screening of the extracts confirmed the presence of alkaloids, carbohydrate, tannins and saponin in HSME and HSAE, whereas HSPE and HSCE confirmed the presence of solely carbohydrate and phytosterol with the absence of other compounds. Quantification of phytochemicals of different extracts of *H.*

scandens revealed that HSME exhibited the highest phenolic content, measuring at 27.2 ± 0.6 mg of Gallic Acid Equivalent (GAE) per gram. This was followed by HSAE with a TPC of 23.6 ± 0.7 mg GAE/g. HSPE displayed a TPC of 14.5 ± 0.6 mg GAE/g, while HSCE had the lowest TPC at 13.7 ± 0.5mg GAE/g. The highest TFC content of *H. scandens* was shown by HSME, which had a value of 29.9±0.3 QE mg/g, followed by HSAE which had 27.4 ± 0.3 QE mg/g of flavonoid. HSPE possessed a TFC of 25.1 ± 0.2 QE mg/g and HSCE on the other hand had the lowest flavonoid content at and 24.6 ± 0.2. The DPPH scavenging activity of *H. scandens* extracts increased proportionally with higher concentration of the extract (10-100 µg/ml). The scavenging activity was effective in the order: HSME> HSAE> HSPE > HSCE. However, standard BHT had the highest effect. The ability of *H. scandens* extracts to scavenge $ABTS^{+}$ cations increased as concentration increased (10-100 g/ml). Standard BHT had the highest scavenging activity. The order of effectiveness for the scavenging activity of the extracts against superoxide ion was as follows: HSME > HSAE > HSPE > HSCE. HSPE, HSME and HSAE showed scavenging potential against superoxide ion.

However, HSCE could not scavenge the radical within the tested range of concentrations. The superoxide scavenging activity of the extracts displayed an incremental increase in activity with rising concentrations (10-100 µg/ml). The following was the order of the scavenging activity's effectiveness: HSME > HSAE > HSPE. Standard Ascorbic acid had the highest scavenging potential. HSPE and HSCE were unable to effectively scavenge nitric oxide at the tested concentration range of 10-200µg. In contrast, HSME and HSAE exhibited scavenging activity that increased as their concentration increased. HSME had higher scavenging potential than HSAE**.** Ascorbic acid was used for standard reference which had the highest scavenging activity. the antioxidant activity of petroleum ether and chloroform extracts of *Hedyotis scandens* was evaluated using total antioxidant and reducing power method. HSPE, HSCE, HSME and HSAE were tested against two gram-positive and three gram-negative bacteria. However, it was observed that HSPE and HSCE did not exhibit any activity against all the tested microorganisms even at high concentration, therefore it was concluded that both the extract did not possess

antibacterial property against the tested organisms. HSME however showed pronounced inhibition activity compared to HSAE. HSME had highest activity against *Micrococcus luteus* and *Salmonella typhi* followed by *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus subtilis* and lastly, *Pseudomonas aeruginosa*. HSAE on the other hand showed highest inhibition against *Klebsiella pneumoniae* followed by *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Micrococcus luteus*, *Salmonella typhi* and *Escherichia coli*. The Minimum Inhibitory Concentration (MIC) of HSME was determined as 4mg/ml for *Micrococcus luteus*, 6mg/ml for *Salmonella typhi*, and 8mg/ml for *Klebsiella Pneumoniae*. In contrast, the MIC for the remaining tested bacteria was identified as 10mg/ml. HSAE had an MIC of 8mg/ml for *Klebsiella Pneumoniae* whereas for the other bacteria MIC was found to be 10mg/ml.

Based on preliminary study, experimental results and a comprehensive literature review, we selected two extracts, HSME and HSAE, for *in vivo* studies, specifically to assess their diuretic effects. During the entire 14-day observation period, in compliance with OECD guideline 423, all animals participating in the acute toxicity study consistently remained alive and did not exhibit any observable neurological or behavioral abnormalities. The results of the acute toxicity test confirmed the safety of HSME and HSAE up to the highest tested dose of 5000mg/kg. The extracts at all administered doses did not induce any adverse effects on the urinary pH of the tested animals when compared to the control group. Nevertheless, the urinary pH of the standard furosemide exhibited the highest value, closely resembling the pH of HSME at 1000 mg/kg + F, 10mg/kg and HSAE 1000mg/kg + F, 10mg/kg. In contrast, the control group recorded the lowest urinary pH. Both the extract produced diuresis which appeared to be a function of dose and time. The extracts at all administered doses exhibited significant diuresis at the 5^{th} h and there was an absence of diuresis at the 24^{th} h, consistent with the reference drug, except for HSME at 250mg/kg and HSAE at 250, 500, and 1000mg/kg, which displayed diuresis even at the 24^{th} h. The diuretic action of HSME and HSAE was pronounced, with HSME demonstrating a rapid surge in urine output within 5 hours, while HSAE exhibited some activity within 24 hours. The extracts at all administered

doses exhibited heightened diuresis, diuretic action, and diuretic activity compared to the control group, which recorded the lowest outcomes. Values above 1.50 indicate a robust diuretic effect, while those between 1.00 and 1.50 suggest moderate activity. Diuretic actions in the range of 0.72 to 1.00 imply mild effects, and values below 0.72 denote no diuretic action. In our research, both HSME and HSAE consistently showed strong diuretic effects with diuretic action values exceeding 1.50. However, HSAE at 250 mg/kg after 5 hours demonstrated a diuretic action of 1.23 which falls in the moderate effect range, revealing subtle aspects of diuretic activity even at the lowest tested dose. When paired with furosemide, HSAE and HSME demonstrated increased diuretic activity, diuretic action and diuresis as opposed to when the extracts were administered independently.

HSME at 500 and 1000 mg/kg doses, as well as HSME 500+F and HSME 1000+F, showed a significant rise in sodium (Na) and chloride (Cl^-) levels as compared to the control group. However, when compared to the activity of HSME, HSAE showed a dose-dependent increase in sodium (Na) and chloride (Cl^-) production, although to a smaller degree. The increase in diuresis caused by HSME and HSAE might partially be explained by the rise in natriuresis (Na^+/K^+) caused by the administration of the extracts. The assessment of urinary carbonic anhydrase inhibition showed no discernable increase in inhibition. This suggests that the inhibition of carbonic anhydrase is not the primary mechanism by which HSME and HSAE exert their diuretic action. Both the extracts appear to facilitate the excretion of sodium ions more prominent than potassium ions, as indicated by the significant rise in the ratio of sodium-to-potassium ion excretion. This characteristic is extremely advantageous in an efficient diuretic since it diminishes the risk of hyperkalemia, a typical electrolyte imbalance frequently linked with conventional diuretics.

Positive results from the analysis of various biochemical markers were obtained. Deviations from these biochemical proportions can lead to an organ's normal function to be disrupted. The results of the biochemical analysis of serum indicated that the administered extracts, across various doses, did not result in significant increases in tested parameters when compared to the control group.

HSME 250 mg/kg+F and HSAE 250 mg/kg+F, 10 mg/kg, exhibited a minor increase in urea concentration within the normal range. Furthermore, HSAE 500 mg/kg+F, 10 mg/kg, resulted in a slight elevation in creatinine levels, though still within the normal range. The histopathological analysis of kidney tissue indicated a consistent preservation of normal histoarchitecture across all treatment groups, even in the group administered the highest dosage. The absence of any discernible signs of toxicity in the treated groups, when compared to the control group, underlined the safety profile of the administered substances. Both HSME and HSAE demonstrated a remarkable increase in Glomerular Filtration Rate (GFR) in comparison to the control group, suggesting their potential positive influence on renal filtration processes. Remarkably, furosemide, a well-known diuretic, exhibited the highest GFR value within the tested groups, signifying its robust impact on renal filtration. Following furosemide, HSAE administered at a dosage of 1000 mg/kg emerged as the second-highest GFR enhancer, indicating its potential in promoting efficient kidney function. The GC-MS analysis of *H. scandens* extracts revealed the presence of several bioactive compounds, most of which are being reported for the first time in this study. HSPE revealed the existence of 24 bioactive compounds. HSCE on the other hand revealed the presence of 26 compounds. 11 bioactive compounds were identified from HSME and HSAE documented the highest bioactive compounds, with a total of 31 compounds. GC-MS analysis revealed the presence of several compounds which were reported to possess various properties like antioxidant, antimicrobial as well as diuretic. These findings collectively underscore the potential renal benefits of HSME and HSAE, further solidifying their promising role in promoting renal health.