PHYTOCHEMICAL ANALYSIS AND EVALUATION OF THE MEDICINAL PROPERTIES OF *HELICIA* SP. (PROTEACEAE)

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

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Submitted

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

CERTIFICATE

I certify that the thesis entitled **"Phytochemical Analysis and Evaluation of the Medicinal Properties of *Helicia* sp. (Proteaceae)"** submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Life Sciences by **Lalbiakngheti Tlau** is a record of the research work carried out during the period 2020 - 2025 under my supervision and guidance and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning.

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DECLARATION

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November, 2025

I **Lalbiakngheti Tlau**, 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 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 Life Sciences.

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Abbreviations

ºC	: Degree celcius
BHA	: Butylated hydroxy
BHT	: Butylated hydroxytoluene
DMSO	: Dimethylsulfoxide
DNA	: Deoxyribonucleic acid
DPPH	: Diphenyl-1-picrylhydrazyl
DPX	: Distyrene plasticizer xylene
FRAP	: Ferric reducing antioxidant power
I.e.	: Id est
Mg	: Miligram
ml	: Mililitre
ML	: Maximum likelihood
mM	: Milimolar
MW	: Molecular weight
N	: Normality
NaOH	: Sodium hydroxide
NCBI	: National Center for Biotechnology Information
PBS	: Phosphate-buffered saline
pH	: Potential of Hydrogen
pNPP	: p-nitrophenyl phosphate

RA : Relative abundance

Rpm : Revolutions per minute

RT : Retention time

TAC : Total antioxidant capacity

TBHQ : Tert-butylhydroquinone

TFC : Total flavonoid content

TMS : Tetramethyl silane

TPC : Total phenolic content

Viz : Videlicet

μM : Micromolar

CHAPTER - 1

GENERAL INTRODUCTION

Humans have learned the values of medicinal plants since pre-historic times in the management of health and diseases. Paleontological evidence indicates the definitive use of certain plants as early as 60,000 years ago since pollen from plants with known medical characteristics, such as *Achillea* and *Ephedra*, was found in Neanderthal burial sites in Iraq, such as Shanidar IV (Solecki, 1975). In addition, opium, thyme and several hundreds of medicinal plants were also recorded on Sumerian clay tablet around 1550 BCE in Ancient Mesopotamia (Scurlock & Anderson, 2010). Around the same time, uses of 850 phytomedicines, among them garlic, juniper, and aloe were used in Ancient Egypt, as documented in the Ebers Papyrus, for treating diverse arrays of illnesses, such as intestinal worms, cough, skin, stomach, eyes, and several others (Smith, 1930; Nunn, 2002).

In Ancient India, Ayurveda, which dated back to more than 1500 BCE, methodically classified medicinal plants such as Turmeric (*Curcuma longa*) and Ashwagandha (*Withania somnifera*) serving as the basis of the world's ancient system of healing practices (Narayanaswamy, 1981). Meanwhile, in Chinese traditional medicine, the applications of ginseng (*Panax ginseng*), which is described as "superior tonic", were recorded over 3000 years ago for treating different types of illnesses such as chest pain, cold, vomiting, and diarrhea, and its wide application has been extended till today in several chronic conditions (Park *et al*., 2012).

In the Mizo community, residing in Mizoram, Northeast India, medicinal plants, known as "*ramhmul damdawi*" (natural medicine), play pivotal roles since their earliest known history. Back when modern healthcare system was unheard of, Mizo people relied entirely on traditional healers and home remedies using local plants available to them. Since traditional healthcare practices rely upon the knowledge inherited orally through generations, it remains serve as the primary

resources in some parts of the state as they not only culturally acceptable and easily accessible, but also because of their availability in nature in addition to having little or no adverse effects in comparison to modern pharmaceutical drugs. To the Mizo people, this healthcare practice is a component of their tradition and cultural heritage (Lalruatpuii *et al*., 2024).

As per the World Health Organization (WHO), it is estimated that about 80% of the human population utilize plants and their products in the first-line healthcare requirements and more than 30% of the total botanical species are accounted to have therapeutic properties. Revered as "the botanical garden of the world," India is the foremost producer of herbal medicines globally (Anand *et al.,* 2019). In addition, Mizoram, situated within the Indo-Burma biodiversity hotspot, is known for its abundant flora and offers a wide variety of medicinal plant species. It was documented that 159 plant species from 134 genera across 56 families are utilized in ethnomedicinal practices in Mizoram (Rai & Lalramnghinglova, 2010).

According to WHO, a medicinal plant is one that has therapeutic qualities or has positive pharmacological effects on people or animals (Kariuki, 2024). An interdisciplinary field that studies how various ethnic or cultural groups have historically used natural chemicals derived from plants for medicinal purposes while assessing their pharmacological qualities using contemporary scientific methods. This is termed Ethnopharmacology (Calixto, 2005). Consequently, the fundamental strategy of pharmaceutical ventures is to exploit all possible sources, natural or synthetic, to acquire innovative techniques in new drug developments. As such, medicinal plants play a major role in investigating potential lead molecules for novel medications. Therefore, ethnopharmacology remains the essence of drug discovery and management of the most critical diseases (Süntar, 2020). However, despite its wide application and great contribution into the discovery of conventional drugs, the therapeutic properties of many medicinal plants are still lacking adequate scientific validation (Ekor, 2014). Meanwhile, an increasing scholarly focus on traditional medicinal plants is rapidly gaining recognition in the sector of modern research, causing these plant resources have been excessively exploited and harvested in an unsustainable manner, leading certain species toward the verge of extinction and

making them highly vulnerable. It is thereby necessary to emphasize the importance of safeguarding and preserving these invaluable ethnomedicinal systems and practices for future generation (Hamilton, 2004). With the aim of achieving this, actions can be undertaken by more documentation of traditional knowledge and encouraging the people by raising awareness to prevent over harvesting and exploiting valuable plants from their native environment (Akerele *et al*., 1991).

The extraction of bioactive compounds from raw herbal remedies, which marked the onset of modern pharmaceutical chemistry, was started when morphine was successfully isolated from the opium poppy (*Papervum somniferum*) by Friedrich Serturner in 1804 (Krishnamurti & Chakra, 2016). Shortly thereafter, two other alkaloids were isolated in 1820, quinine as an antimalarial from cinchona bark (*Cinchona officinalis*) and caffeine as a stimulant from coffee (*Coffee arabica*) and tea (*Camellia sinensis*), respectively (Solomon & Lee, 2009; Ashahira *et al*., 2008). Additionally, later in 1830, atropin was purified from deadly nightshade (*Atropa belladonna*) (Lee, 2007). These achievements set a milestone for modern drug discovery, signifying that ethnomedicinal plants might be standardized and used as efficacious pharmaceuticals (Pirintsos *et al*., 2022). However, the primitive isolation techniques were strictly restricted to a few pioneering chemists since they were primarily based on crystallization, acid-based extraction, and evaporation which required lengthy, expensive and labor-intensive processes (Newman & Cragg, 2016). But this challenge was overcome by the appearance of chromatography in the early 20th century that subsequently led to the development of more sophisticated technique like gas chromatography in the 1950s and the emergence of gas chromatography-mass spectrophotometry (GC-MS) over the next decade. Afterward, the establishment of high performance liquid chromatography, revolution of FT-NMR and 2D NMR, and LC-MS/MS took over the isolation technique which now advances the analysis of molecular elucidation of isolates from complex extracts of the plants in a short period of time (Ettre, 2006; Smolková-Keulemansová, 2000; Himmelsbach, 2012).

The understanding of the structures of biologically derived chemicals, even when not directly utilized, has provided a conceptual foundation for the development

of novel or enhanced pharmaceuticals. This greatly improved synthetic methods in organic chemistry for making analogues of the original compounds that are better at treating diseases and working as drugs (Newman, 2008; Sunazuka *et al.*, 2008). The semi-synthetic compounds are conveniently modified to obtain target-specific and more effective drugs, and are utilized to develop a patentable molecules and techniques in drug development. The innovative system is used to achieve more complex compounds that are required for complicated diseases (Najmi *et al*., 2022).

Phytochemical or bioactive compounds has been a main focus of modern scientists to explore their potent medicinal properties that preserve or enhance human health and prevent physiological disorders (Prakash *et al*., 2012). These compounds acts as antioxidant and help prevent or slowing the occurrence of the cell damage. Several categories of plant secondary metabolites like alkaloids, cardiac glycosides, carbohydrates, polyphenols (such as flavonoids and phenols), saponins, terpenes and terpenoids have been experimentally investigated for important medicinal properties including antidepressant, cough suppressant, cancer, inflammation, diuretics, hypertension, coronary heart disease, high cholesterol and diabetes and so on (Bahramsoltani *et al*., 2015; Mendoza & Silva, 2018).

The majority of the health challenges the world is currently dealing with are predominantly caused by the occurrence of oxidative stress in the imbalance equilibrium between free radicals and endogenous antioxidant defense system which causes variation in DNA, proteins and lipids. Under these circumstances, secondary metabolites of plants like flavonoids and terpenoids operate as antioxidants to prevent or protect free radicals from accumulating which, as a result, can induce impairment in our cells (Lalnunfela *et al*., 2020). Thus, plant-based foods and products rich in antioxidants become very vital and have positive impacts to human health (Prakash *et al*., 2016). Different plant parts across different genus of Proteaceae revealed promising antioxidant properties. Consequently, *Hakea sericea* exhibited remarkable total phenolic content and ferric reducing antioxidant capacity, which showed even higher activity compared to several Australian native fruits such as bush tomato, limes, lemon aspen, pepper berry, and riberry. It is suggested that the presence of ellagic acid in *H. sericea* fruit is the chief agent to its high antioxidant

activity (Luis *et al*., 2011). Likewise, quinones like 2-Methoxyjuglone found in *Lomatia hirsute*, and graviquinone in *G. robusta*; arbutin and its derivatives in *Persoonia* sp., bisresorcinol in *Heliciopsis terminalis*; tropane alkaloids, anthocyanins, heliciopsides (A-E); ursolic acid and many others are responsible for diverse bioactive properties of Proteaceae (Zhang *et al*., 2023).

Infectious diseases cover a range of ailments induced by various types of pathogens such as bacteria, fungi, viruses, protozoa, and helminth parasites, which are transferred from one person to another or from animals to humans via physical contact with infected individuald, or by means of food, air, soil, and fluid. Diseases caused by bacterial and viral infections, including helminth infections, collectively called as Neglected Tropical Diseases, are among the major factors of mortality and socio-economic backwardness in many countries (Boutayeb, 2006). Moreover, it is assumed that cases of infectious diseases will be extensively increasing globally, much greater than currently recognized, arising from climate change, rapid population growth which cause environmental disruption that will adversely affect human, wildlife and domestic animals in the food chain (Wu *et al*., 2017). Due to limited treatment options and increasing resistance (van Rhijn, 2024), the plants have been investigated for their rich source of structurally diverse compounds to overcome this challenge (Quave, 2016). The leaf and flower of *Macadamia integrifolia* exhibited strong antibacterial activity against *Candida albicans*, *Bacillus cereus*, and *Escherichia coli* showing a range of minimum inhibitory concentration (MIC) from 2.4 to 22.1 µg/ml; in addition to strong fungicidal effect against *C. albicans* and *Saccharomyces cerevisiae* (Boyer & Cock, 2013). *H. sericea* also indicated antimicrobial efficacy against *Candida tropicalis*, *C. albicans*, *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and antibiotic-resistant *S. aureus* (Luis *et al*., 2013; Madureira *et al*., 2012). *Persoonia* such as *P. pinifolia, P. linearis*, and *P. junipera* showed inhibition against *B. subtilis*, *E. coli*, *Bacillus* sp. and *Staphylococcus* sp. (Cock, 2019; Cleland, 1950). Another plants from Proteaceae such as *Dilobeia thouarsii*, and *G. robusta* also exhibited considerable antibacterial activity. *D. thouarsii* showed MIC of 15.6 µg/ml against *C. albicans* and 1 mg/ml against *Cryptococcus neoformans* (Razafintsalama *et al*., 2013). Effective activity

against *E. coli* was also exhibited by *L. Hirsute* and *Knightia excelsa* with MIC of 8 and 22 µg/ml, respectively (Brady *et al*., 2004; Simonsen *et al*., 2006). Anthelmintic activity surveyed on plants from Proteaceae family like *R. montana*, *G. robusta*, and *Oreocallis grandiflora* expressed activity against Platyhelminthes (*Schistosoma mansomi*), and protozoan parasite (*Leishmania donovani*) (Cunha *et al*., 2012; Takahashi *et al*., 2004). The antimicrobial and antioxidant activities of Proteaceae due to the presence of crucial bioactive compounds underscore their true potential for use in pharmaceutical products. However, further research into their diversity and investigation into their chemical components are still major challenges to establish novel compounds that could be lead molecules in drug development.

PROTEACEAE - AN OVERVIEW ON ITS MORPHOLOGY AND ETHNOMEDICINAL IMPORTANCE

The Proteaceae family has a varied array of flowering plants, consisting of around 83 genera and exceeding 1,600 species. They are primarily located in the Southern Hemisphere, exhibiting the highest variety in Australia, South Africa, and South America (Christenhusz & Byng, 2016). Prominent genera within this family include *Protea*, *Grevillea*, *Hakea*, *Banksia*, *Leucadendron*, and *Macadamia*. Additionally, *Helicia,* a lesser-known genus, is one of the prevalent Proteaceae found in the Southeast Asia.

Plants belonging to the Proteaceae family consists of varied plants ranging from shrubs to trees, measuring 0.2 to 40 meters in height, exhibiting a diverse array of leaf structure that are often leathery, and occasionally meaty, spiny, or serrated. The venation of the species is typically brochidodromous, palmate, parallel or pinnate. These plants are mostly bisexual, with floral structures that are either actinomorphic or distinctly zygomorphic. Many taxa within the Proteaceae family exhibit self-pollination, resulting in a diminished flower-to-fruit ratio attributable to inbreeding depression. Specifically, certain species like *Hakea pulvinifera* and *Lomatia tasmanica* are sterile and exhibit natural vegetative proliferation. Their inflorescences can be either compound or simple and frequently arranged in racemes.

Their fruits are categorized into dehiscent or leathery follicles, indehiscent drupes or pseudodrupes, and are formed from a solitary carpel.

The Proteaceae family has been utilized as substantial economic value and provide diverse products in the nutritional, medicinal, horticultural, and structural materials sectors. Indigenous Australians utilized the fruit, seed, and kernel of *Persoonia* spp. (Corbyn, 2006), *P. falcata* is utilized in the management of cold/flu, ocular, and gastrointestinal ailments (Webb, 1969). *Macadamia integrifolia* and *M. tetraphylla* are consumable and offer nutritional and potential pharmacological benefits (Insanu *et al*., 2021). Flavonoids obtained from the flowers and leaves of *Oreocallis grandiflora* have promising applications in anti-inflammatory and diabetes therapies (Vinueza *et al*., 2018). Given that the majority of Proteaceae are trees or shrubs, they serve as a valuable source of firewood and timber, enhancing their use as utility materials. For instance, *Grevillea* and *Hakea* are utilized as ideal materials for making boomerangs (Wightman *et al*., 1991). Australian aboriginals consumed the seeds, nuts, gum, or fruits of *Brabejum*, *Finschia*, *Floydia*, *Gevuina*, *Hakea*, *Hicksbeachia*, *Macadamia*, *Oreocallis*, *Panopsis*, and *Persoonia* (Kubitzki, 2014; Weston, 1995). Javanese people consume the young shoots of *Helicia serrata* and *H. robusta* (Kubitzki, 2014), while *Telopea*, *Lambertia*, *Grevillea*, *Banksia*, and *Macadamia* provide as sources of honey (Weston, 1995; Hansen & Horsfall, 2016). *Banksia* and *Persoonia* are utilized for the management of coughs and pharyngitis (Hansen & Horsfall, 2016). In Chilean traditional medicine, the leaves of *Lomatia hirsuta* are utilized for the treatment of chronic respiratory diseases such as asthma and bronchitis (Erazo *et al*., 1997). *Faurea saligna* is utilized for diarrhea therapy, while *Grevillea*, *Hakea*, *Persoonia*, *Roupala*, and *Xylomelum* are employed for skin infections. *Heliciopsis* is indicated for ocular infections, and *Protea* is used for skin infections or hyperpigmentation (Kubitzki, 2014; Erazo *et al*., 1997; Twilley & Lall, 2014; Chinsembu *et al*., 2019; Palombo & Semple, 2001). *Oreocallis* for the treatment of hepatic disorders, hemorrhage, or inflammatory conditions. Species of *Helicia* and *Grevillea* are largely employed for dermal or oral lesions; while those of *Banksia*, *Aulax*, *Grevillea*, *Hakea*, *Heliciopsis*, *Leucadendron*, *Leucospermum*, *Mimetes*, *Paranomus*, *Persoonia*, *Roupala*, *Toronia*, and *Xylomelum* serve as dermal

cosmetics as skin toners owing to the presence of arbutin derivatives. *Banksia*, *Grevillea*, *Hakea*, *Isopogon Leucadendron*, *Leucospermum*, *Persoonia*, *Protea*, *Serruria*, *Telopea*, and *Waratah*, are used for ornamental and horticultural purposes. Species of *Athertonia*, *Buckinghamia*, *Darlingia*, *Grevillea*, *Hakea*, and *Protea*, are utilized as timber (Vinueza *et al*., 2018; Cock, 2011; Palombo & Semple, 2001; Xu *et al*., 2015; Criley, 2000; Wrigley & Fagg, 1989; Wightman *et al*., 1991).

The five primary bioactive properties of the Proteaceae family across several genera have been documented, which are antioxidant activity, antibacterial activity, cytotoxicity, anti-inflammatory effects, and antiviral activity. The leaves have predominantly served as the study material in bioactive assays, followed by barks, stems, flowers, and other plant tissues (Zhang *et al*., 2023).

GENUS *HELICIA* - AN OVERVIEW

Helicia is a genus within the family Proteaceae, comprising over 110 species of trees and shrubs. *Helicia* species are distinguished by spirally arranged leaves featuring whole or regularly or irregularly serrated margins. The flowers appear in grevilleoid pairs within raceme-like axillary and/or ramiflorous inflorescences. The blooms are linear with four tepals that become revolute at anthesis, though they often do not fully separate. There exist four epipetalous anthers and four disc (hypogynous) glands, which may be either separate or conjoined (Foreman, 1983). They are found from the southwestern Ghats and Sri Lanka, extending across Southeast Asia to the southeastern regions of China and the northeastern areas of Australia (Chung, 2001).

The most extensively researched species under the genus *Helicia*, frequently misdiagnosed as *H. robusta* or *H. excelsa* by local taxonomists, is *H. nilagirica,* referred to as “Pasaltakaza” in Mizo. Certain species of *Helicia* possess therapeutic properties, while others produce significant wild fruits and vegetables. However, the the use of plant parts, methods of applications, and species utilized for medicinal purposes differ across various places. Simultaneously, while some species are endemic to particular regions, others are classified as highly endangered group. For instance, *H. shweliensis*, an important wild fruit tree in Yunnan, China, is considered

under “Endangered” category of the International Union for Conservation of Nature (IUCN) Red List (Wang *et al*., 2019). *H. nilagirica* also constitutes a valuable component in the traditional herbal medicine in China, which has been utilized for millennia in the treatments of migraine and insomnia (Yue *et al*., 2014). In Thailand, *H. nilagirica* is also recorded as significant ethnomedicinal plants (Phumthum, 2020). In some parts of India, particularly in the Northeast region, some plants of *Helicia* have traditional importance. In Meghalaya, the mature fruit of *H. erratica* is consumed as fruit (Seal & Chaudhuri, 2014), and in Sikkim, *H. nilagirica* fruits are used as analgesic and anti-inflammatory agents in viral infections like cough and cold (Chauhan, 2004). The Mizo people from Mizoram also use different *Helicia* plants as medicine for several diseases. The oral administration of decoction of *H. erratica* has been used for alleviating stomach pain and ulcers (Lalramnghinglova, 2016). *H. nilagirica* is a popular ethnomedicine used for the treatments of different health problems including gynecological disorders, peptic ulcers, dyspepsia, gastrointestinal problems, oral ulcers, urinary tract infections, scabies, and other dermatological conditions (Jagetia & Zoremsiami, 2018). The application of root, leaves, and flowers for the remedy of removing placenta after giving birth, kidney problems, intestinal, and pile problems was also documented.

Some plants from the genus *Helicia* have been investigated on their phytochemical compositions and bioactive properties, which revealed the presence of crucial chemicals that serve these plants achieved their true potent and biological effect in curing several diseases. Various group of compounds, such as flavonol glycosides, phenolic glycosides, benzenoid glycosides, and their derivatives are widely distributed in the genus *Helicia* (Ming & Sheng, 2011). Important groups of phytochemical compounds such as steroids, flavonoids, carbohydrates and glycosides were identified from the methanol extract of *H. nilagirica* bark (Lalawmpuii *et al*., 2014). When the same extract was subjected to sub-acute experimental model to assess the anti-inflammatory property, it revealed a substantial efficacy similar to the standard drug, diclofenac (Lalawmpuii *et al*., 2016). It was suggested that the occurrence of crucial compounds such as sterols and flavonoids is the basis for this activity as their bioactivities include inhibiting inflammatory

enzymes and pathways, as well as transcription factors involved in antiinflammation (Al-Khayri *et al*., 2022; Plat *et al*., 2014). Wu *et al*. (2010) identified and purified 17 compounds from *H. nilagirica* such as several kaempferols, loliolide, sawaranin, rutin, aromadendrin, helicid, helicidol, bergenin, lucidol, 4-hydroxy benzoic acid, β-sitosterol, daucosterol, lignoceric acid-2,3-dihydroxy-propanenyl ester, melissanol and stearic acid. Additionally, an important active constituent, helicid, isoalated from the seed was experimentally shown to possess neurological effects including analgesic, hypnotic, and sedative effect, for which the compound has been used in neurasthenia syndrome and vascular headache (Zhang *et al*., 2017). A clinical study on helicid revealed that it exhibits a potent neuroprotective activity as it influences several intracellular processes like apoptosis, biomembrane organization, excitotoxicity, neuroglia, nitric monoxide system, and oxidative neurotoxicity. Since it has excellent effectiveness with low toxicity, it is widely employed in numerous pharmaceutical products and have been included in several commercial products (Yue *et al.,* 2014). Chronic administration of helicid for depression significantly restored experimentally induced depression, validating its antidepressant-like properties (Tong, 2019). Hepatic protection by helicid was investigated on acute liver injury induced by carbon tetrachloride (CCl_4) and the results showed considerable beneficial effects indicated by the levels of alanine aminotransferase, aspartate aminotransferase, diagnostic biomarkers of liver toxicity, liver disease, or liver damage, and hepatic pathological damage. It was suggested that the observed protective property of helicid may attribute to its potent antioxidative and anti-inflammatory actions by activating the signalling molecules of the NF-κB pathway (Chen *et al.,* 2020). *H. erratica* extracted by four different solvents also expressed certain amounts of flavonoids and flavonols that are associated with high activity of free radical scavenging at the same time (Tapan & Kaushik, 2015).

Due to the significant contribution of *H. nilagirica* to pharmaceutical science as mentioned above, it is strongly believed that the other species of *Helicia* would also have some relevant medicinal properties. Despite numerous records of ethnomedicinal significance, yet relatively limited number of investigations and literature evaluations, *H. excelsa* was chosen for this study. Hence, this thesis aimed

to provide the phytochemical profiling along with scientific-based understanding of *H. excelsa* from Mizoram, Northeast India based on the following objectives:

1. To identify and analyze the chemical constituents of *Helicia* sp.
2. To determine and quantify the antioxidant property of *Helicia* sp.
3. To study the antimicrobial property of *Helicia* sp. against pathogenic bacteria and fungi.
4. To determine the antiparasitic property of *Helicia* sp. against intestinal helminths.

CHAPTER - 2

IDENTIFICATION, AUTHENTICATION, AND EXTRACTION OF THE PLANT

INTRODUCTION

Plants, particularly under the same genus, usually have morphologically similarities which often lead to misidentification. To avoid such complications, according to WHO, authentication, identification, and documentation of the plant shall be obligatory to guarantee a security, potency, and accuracy of medicinal plant before research (WHO, 2003). Furthermore, it is highly probable that other researchers trivialize experimental observations without proper taxonomic identification to ensure scientific validity. In that matter, herbarium prepared from different parts of the plant is used to verify with voucher specimen (Eisenman *et al.,* 2012). However, sometimes morphological identification of the plant is insufficient to support the accuracy of the inter-species differentiation since morphological features can be altered by various factors such as climate change, soil pH, temperature, age, and others which may cause taxonomic identification less authentic (Hassan *et al*., 2020). Therefore, molecular identification is conventionally employed to differentiate closely related taxa using different types of sequence-based markers including Internal Transcribes Spacer (ITS), maturase K (matK), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL), and many others (Chen *et al*., 2010). In addition, the molecular data provides significant key to phylogenetic analysis which reveals the relationships between degrees of genetic evolution and convergence in the group of taxa (Webster *et al*., 2003). Therefore, the integration of morphological and molecular phylogenetic approaches enhances the precision of elucidating evolutionary relationships among organisms (Patwardhan *et al*., 2014).

Plant extraction is a crucial process that link ethnobotanical knowledge and advanced scientific approaches, contributing to the establishment of systematized, effective, and sustainable products (Abubakar & Haque, 2020). However, the yield

and activity of the extracted compounds are significantly determined by sample processing method, extraction technique, and solvent types (Zhang *et al*., 2023). Consequently, grinding is an effective procedure generally performed to reduce specimen sizes so that increased surface area could be obtained. The soxhlet apparatus is the most preferred technique for bioactive compounds extraction from plant materials mainly due to less time-consuming, complete, required smaller solvent volumes (Handa *et al*., 2008). It also provides continuous recycling of the fresh solvent that constantly gets in touch with plant material and better penetration into plant tissues which secure maximum solubility and recovery of phytochemical compounds (Azwanida, 2015; Tiwari *et al*., 2011). Since this process involves alternate heating and condensation of solvents, it extracts more heat-stable compounds such as steroids, flavonoids, and alkaloids compared to other conventional procedures including maceration and decoction (Azwanida, 2015).

HELICIA EXCELSA - ABOUT THE PLANT

Helicia excelsa, locally known as "*sialhma*", is an interesting yet unexplored important ethnomedicinal plant in Mizoram. However, due to its resemblance with other members of the genus *Helicia*, the plant is always misidentified, especially by the local taxonomists. Foreman (1983) and Sleumer (1955) also described the complications of specific divergence in *Helicia* which required careful taxonomic scrutiny. However, *H. excelsa* has certain distinctions in their habitats, morphological features, and distribution patterns. It has a habit of an evergreen medium tree; approximately 10-15 m tall; young shoots are rust-colored tomentose. Leaves obovate or oblanceolate to elliptic; always coarsely serrated or nearly entire when mature; pink to reddish-purple at the tip and deeply serrated at young stage. Young shoots brown-colored tomentose. Inflorescence apical or axillary racemes; rusty-tomentose. Flowers milky to yellowish; 20-35 flowers per inflorescence. Fruit subglobose to ellipsoid, becomes black and rigid when dry. It is mainly distributed in Northeast India, mainland Southeast Asia, lowland to montane forests of Borneo and Sumatra (Royal Botanic Gardens, Kew, 2025).

MATERIALS AND METHODS

Collection of the plant:

Helicia excelsa was collected from its natural habitat in Hmuifang, Aizawl district, Mizoram, India (23.4488° N, 92.7590° E).

Identification and authentication of the plant:

The flower, leaves, and fruit were prepared in herbaria and identified at the Botanical Survey of India, Eastern Regional Center, Government of India, Shillong, India. A voucher specimen was also submitted to Pachhunga University College with accession code: PUC-H-21-01.

DNA extraction and amplification:

The genomic DNA was extracted from the leaves of the plant following a standard 2X CTAB procedure (Doyle and Doyle, 1987). The desired genes from the genomic DNA were amplified in a ProFlex^{TM} 3 x 32-well polymerase chain reaction (PCR) system (Applied Biosystems, Waltham, Massachusettes, USA). An aliquot of of 12.5 µl of EmeraldAmp® GT PCR Master Mix (2X) (Takara Bio, Japan), 2 µl of genomic DNA, 1 µl of each forward and reverse primer, and 9.5 ml of molecular grade H_2O was prepared. Two primers, 5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3' (forward) and 5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3 (reverse) were used for amplifying *ITS* gene (Douzery *et al*., 1999). The conditions used in the amplification was: denaturation at 94°C for 5 mins, followed by 35 cycles at 96°C for 15 sec each, annealing at 50°C for 1 min and extension at 60°C for 4 min followed by at 72°C for 7 min. The PCR product was sequenced at the Barcode Biosciences Pvt. Limited, Bangalore, India.

Extraction of the plant:

The collected healthy leaves were air-dried in the shade at room temperature (23 - 25°C), instead of forced drying at high temperature to preserve heat-labile compounds. To amplify the surface contact of the sample with the solvents, the dried leaves were ground into pieces using electric grinder. The samples were loaded in 2-

litre Soxhlet apparatus and the bottom and upper surfaces were shielded with cotton pads to avoid scattering of the sample during the process. Solvents of varying polarities were used, namely petroleum ether (nonpolar), chloroform (moderately polar), and methanol (highly polar) since different compounds are specifically extracted at different polarities. The solvent in each extraction was added to the sample until it overflowed, the extracted solutions were collected in the round-bottom flasks for each cycle of the extraction. For each extraction, the complete process took approximately 72 hours. The crude extracts were concentrated through solvent evaporation and recovery using a rotary vacuum evaporator (Buchi Rotavapor® R-215). In addition, the aqueous extract was also prepared using maceration process. The pieces were loaded into a clean beaker and poured distilled water till the sample was completely submerged. After one week of continuously stirring at ambient temperature, the content was filtered by filter paper and the supernatant was collected in a beaker. The crude extract of the plant was achieved using continuous hot water bath.

The yield of extraction is expressed as a percentage of the initial dry weight. It is calculated using the formula:

Yield (%) = (weight of dry extract / weight of dry sample) x 100

RESULTS

Identification and authentication of the plant:

BSI identified the plant as *Helicia excelsa* with identification number: BSI/ERC/Tech/2021-22/389. The photograph of *H. excelsa* is shown in **Figure 2.1**. Additional authentication was performed with specimens maintained at the Natural History Museum, London (herbarium numbers BM013822430 and BM000951124) and the Royal Botanic Gardens, Kew (numbers K000009323 and K001110658). Moreover, a renowned local taxonomist, Sawmliana (2024) also identified it as *H. excelsa* in his book and the systematic classification can be written as below:

Taxonomy:

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Proteales
Family	Proteaceae
Genus	*Helicia*
Species	*excelsa*
Authority	(Roxb.) Blume

DNA sequencing and phylogenetic analysis of the plant:

The full length of the generated sequence was 800 bp. Sequence analysis was performed using FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA, USA; http://www.geospiza.com) and alignment was performed in MEGA X (Kumar *et al*., 2016). The generated sequence was compared with the closest available sequences in NCBI GenBank using the BLASTN algorithm. Four nearest genera under the family Proteaceae, *viz*, *Helicia* sp., *Grevillea* sp., *Virotia* sp., and *Protea* pp. were retrieved for phylogenetic analysis. Following the lowest Bayesian Information Criterion (BIC) score, the maximum likelihood tree (ML) was constructed based on the best-fit model using Tamura 3-parameter (T92) with gamma distribution. All branches were supported statistically by a replicate of 1000 bootstraps. The entire positions having

gaps and missing data were discarded. The final sequences were deposited to NCBI GenBank under accession no PX245743.

The maximum likelihood (ML) tree of the generated sequence and database sequences retrieved from NCBI GenBank is shown in **Figure 2.2**. Based on constructed ML tree, each genus under Proteaceae clustered cohesively forming a monophyletic group. The generated sequence showed closest similarity with *H. nilagirica* (MW044388). However, the genetic divergence showed minimal value indicating that the species has not attained complete genetic isolation based on *ITS* gene alone. This is to be expected among cryptic and closely related species as taxonomic incongruence can arise between structural and molecular identifications.

Yield of extraction:

The yield of the four extracts, namely *H. excelsa* petroleum ether extract (HEP), *H. excelsa* chloroform extract (HEC), *H. excelsa* methanol extract (HEM), and *H. excelsa* aqueous extract (HEA) were

HEP = 1.26%

HEC = 3.14%

HEM = 1.25%

HEA = 1.37%

DISCUSSION

With respect to the global challenge in identifying *Helicia*, it applies to Mizoram as well. When it comes to Sialhma, it is constantly identified as either *H. erratica*, or *H. robusta.* Fortunately, this paradox was resolved when the herbaria prepared from leaves, inflorescence, and fruit of the specimen were analyzed at the Botanical Survey of India (BSI), Shillong, India, and identified the species as *H. excelsa*. As it is predominantly found in high altitude ranges from 900 - 1,500 m (Sawmliana, 2024), it is consistently a dominant tree species in mountain regions in Mizoram such as Hmuifang, Reiek, and Phawngpui. Remarkably, during field work, it was discovered that Sialhma is an important biological indicator of air quality since it is also found in various reserve forests in villages including N. Mualcheng and Khumtung of Serchhip District, Mizoram.

The Mizo people extensively used the plant for diverse means in many regions. It is predominantly traditionally used for the treatment of stomach ulcers in most parts of Mizoram (Khiangte & Lalramnghinglova, 2017). While the leaves and stem bark are applied for fever and diarrhea (Lalrinzuali *et al*., 2015), the application of decoction made from the juices of bark and leaves are used for treating stomach pain and urinary problems in several regions of the state has been recorded (Lalhmingsangi & Sahoo, 2018; Rai & Lalramnghinglova, 2010). Sharma et al. (2001) reported that a necklace prepared from the fruit is known to be an effective remedy to cure convulsions, moreover, the decoction of the rhizome is applied in parasitic infection like scabies, while decoction of the root bark are used in removing retained placenta. Uterine disorders and diabetes are also treated through oral administration of the decoction prepared from these parts of the plant. Moreover, through first-hand information from the northeast part of the state, the seed coat (coriaceous testa) is eaten raw to treat stomach problems. In Meghalaya, *H. excelsa* is also used as medicine (Laloo *et al*., 2006), while in Indonesia, it is classified as an important ethnomedicinal plant (Caniago & Stephen, 1998).

Therefore, considering ethnomedicinal importance of *H. excelsa* among Mizo people, after careful morphological and molecular identification, experiments were

then conducted by using the four extracts of *H. excelsa* leaves based on objectives as mentioned in the previous chapter.

Figure 2.1: Photograph of *Helicia excelsa* from its natural habitat in Mizoram, India.

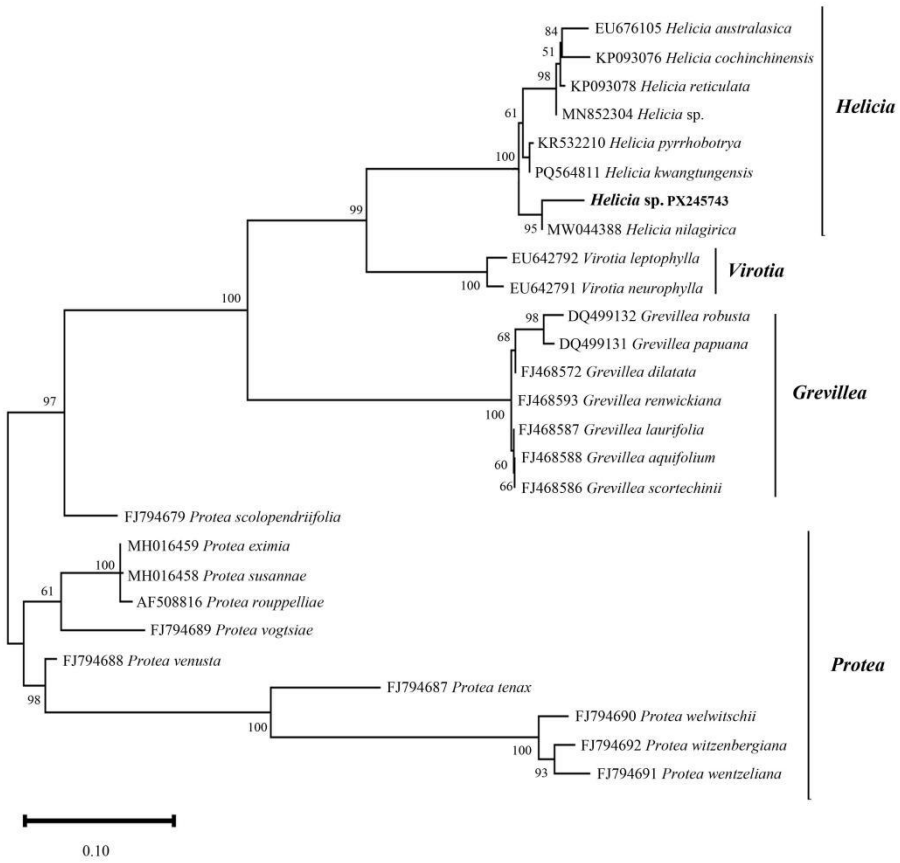

Figure 2.2: Maximum likelihood tree of *Helicia* sp. along with the closest genus under the family Proteaceae. The numbers at the nodes correspond to the bootstrap support (1000 replicates, 50% or more). The NCBI accession numbers were given along with each species.

CHAPTER - 3

IDENTIFICATION OF PHYTOCOMPOUNDS OF *HELICIA EXCELSA*

INTRODUCTION

Medicinal plants, or medicinal herbs are any parts of plant that contain naturally occurring complex compounds with therapeutic properties which are used as the main treatment for a vast array of diseases since antiquity. WHO stated that 80% of the global population depends on traditional healing practices for principal healthcare requirements, and approximately 21,000 which contributes over 30% of the total plant species, have potential therapeutic properties for being used as medicinal plants. From which, India, well-known for its medicinal plants contribution to the world, offers about 2,500 species, and approximately 1,200 are utilized in the drug industries and 150 of those plants are commercialized annually as pharmaceutical products (Anand *et al*., 2019; Ravi & Bharadvaja, 2019). The ethnomedicinal properties of plants are primarily associated with several phytocompounds such as alkaloids, flavonoids, glycosides, saponins, terpenoids,and tannins which are believed to be accountable for their therapeutic properties (Wadood *et al*., 2013; Msomi & Simelane, 2018). In conventional healthcare practices, the application of herbal medicines thrive mainly due to affordability, cultural acceptibility, availability, efficacy, and the perception in the relatively less side effects than synthetic drugs (Msomi & Simelane, 2018; Chikezie *et al*., 2015).

The word “phytochemicals” is a Greek-origin word “phyto”, which means plant that are bioactive, yet non-nutritional compounds naturally present in the plant-based products such as vegetables, grains, fruits, and other parts of the plant (Liu, 2004). Although not belonged to essential nutrients, they are the secondary metabolites produced by plants which have earned prominent modern research interest mainly due to their extensive biological effects such as anticancer, antimicrobial, anti-inflammatory, antioxidant, and anthelmintic properties. Numerous studies has validated that these plant-based compounds are the fundamental cause to

the impediment and effective management of several chronic diseases and infections (Manach *et al*., 2004; Shahidi & Ambigaipalan, 2015; Scalbert *et al*., 2005).

The discovery of antibiotics is one of most revolutionary achievement in the history of modern healthcare industry and the mass production of penicillin in the 1940s indicated the beginning of new generation to control infectious diseases. Diseases such as tuberculosis, pneumonia, sepsis, and syphilis which once were lethal are now treatable that further wide-opened the access of modern medical system (Aminov, 2010). However, the dramatic increase in drug resistance has put modern pharmacotherapy at risk, thereby becoming a driving force for the search of novel phytochemical compounds from natural products such as medicinal plants. Their simple processes, budget-friendliness and quick execution provide detection of phytochemical composition of plant extracts a valuable insights (Sasidharan *et al*., 2011). However, due to this technique alone lacks specificity, it heightens the risk of observation outcomes and may lead to misidentification of phytochemical classes as it lacks molecular and structural information which limits their uses in extensive phytochemical characterization (Tiwari *et al*., 2011; Abubakar & Haque, 2020). Accordingly, in phytochemical investigations, it is crucial to focus on precision outcome as many essential compound classes such as phenolic acids, terpenoids and flavonoids have similar reactive groups but vary in molecular structure and physiological functions (Akinmoladun *et al*., 2007).

To minimize these risks, GC-MS is recruited in most cases. With its advancement in analytical technique, it paves the way for accurate detection, structural classification, and quantification of the compounds in complex plant extracts from spectral databases like National Institute of Standards and Technology (NIST) and Wiley (Patti, 2011; Sparkman *et al*., 2011). For instance, despite belonging to different compound classes, bioactive compounds such as thymol, eugenol and α-pinene are always identified identical since they are commonly found in essential oils and shared similar structure and biological activities. This obstacle has been successfully overcome with identification and characterization provided by GC-MS in various medicinal plants (Sharifi Rad *et al*., 2017).

Since plant-derived metabolites always exhibit novel mode of action or synergistic effect, combining preliminary phytochemical screening with GC-MS analysis enables a vigorous approach to natural-based drug discovery (Cowan, 1999; Hoste *et al*., 2006). Hence, this combined approach encourages systematic phytochemical profiling of *H. excelsa* leaf, which remains limited, with the objective of not only assessing potential biological activities but also addressing the global imperative to develop alternatives to conventional drugs that are facing resistance.

MATERIALS AND METHODS

Reagents preparation for phytochemical tests :

1. Mayer's Reagent: Solution A (1.358 g of mercuric chloride + 60 ml of distilled water) and solution B (5 g of potassium iodide + 10 ml distilled water) were mixed together and the total volume was made up to 100 ml with distilled water.

2. Wagner's Reagent: A mixture of 2 g of potassium iodide and 1.27 g of iodine in 100 ml with distilled water was used ad Wagner's reagent.

3. Dragendorff's Reagent :

Stock Solution: 50 ml of glacial acetic acid, 4 g of sodium iodide, and 5.2 g of bismuth carbonate were mixed together and allowed it to boil for 2 minutes. The sodium acetate crystals formed were filtered out after 12 hours, and 40 ml of the filtrate was blended 16 ml of ethyl acetate and 1 ml of distilled water, and then preserved it in an amber bottle for further use.

Working Solution: A mixture of 10 ml of the stock solution and 20 ml of acetic acid to 100 ml of distilled water was employed as Dragendorff's reagent.

4. Hager's Reagent: Picric acid dissolved in distilled water until saturated was used as Hager's reagent.

5. Millon's Reagent: 1 g of mercury was dissolved in 9 ml of fuming nitric acid was mixed with 10 ml of distilled water.

Chemicals:

Acetone, ethanol, chloroform, ethyl acetate were purchased from Oxford Lab Fine Chem LLP, Maharashtra, India. Hydrochloric acid, glacial acetic acid, sodium nitroprusside, sodium hydroxide, ninhydrin, sodium bicarbonate, sodium carbonate, sodium citrate, copper sulfate, potassium sodium tartrate, sodium chloride, sodium acetate, mercuric chloride, potassium iodide, sodium carbonate, gelatin, mercuric chloride, bismuth carbonate, sodium iodide, and lead acetate were procured from Merck Life Science Private Limited, Mumbai, India. Fehling's solution A, fehling's solution B, magnesium ribbon, zinc dust, copper sulphate, potassium hydroxide,

benedict's reagent, iodine, picric acid, and sodium phosphate were obtained from Himedia Laboratories Private Limited, Mumbai, India. Potassium dichromate was purchased from Sisco Research Laboratories Private Limited, Mumbai, India. Ferric chloride and α-naphthol were acquired from Avantor Performance Materials India Limited, Maharashtra, India.

Preliminary phytochemical screening:

Four different *H. excelsa* extracts (HEM, HEC, HEP and HEA) were exposed to preliminary phytochemical screening using standard experimental procedures with slight changes to observe the presence or absence of different classes of phytoconstituents as follows (Vimalkumar *et al*., 2014, Kokate *et al*., 2008, Shaikh and Patil, 2020):

Test for Alkaloids:

a) Mayer's test: To the mixture of 0.2 ml of diluted hydrochloric acid and 2 ml of the extract solution in a test tube, 0.1 ml of Mayer's reagent was added. The alkaloid in the sample was detected by the formation of a yellowish buff precipitation.

b) Dragendorff's test: The appearance of an orange-brown precipitation in a mixture of 0.1 ml diluted hydrochloric acid, 0.1 ml Dragendorff's reagent, and 2 ml of the sample solution was used to detect the presence of alkaloids.

c) Wagner's test: 2 ml extract solution was mixed with diluted hydrochloric acid and 0.1 ml Wagner's reagent and the appearance of a reddish-brown suggested the presence of alkaloids.

d) Hager's test: 0.2 ml of diluted hydrochloric acid and 2 ml of extract solution were added to 0.1 ml of Hager's reagent. The occurrence of a yellowish precipitation suggested the detection of alkaloids.

Test for flavonoid:

a) Alkaline test: To about 2 ml of the extract, 2-3 drops of sodium hydroxide solution and diluted hydrochloric acid were added. The occurrence of an intense yellow color that becomes colorless upon the addition of acid confirmed the presence of flavonoids.

b) Lead acetate test: About 5 mg of the crude extract was mixed with a few drops of 10% lead acetate solution. The formation of a yellow precipitation indicated the detection of flavonoids.

c) Ferric chloride test: To 5 ml of the extract solution, 2 ml of glacial acetic acid, a drop of ferric chloride solution, and 1 ml of concentrated sulfuric acid were mixed. The appearance of a purple ring below the brown ring suggested the presence of flavonoids.

d) Shinoda test: To a sample solution, a fragment of magnesium turnings and 1-2 drops of concentrated hydrochloric acid were added. The appearance of pink color in the mixture indicated the presence of flavonoids.

e) Zinc-HCl test: To the sample solution, a pinch of zinc dust and concentrated hydrochloric acid were supplied. The detection of flavonoids was confirmed by the development of red color.

Test for phenolics:

a) Lead acetate test: A small crude extract of the plant was mixed with 0.5 ml of 1% lead acetate. A precipitate formation indicated the participation of phenolic compounds.

b) Ferric chloride ($FeCl_3$): 2-3 drops of ferric chloride solution was added to a sample solution, and the occurrence of color change such as green, blue, red, or purple suggested the presence of phenols.

c) Potassium dichromate test: 5 ml of the extract was treated with 1 ml of 10% potassium dichromate. The appearance of a yellowish-brown precipitate showed the detection of phenolic compounds.

d) Iodine solution test: 2-3 ml of the sample was mixed with a few drops of dilute iodine solution. A transient red color development suggested the presence of phenolic compounds.

e) Ellagic acid test: When the extract solution treated with 5% glacial acetic and sodium nitrite solutions showed a muddy or niger brown precipitation suggested the presence of phenolic compounds.

f) Gelatin Test: To 5 ml extract, 1% gelatin solution and 10% sodium chloride were added. The development of white precipitation indicated the presence of phenolic compounds.

Test for carbohydrates:

a) Molisch's test: A formation of violet ring when 2 ml of the filtrated extract was treated with 2-3 drops of α-naphthol and 1 ml of concentrated sulfuric acid confirmed the presence of carbohydrates.

b) Benedict's test: 0.5 ml of Benedict's reagent was added to the extract filtrate, and the mixture was boiled for 2 minutes in hot water bath. The development of a green, yellow or red colour signified the presence of carbohydrates.

c) Iodine test: 2 ml of iodide solution with potassium iodide was added to 2 ml of the test extract. The appearance of a blue color denoted the detection of carbohydrates.

d) Fehling's test : 1 ml each of Fehling's solution A and B were added to 1 ml of the filtrated extract. After the mixture was boiled in a hot water bath for about 2-3 minutes, the development of a red precipitate suggested the presence of carbohydrates.

Test for glycosides:

a) Libermann-Burchard test: To 1 ml of the sample dissolved in chlorofom, 2 ml of acetic acid and 1-2 drops of concentrated sulfuric acid were carefully added alongside of the test tube and gently shook. A color change from pink to blue, green and deep green indicated the presence of a form of glycosides such as sterol and triterpenoid.

b) Salkowski's test: About 2 ml of the extract dissolved in chloroform was treated with 2 ml of concentrated sulfuric acid. Since the solutions did not mix, a reddish-brown or red color appeared at the interface suggested the presence of sterol glycosides.

c) Keller-Killiani test: 5 ml of the extract dissolved in distilled water was mixed with 2 ml of glacial acetic acid, a few drops of ferric chloride and 1 ml of concentrated sulfuric acid. A brown ring, accompanied by a purple ring below it indicated the presence of cardiac glycosides.

d) Borntrager's test: To 2 ml of the sample dissolved in distilled water, 2 ml of sulfuric acid was added. The mixture was then boiled and filtered. The filtrate was mixed with an equal amount of chloroform and shaken vigorously. The separated organic layer was treated with ammonia and the development of a pinkish-red color in the upper layer confirmed the presence of anthraquinone glycosides.

e) Legal's test: The sample dissolved in pyridine was treated with a few drops of sodium nitroprusside solution, followed by the addition of 2-3 drops of sodium hydroxide. A pink or red occurrence signified the presence of glycosides.

Test for saponins:

a) Froth/Honeycomb test : About 2 ml of the extract was mixed with 2-3 drops of 5% sodium bicarbonate and the mixture was shaken vigorously. The occurrence of honeycomb-like froth after allowing the mixture to stand for 5 minutes suggested the presence of saponins.

b) Foam test: About 0.5 mg of the extract was mixed with distilled water and shaken vigorously for about 15 minutes. The appearance of a stable foam layer about 2 cm in height confirmed the presence of saponins.

Test for tannins:

a) Braymer's test: 1 ml of the filtrate sample was mixed with 3 ml of distilled water and 3 drops of 10% ferric chloride. A blue-green color formation indicated the presence of tannins.

b) Gelatin test: To 5 ml of the extract solution, 10% sodium chloride and 1% gelatin solution were added. A white precipitate development indicated the presence of tannins.

c) 10% NaOH test: About 0.5 ml of the plant extract solution was treated with 5 ml of 10% sodium hydroxide solution. The formation of emulsion suggested the presence of hydrolysable tannins.

Proteins and amino acids test :

a) Biuret test: A drop of 2% copper sulfate solution and 1 ml of 95% ethanol were added in to 2 ml of the filtrated plant sample. The development of a pink-colored solution in the ethanolic layer suggested the presence of proteins and amino acids.

b) Millon's test: 2 ml of the sample filtrate was mixed with a few drops of Millon's reagent. A white precipitate turned red upon heating confirmed the presence of proteins.

c) Ninhydrin test: To 3 ml of the extract solution, about 2-3 drops of ninhydrin solution was added. The appearance of a purple or blue color development signified the presence of amino acids.

d) Xanthoproteic test: About 3 ml of the sample solution was mixed with a few drops of concentrated nitric acid. The appearance of yellow color confirmed the presence of proteins and amino acids.

Test for phytosterol:

a) Salkowski's test: To 3 ml of the filtrated sample, a few drops of concentrated sulfuric acid was added. After allowing to stand for about 5 minutes, a red color development in the lower layer suggested the presence of phytosterols.

b) Libermann-Burchard: To about 5 mg of the plant extract, 2 ml of acetic anhydride and 1-2 drops of concentrated sulfuric acid were carefully added along the side of the test tube. The presence of phytosterol was confirmed by a series of color changes in the mixture solution.

Identification of compounds of *H. excelsa* using gas chromatography - mass spectrometry (GC-MS):

The mass chemical identification of the *H. excelsa* extracts for four different extracts (HEM, HEC, HEP and HEA) was done using GC-MS system (Thermo Scientific TRACE™ 1300 ISQ™ LT) by dissolving the samples in acetonitrile solution. The identification of the chemicals for the samples were initiated using TR-5MS (260F142P) with a dimension and film thickness of 30 m × 0.25 mm × 0.25 µm and 0.25 µm, respectively. The injector port and the oven optimal temperatures were set to 250°C and 70°C, respectively, and the oven temperature was moderately raised up to 250°C. The flow of helium gas, also known as a carrier gas, was constantly set at the rate of 1 ml/minute. At that rate, 1 µl of the sample was injected in the split mode with a ratio of 1:50. The ionization of the mass spectrophotometer was then started with an electron energy of 70 eV and both the temperatures for ion source and transfer line were set at 250°C. After 55 minutes of running the instrument/device,

the data gave out by Thermo Scientific™ Xcalibur™ software were then identified as compounds from databases of Wiley Registry™ and the National Institute of Standards and Technology (NIST) based on their chemical formula, molecular mass and retention time. The percentage abundance of each compound was also considered to show the efficacy of the sample in each test.

Thin layer chromatography (TLC)

The four *H. excelsa* extracts were analyzed with thin layer chromatography (TLC). TLC is the fundamental technique in identifying compounds present in complex mixtures such as medicinal plant extracts. It identifies the exact chemical compounds and is thus the first critical procedure in compound isolation (Marston, 2011). Each plant extract was dissolved in its respective solvent. The compound was spotted on pre-coated Sigma-Aldrich® (Darmstadt, Germany) TLC Plates (20 × 20 cm, 200 µm thick) using silica gel (particle size 5-17 µm, pore diameter 60 Å, with specific pore volume 0.75 ml/g). A transverse line was marked at 1 cm from the base edge of the TLC plate. The plant extracts were spotted on the line using glass microcapillaries. The samples were left to dry in open air and then introduced into a TLC chamber containing the mobile phase made up of a solvent mixture, toluene and ethyl acetate (8:2) for petroleum ether and chloroform extracts, *n*-butanol, acetic acid and water (4:1:5) for methanol and aqueous extracts. The TLC chamber was closed and the mobile phase was allowed to migrate by capillary action. The migration was stopped when the solvent front reached ~5 cm from the point of origin. The TLC plates were taken out and visualised in an ultraviolet light cabinet (under the intensity of 254 and 366 nm) and iodine chamber. The retention factor (R_f) was calculated from the ratio of the distance travelled by the compounds against that of the mobile phase.

RESULTS

Preliminary phytochemical screening of the plant extracts:

The primary phytochemical screening of *H. excelsa* extracts using methanol, chloroform, petroleum ether, and aqueous solvents were performed on nine different chemical groups was shown in **Table 3.1.** Alkaloid was detected in all extracts except negative Hager's test and Wagner's test observed in petroleum ether and chloroform extracts, respectively. Flavonoid was observed to be present mainly in aqueous extract, while chloroform appeared to be negative for all the tests; Zn-HCl reduction test was negative in all the extracts; alkaline and Shinoda's tests were also negative in all the extracts except aqueous extract; apart from chloroform extract, ferric chloride was also tested negative in methanol extract. Moreover, all phenolic compound tests were predominantly detected in methanol extract, while ferric chloride and gelatin tests were positive in all the extracts, lead acetate and iodine solution tests were detected in methanol extract only. Carbohydrate tests were weakly detected across all the extracts; while methanol extract did not respond to any tests, Molisch's and Benedict's tests were positive in aqueous extract, Fehling's and Benedict's tests were positive in chloroform extract, Benedict's test was also found positive for petroleum ether extract. Additionally, the presence of glycosides was confirmed variably in the extracts, yet Borntrager's and Legal's test were negative across all the extracts. The Liebermann-Burchard test was positive in petroleum ether and chloroform extracts, Salkowski's and Keller-Kilani's test were positive in methanol and aqueous extracts, respectively. Saponin's forth and foam tests were positive only in water soluble extracts such as methanol and aqueous extracts. In regard to tannins, Braymer's test was detected only in aqueous extract, gelatin test in methanol and aqueous extracts, NaOH test in petroleum ether and chloroform extracts. In protein and amino acid test, while Millon's test was positive in the three extracts except chloroform, the other two tests such as biuret and ninhydrin tests were negative in all the extracts, and xanthoproteic acid test was positively detected in the aqueous extract. Lastly, phytosterol's Liebermann-Burchard's test gave positive result in all the extracts, while Salkowski's test was positive in methanol and aqueous extracts.

Gas chromatography - mass spectrometry (GC-MS) profiling of the phytoconstituents of the extracts of *H. excelsa*:

The profiling of phytochemical compounds of the four extracts of *H. excelsa* using GC-MS was carried out based on their molecular weights (MW), retention times (RT), molecular formulas, and relative abundances (RA) or percentage areas (%). The detected compounds were identified from databases of Wiley Registry™ and the National Institute of Standards and Technology (NIST).

The chromatogram of the methanol extract shown in the **Figure 3.1** revealed a total of 90 compounds belonging to various phytochemical classes, and the detected corresponding compounds are presented in **Table 3.2**. Depending on the total relative abundance, the first 10 compounds identified were as listed below. The analysis identified the highest abundance as a monoterpenoid compound class of Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene ($C_{10}H_{16}$, MW : 136, RT : 9.832) with 5.76%, followed by 1,3,6-Octatriene, 3,7-dimethyl-, (Z)- ($C_{10}H_{16}$, MW : 136, RT : 11.575) with 5.57%, D-Limonene ($C_{10}H_{16}$, MW : 136, RT : 11.045) with 5.12%, 2-Propenoic acid, 3-phenyl- ($C_9H_8O_2$, MW : 148) at two different interval times of 23.34 and 21.31 minutes with relative abundance of 4.82%, n-Hexadecanoic acid ($C_{16}H_{32}O_2$, MW : 256) appeared at 34.675 minutes with % abundance of 4.03%, Neophytadiene ($C_{20}H_{38}$, MW : 278) at two different retention times 32.995 and 33.56 minutes with total relative abundance of 3.38%, Phytol ($C_{20}H_{40}O$, MW : 296) at retention time of 36.83 minute with 3.92%, trans-.beta. -Ocimene ($C_{10}H_{16}$, MW: 136, RT: 11.245) with a total abundance of 2.95%, Hexadecanoic acid ($C_{17}H_{34}O_2$, MW: 270) at a retention time of 34.125 with a percentage abundance of 2.64, and lastly, Benzyl .beta. -d-glucoside ($C_{13}H_{18}O_6$, MW: 270) at 40.575 minute was found with a relative abundance of 2.57%. Additionally, numerous molecules from other classes exhibiting substantial biological activity have been identified. Some of these compounds are 2-Acetoxy-1,1,10-trimethyl-6,9-epidioxydecalin ($C_{15}H_{24}O_4$, MW: 268, RT: 32.12, RA: 2.54), 13-Docosenamide, (Z)- ($C_{22}H_{43}NO$, MW: 337, RT: 45.475, RA: 2.11), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester ($C_{19}H_{38}O_4$, MW: 330, RT: 42.86, RA: 1.99%), 8,11,14-Docosatrienoic acid, methyl ester ($C_{23}H_{40}O_2$, MW: 348, RT: 36.625, RA: 1.39), and so on. The chemical with the

lowest relative abundance was Pentanedioic acid ($C_5H_8O_4$, MW: 132), which had 0.09% at a retention time of 9.113 minutes.

The GC-MS analysis of chloroform extract of *H. excelsa* shown in **Figure 3.2.** identified 23 different compounds and those compounds were listed in **Table 3.3**. Nonacos-1-ene ($C_{29}H_{58}$, MW : 406), detected at 18.95 and 20.65 minutes (with abundances of 6.88 and 4.65%) had the highest total abundance with 11.53%. Based on the relative abundance, Heptacos-1-ene ($C_{27}H_{54}$, MW : 378) came second with 9.30 at 17.09 minute, followed by 9-Eicosene, (E)- ($C_{20}H_{40}$, MW : 280, RT : 15.06) with 9.12%, 2,4-Di-tert-butylphenol ($C_{14}H_{22}O$, MW : 206) detected at retention time of 11.9 with abundance of 9.02%, Hexadecen-1-ol, trans-9- ($C_{16}H_{32}O$, MW : 240, RT : 12.81) with 5.67% abundance, n-Hexadecanoic acid ($C_{16}H_{32}O_2$, MW : 256, RT : 16.81) with 5.58%, Tetracyclo[5.1.0.02,4.03,5]octane, 8,8-dichloro-exo-6-methoxy- ($C_9H_{10}Cl_2O$, MW : 204) detected at 18.19 minute and 20.41 with total relative abundance of 5.39%, 1,1-Dichloro-2-methyl-3-(4,4-diformyl-1,3-butadien-1-yl)cyclopropane ($C_{10}H_{10}Cl_2O_2$, MW : 232) at two different retention times of 23.52 and 24.01 minutes with total abundance of 4.55%, Dodecyl acrylate ($C_{15}H_{28}O_2$, RT : 13.98) having MW of 240 with abundance of 4.09%, Phytol ($C_{20}H_{40}O$, MW : 296, RT : 18.26, % area : 3.81), and Octadecane, 6-methyl- ($C_{19}H_{40}$, MW : 268) detected at 17.14 minute with 3.35% abundance. Other important compounds such as 2,4,6,8,10- Tetradecapentanoic acid, 9a- (acetyloxy)- 1a, 1b, 4,4a, 5,7a, 7b, 8,9a- decahydro- 4a, 7b- dihydroxy- 3- (hydroxymethyl)- 1, 1,6,8- tetramethyl- 5- oxo- 1H- cyclopropa[3,4] benz[1,2- e] azulen- 9yl ester, [1aR- (1aa, 1ba, 4aa, 7aa, 7ba, 8a, 9a, 9aa)] ($C_{36}H_{46}O_8$, MW : 606, RT : 20.73, % are : 2.98), [1,1'-Biphenyl]-2,3'-diol, 3,4',5,6'-tetrakis(1,1-dimethylethyl)- ($C_{28}H_{42}O_2$, MW : 410, RT : 20.46, % area : 3.16), Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester ($C_{19}H_{25}NO_5$, MW : 347, RT : 15.94, % area : 2.98), 9-(2',2'-Dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethylamino)-ethoxy]fluorene ($C_{30}H_{42}Cl_2N_4O_3$, MW : 576, RT : 21.96, % area : 2.79), Pentadec-7-ene, 7-bromomethyl- ($C_{16}H_{31}Br$, MW : 302, RT : 22.24, % area : 2.98), 3-Trifluoroacetoxypentadecane ($C_{17}H_{31}F_3O_2$, MW : 324, RT : 12.96, % area : 1.67) and others were also detected.

The analysis of petroleum ether extract of *H. excelsa* by GC-MS (**Figure 3.3**) detected 9 different compounds of different classes was shown in **Table 3.4**. Among them, according to the relative abundance (% area), Phytol ($C_{20}H_{40}O$, MW : 296) detected at 18.26 minute was found to be the highest with 18.45%, followed by Pentadecanoic acid, 14-methyl-, methyl ester ($C_{17}H_{34}O_2$, MW : 270, RT : 16.44) with 14.02% abundance, 2,4-Di-tert-butylphenol ($C_{14}H_{22}O$, MW : 206, RT : 11.89) having abundance of 12.92%, Phthalic acid, 5-methylhex-2-yl heptadecyl ester ($C_{32}H_{54}O_4$, MW : 502, RT : 21.96) with 9.59%, cis,cis,cis-7,10,13-Hexadecatrienal ($C_{16}H_{26}O$, MW : 234, RT : 18.16, % area : 9.41), 2-Piperidinone, N-[4-bromo-n-butyl]- ($C_9H_{16}BrNO$, MW : 233, RT : 18.99, % area : 9.23), 9-Tetradecen-1-ol, acetate, (E)- ($C_{16}H_{30}O_2$, MW : 254, RT : 18.79, abundance : 9.04%), 9-octadecenoic acid, 2,2,2-trifluoroethyl ester ($C_{20}H_{35}F_3O_2$, MW : 364, RT : 20.55, abundance : 8.86%), and lastly, the least abundant compound 9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione, having molecular formula of $C_{11}H_{16}O_4$ detected at 16.85 minute with 8.49% was also recorded.

The chromatogram of the aqueous extract of *H. excelsa* showed 13 important compounds shown in **Figure 3.4**. As shown in **Table 3.5**, among the compounds, Silicic acid, diethyl bis(trimethylsilyl) ester ($C_{10}H_{28}O_4Si_3$, MW : 296), detected at four different retention times such as 22.83, 23.04, 24,40 and 27.07, was found to have the highest total abundance with 18.51%, followed by 3-O-Methyl-d-glucose ($C_7H_{14}O_6$, MW : 194) with abundance of 15.68 detected at two different retention times (14.32 and 14.60), Isosorbide Dinitrate ($C_6H_8N_2O_8$, MW : 236) having total abundance of 9.26% with retention times of 11.41 and 11.44, 5-Hydroxy-4,4,6-trimethyl-7-oxabicyclo[4.1.0]heptan-2-one ($C_9H_{14}O_3$, MW : 170, RT : 14.97) and Hydroxyacetic acid, hydrazide ($C_2H_6N_2O_2$, MW : 90, RTs : 3.31 and 3.61) having the same relative abundances of 8.72%, Octan-2-one, 3,6-dimethyl- ($C_{10}H_{20}O$, MW : 156, RT : 15.05) with 6.23% relative abundance, 2-Methyl-d-glucose ($C_7H_{14}O_6$, MW : 194) detected at 15.18 minute with 5.34% abundance, Thymol, TBDMS derivative ($C_{16}H_{28}OSi$, MW : 264, RT :23.94) with percentage abundance of 5.16, [1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester ($C_{21}H_{38}O_2$, MW : 322, RT : 13.61) with 4.72% relative abundance, 1-acetoxy-p-menth-3-one ($C_{12}H_{20}O_3$, MW :

212) at retention time 13.10 with 4.63 percentage abundance, and the last three compounds namely L-Glucose ($C_6H_{12}O_6$, MW : 180), d-Glycero-d-ido-heptose ($C_7H_{14}O_7$, MW : 210) and Desulphosinigrin ($C_{10}H_{17}NO_6S$, MW : 279) having relative abundance of 4.45, 4.36 and 4.27% were detected at 15.34, 11.35 and 15.96, respectively.

Thin layer chromatography (TLC)

Thin layer chromatography (TLC) using different solvent mixtures showed different spots were visualized for each extract. In HEM, the R_f value of the compound was calculated as 0.22. In HEC, the R_f value was 0.27. In HEP, the R_f values were 0.44, 0.62, 0.7, and 0.88 and in HEA, they were 0.7 and 0.8. The precise identification of these compounds using TLC will help in the complete isolation and purification of each compound, which will be essential for further chemical and pharmacological studies.

DISCUSSION

The phytochemical studies of the family Proteaceae revealed extensive variety of bioactive compounds. However, Proteaceae is one of the most unexplored plant families. Although it has been extensively used in ethnomedicine since ages, phytochemical profiling covered only 30% of the species, among which, compound isolation and purification was achieved for less than 10% of species, and additionally, more than 400 identified compounds were consisted of mainly by phenolic (69%), alkaloids (13%), and quinones (8%), which are potent antimicrobial, anti-inflammatory, and antioxidant effects (Zhang *et al*., 2023). However, chemical characterization using Nuclear Magnetic Resonance (NMR) spectroscopy performed for *Protea cynaroides* provided evidence for the presence of six compounds namely 3,4-bis(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol, 2-(hydroxymethyl)-4-oxo-4H-pyran-3-yl-6-O-benzoate-β-Dglucopyranoside, 3-hydroxy-7,8-dihydro-β-ionone-3-O-β-D-glucopyranoside, 3,4-dihydroxybenzoic acid, and 3-hydroxykojic acid (Yalo *et al*., 2022), while chemical investigation of *Grevillea robusta* using 1D and 2D NMR recently reported the identification of 3,5-dihydroxy cinnamate, 6-hydroxy coumarin, p-hydroxy benzeldahyde, methyl gallate, ethyl gallate arbutin 6”-O-3,5 dihydroxycinnamic acid ester, and robustaside D (Ahmed and Makboul, 2021). Furthermore, investigation of new compounds on methanolic extract of *Heliciopsis terminalis* by employing Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) observed the presence of six phenolic glycosides (Heliciopside A-E, 3,4,5-trimethoxyphenyl-β-D-glucopyranoside), triterpene glycoside (Clemochinenoside D) and other compounds (Ryu *et al*., 2022). GC-MS analysis of *Stenocarpus sinuatus* demonstrated the presence of biologically important compounds, such as 3,7,11,15-Tetramethyl-2-hexadecene, Neopytadiene, Phytol, α-Tocospiro A, 2-Methyloctacosane, β-tocopherol, Hentriacontane, and γ-sitosterol , which are widely used in skincare products and cosmetic formulations (Younis *et al*., 2022). Interestingly, phytol was also detected in the methanol, chloroform and petroleum ether, neophytadiene and tocopherol in methanol extracts of *H. excelsa.*

Preliminary phytochemical screening of the extracts of *H. excelsa* leaf indicated the presence of several classes of phytocompounds such as alkaloids,

phenolics, flavonoids, saponins, glycosides, tannins, phytosterols, and carbohydrates with considerable differences according to the polarity of solvent used for extraction. Among the extracts, the broadest spectrum of phytochemicals was exhibited in methanol and aqueous extracts, which validates the previous findings that polar solvents usually extract a greater variety of bioactive compounds from plants when compared to non-polar solvents like chloroform and petroleum ether (Tiwari *et al*., 2011).

Alkaloid, a multifunctional group of compounds well-known for their robust biological effects such as anti-inflammatory, antimicrobial, and anticancer, were fundamentally detected in methanol, chloroform, and aqueous extract, which indicate their favourable presence of this particular compound associates with alkaloids' high solubility in polar solvents (Jeliński *et al*., 2019). A prominent presence of flavonoids and phenolics, two broad compound groups, known for their strong antioxidant property (Rice-Evans *et al*., 1997), detected in methanol and aqueous extracts suggesting the potent antioxidant of *H. excelsa*, which is further supported by significant positive results in ferric chloride and Shinoda's tests, and additionally, similar results were also observed in related species of the Proteaceae family (Chinsembu, 2016).

Carbohydrates, mainly reducing sugars, were primarily detected in aqueous extracts, indicating the presence of mono- and disaccharides participated in bioenergetics and glycoside synthesis (Trethewey *et al*., 1999). Glycosides detected in all the extracts indicating that a wide range of biological activites such as inhibition of Na^+/K^+-ATPase with potent anticancer effects (Newman *et al*., 2008). Saponins, recognized for their membrane-disrupting and cholesterol-affinity activates which suggesting antifungal and antimicrobial properties (Sparg *et al*., 2004), were chiefly found in methanol and aqueous extracts. Tannins, known for their astringent and antimicrobial activities (Scalbert, 1991), were also weakly present across all extracts. Additionally, phytosterol, well known for its anti-inflammatory and hypocholesterolemic property, supporting their functions in nutraceutical formulations (Piironen *et al*., 2000), was detected across all the extracts specifically via Liebermann-Burchard test. Although minutely detected in petroleum

ether, methanol and aqueous extracts, the presence of proteins and amino acids somewhat suggests a potential bioactive peptides.

The employment of GC-MS for profiling the extracts of *H. excelsa* enriched and supplemented the findings based on phytochemical screening by distinguishing a wide range of phytoconstituents along with the compound classes they belong to. The methanol extract of the plant was identified to possess various notable compounds repleted with biological activities. Compounds including 2-Carene, D-Limonene, and trans-.beta.-Ocimene, 1,3,6-Octatriene, 3,7-dimethyl-, (Z)-, and gamma.Terpinene, classified as monoterpene, are distinguished for their antibacterial, antioxidant, insecticidal, neuroprotective, anti-inflammatory, and antitumor properties (Schmidt *et al*., 2005; Doughari & Bazza, 2010; Wang *et al*., 2024; Balla *et al*., 2017; Asiwe *et al*., 2023; Alves-Silva *et al*., 2020). Various compound classes, including phenolic derivatives such as (Z)-5-(Pentadec-8-en-1-yl)benzene-1,3-diol, 1,3-Benzenediol, 5-pentadecyl-, Demethoxyencecalinol, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 4-Vinylphenol), are recognized for their antioxidant, anticancer, antidiabetic, antimicrobial, and antifungal properties (Terpinc *et al*., 2011; Yue *et al*., 2015; Shah *et al*., 2020; Romagnoli *et al*., 2016). Additionally, fatty acids (Methyl-2-hydroxy-tetracosanoate, Hexadencanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, Octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-, Octadecanoic acid) further suggest the antidiabetic, pesticidal, antioxidant, antimicrobial, anti-inflammatory, antifungal, and antitumor efficacy of the methanol extract of *H. excelsa* (Akpuaka *et al*., 2013; Kadhim *et al*., 2017; Tyagi & Agarwal, 2017; Shilpa *et al*., 2009).

The chloroform extract of the plant is also high in fatty acid esters and hydrocarbons, such as 9-(2',2'-Dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethylamino)-ethoxy]. Fluorene, pentadec-7-ene, 7-bromomethyl, octadecane, and 6-methyl are well-known for their strong antibacterial, antioxidant, cytotoxic, antifungal, and anticancer properties (Osuntokun & Cristina, 2019; Mushtaq *et al*., 2013). Compounds like 9-Eicosene, (E)- and Nonacos-1-ene, classified as insect chemical signals are also appeared to contain insect-repelling properties (Ghalloo *et al*., 2022; Farine *et al*., 2012).

Moreover, the petroleum ether extract revealed its diverse chemical classes. Compounds such as 2-Piperidinone, N-[4-bromo-n-butyl]-, phenolic derivatives of 2,4-Di-tert-butylphenol, lipophilic constituents including pentadecanoic acid, 14-methyl-, methyl ester, and cis,cis,cis-7,10,13-Hexadecatrienal are acknowledged for their antimicrobial, antioxidant, anti-inflammatory, anticancer, and anti-androgenic, antifungal, hepatoprotective, and larvicidal properties (Zhao *et al*., 2020; Al-Salman, 2019; Audi & Wada, 2023, Momodu *et al*., 2022; Ogunlesi *et al*., 2019; Ashoka & Shivanna, 2022).

The aqueous extract was mainly distinguished by sugar derivatives such as 3-O-Methyl-D-glucose, 2-Methyl-D-glucose, and L-glucose, which are broadly acknowledged for their activities including anticancer, anti-inflammatory, antibacterial and antioxidant (Ulqodry *et al*., 2025; Satapathy *et al*., 2023). The identification of Desulphosinigrin, a derivative of glucosinolate, also supports its anticancerous and antiepileptic activities (Wagay & Rothe, 2016; Krupashree *et al*., 2018). Moreover, oxygenated aromatic compounds such as hydroxyacetic acid, hydrazide, and thymol underscore the antibacterial, antifungal, antiparasitic, anti-inflammatory, and antitumor potential of the extract (Sahoo *et al*., 2021; Popiołek, 2017).

Phytochemicals	Name of test	HEM	HEC	HEP	HEA
Alkaloid	1. Hager's test	+	+	-	+
	2. Wagner's test	+	-	+	+
	3. Mayer's test	+	+	+	+
	4. Dragendorff's test	+	+	+	+
Flavonoid	1. Alkaline test	-	-	-	+
	2. Lead acetate test	+	-	+	+
	3. Ferric chloride test	-	-	+	+
	4. Shinoda's test	-	-	-	+
	5. Zn-Hcl reduction test	-	-	-	-
Phenolic	1. Lead acetate test	+	-	-	-
	2. Ferric chloride test	+	+	+	+
	3. Potassium dichromate test	+	+	-	-
	4. Iodine solution test	+	-	-	-
	5. Ellagic acid test	+	+	+	-
	6. Gelatin test	+	+	+	+
Carbohydrate	1. Molisch's test	-	-	-	+
	2. Benedict's test	-	+	+	+
	3. Iodine test	-	-	-	-
	4. Fehling's test	-	+	-	-
Glycoside	1. Libermann's test	-	+	+	-
	2. Salkowski's test	+	-	-	-
	3. Keller-Kilani's test	-	-	-	+
	4. Borntrager's test	-	-	-	-
	5. Legal's test	-	-	-	-
Saponin	1. Froth test	+	-	-	+
	2. Foam test	+	-	-	+

Tannin	1. Braymer's test	-	-	-	+
	2. Gelatin test	+	-	-	+
	3. 10% NaOH	-	+	+	-
Proteins and amino acid	1. Biuret test	-	-	-	-
	2. Millon's tet	+	-	+	+
	3. Ninhydrin test	-	-	-	-
	4. Xanthoproteic acid test	-	-	-	+
Phytosterol	1. Salkowski's test	+	-	-	+
	2. Liebermann-Burchard's test	+	+	+	+

+indicates presence; and – indicates the absence of a particular compound group.

Table 3.1: Phytochemical screening of different compound classes of the extracts of *H. excelsa*.

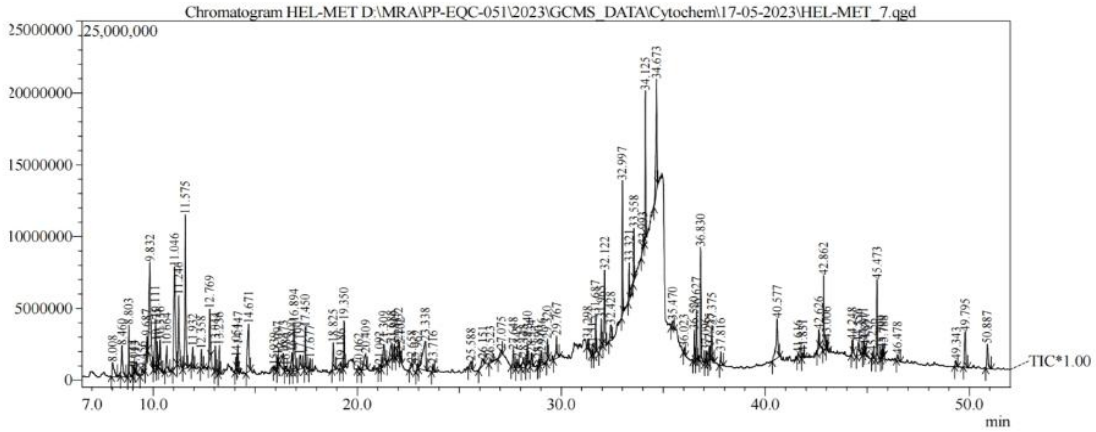

Figure 3.1 : A chromatogram of the methanol extract of *H. excelsa*.

Compound	Molecular weight (g/mol)	Retention Time (Minute)	% Area	Molecular formula
1,2-Cyclopentanedione	98	8.008	0.93	$C_5H_6O_2$
Imidazole-4-carboxamide	111	8.46	1.33	$C_4H_5N_3O$
Bis(4-methylcyclohexyl) ethylphosphonate	302	8.803	1.27	$C_{16}H_{31}O_3P$
2-Furancarboxaldehyde, 5-methyl-	110	9.044	0.16	$C_6H_6O_2$
Pentanedioic acid	132	9.113	0.09	$C_5H_8O_4$
2H-Oxireno[3,4]cyclopenta[1,2-c]furan-2-one / Guaiacylglycerol	214	9.456	0.27	$C_{10}H_{14}O_5$
2,6-Dimethyl-2-trans-6-octadiene	138	9.687	0.82	$C_{10}H_{18}$
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene	136	9.832	5.76	$C_{10}H_{16}$
1,5-Heptadiene, 2,3,6-trimethyl-	138	10.11	2.33	$C_{10}H_{18}$
3-Pyrazolidinone, 1,4-dimethyl	114	10.175	0.83	$C_5H_{10}N_2O$
Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)	136	10.335	1.10	$C_{10}H_{16}$
2-Carene	136	10.665	1.12	$C_{10}H_{16}$
D-Limonene	136	11.045	5.12	$C_{10}H_{16}$

trans-.beta.-Ocimene	136	11.245	2.95	$C_{10}H_{16}$
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	136	11.575	5.57	$C_{10}H_{16}$
gamma.-Terpinene	136	11.930	0.72	$C_{10}H_{16}$
alpha.-Methyl-.alpha.-[4-methyl-3-pentenyl]oxiranemethanol $$ 6-Methyl-2-(2-oxiranyl)-5-hepten-2-ol	170	12.36	0.51	$C_{10}H_{18}O_2$
(+)-4-Carene	136	12.77	1.38	$C_{10}H_{16}$
Butanedioic acid, methylene-, dimethyl ester	158	13.045	0.62	$C_7H_{10}O_4$
Linalool	154	13.235	0.68	$C_{10}H_{18}O$
2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)-	136	14.055	0.29	$C_{10}H_{16}$
Linalool, methyl ether	168	14.145	0.66	$C_{11}H_{20}O$
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	144	14.67	2.49	$C_6H_8O_4$
Thiocarbamic acid, N-cyclohexyl-, S-(2,5-dihydroxyphenyl) ester	267	15.94	0.16	$C_{13}H_{17}NO_3S$
Alpha.-Terpineol	154	16.095	0.23	$C_{10}H_{18}O$
4-Methyl itaconate	144	16.375	0.99	$C_6H_8O_4$
Ethane, 1,1,2-trimethoxy-	120	16.485	0.17	$C_5H_{12}O_3$
2,6-Octadiene, 1-methoxy-3,7-dimethyl-, (E)-	168	16.8	0.67	$C_{11}H_{20}O$
4-Vinylphenol	120	16.895	2.02	C_8H_8O
5-Hydroxymethylfurfural	126	17.2	0.82	$C_6H_6O_3$
2,6-Octadien-1-ol, 3,7-dimethyl-, formate, (Z)-	182	17.45	1.07	$C_{11}H_{18}O_2$
Geraniol	154	17.675	0.35	$C_{10}H_{18}O$
1,3-Dioxolane, 4,5-diethenyl-2,2-dimethyl-	154	18.825	0.93	$C_9H_{14}O_2$
Phenol, 4-(methoxymethyl)-	138	19.185	0.28	$C_8H_{10}O_2$
2-Methoxy-4-vinylpheno	150	19.35	1.24	$C_9H_{10}O_2$

Neric acid	168	20.06	0.15	$C_{10}H_{16}O_2$
Tricyclo[3.2.2.0]nonane-2-carboxylic acid	166	20.409	1.06	$C_{10}H_{14}O_2$
3,7-Nonadien-2-ol, 4,8-dimethyl-	168	21.09	0.16	$C_{11}H_{20}O$
2-Propenoic acid, 3-phenyl-	148	21.31	1.48	$C_9H_8O_2$
Tricyclo[3.2.2.0]nonane-2-carboxylic acid	166	21.69	1.00	$C_{10}H_{14}O_2$
Neric acid	168	21.82	0.52	$C_{10}H_{16}O_2$
2,3-Dimethyltricyclo[2.2.1.02,6]heptane-3-carb	166	22.03	0.71	$C_{10}H_{14}O_2$
Bicyclo[3.2.2]non-8-en-6-ol, (1R,5-cis,6-cis)	138	22.1	0.16	$C_9H_{14}O$
3-Dimethyltricyclo[2.2.1.02,6]heptane-3-carboxylic acid	166	22.66	0.19	$C_{10}H_{14}O_2$
alpha.-Pinene	136	22.96	0.22	$C_{10}H_{16}$
2-Propenoic acid, 3-phenyl-	148	23.34	3.34	$C_9H_8O_2$
Tricyclo[4.4.0.0(2,8)]decan-3-ol	152	23.715	0.30	$C_{10}H_{16}O$
1,6-Octadiene, 3-ethoxy-3,7-dimethyl-	182	25.59	0.41	$C_{12}H_{22}O$
2,3-Dimethyltricyclo[2.2.1.02,6]heptane-3-carboxylic acid	166	26.151	0.53	$C_{10}H_{14}O_2$
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4	180	26.523	0.18	$C_{11}H_{16}O_2$
1,4-Cyclohexadiene, 3,3,6,6-tetramethyl-	136	27.075	0.35	$C_{10}H_{16}$
Phenol, 4-ethenyl-2,6-dimethoxy-	180	27.648	0.65	$C_{10}H_{12}O_3$
7-Oxabicyclo[4.1.0]heptan-3-ol, 6-(3-hydroxy-1)	226	27.845	0.33	$C_{13}H_{22}O_3$
Megastigmatrienone	190	28.148	0.58	$C_{13}H_{18}O$

2-Cyclohexen-1-one, 4-ethyl-3,4-dimethyl-	152	28.340	0.67	$C_{10}H_{16}O$
3-Cyclohexene-1-methanol, 2-hydroxy-.alpha	170	28.534	0.53	$C_{10}H_{18}O_2$
Longipinane, (E)-	206	28.890	0.18	$C_{15}H_{26}$
3-Hydroxy-.beta.-damascone	208	29.035	0.66	$C_{13}H_{20}O_2$
Megastigmatrienone	190	29.32	0.57	$C_{13}H_{18}O$
2-Cyclohexen-1-one, 4-(3-hydroxy-1-butenyl)-3,5,5-trimethyl-	208	29.765	1.02	$C_{13}H_{20}O_2$
Tridecanoic acid, 2-ethyl-	242	31.3	0.25	$C_{15}H_{30}O_2$
2,5-Dimethylhex-5-en-3-yn-2-ol	124	31.515	0.15	$C_8H_{12}O$
Demethoxyencecalinol	204	31.685	0.87	$C_{13}H_{16}O_2$
Tetradecanoic acid	228	31.965	0.83	$C_{14}H_{28}O_2$
2-Acetoxy-1,1,10-trimethyl-6,9-epidioxydecalin	268	32.12	2.54	$C_{15}H_{24}O_4$
3-Butylindolizidine	181	32.43	0.68	$C_{12}H_{23}N$
Neophytadiene	278	32.995	2.45	$C_{20}H_{38}$
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296	33.32	0.79	$C_{20}H_{40}O$
Neophytadiene	278	33.56	0.93	$C_{20}H_{38}$
3-O-Methyl-d-glucose	194	33.995	0.21	$C_7H_{14}O_6$
Hexadecanoic acid	270	34.125	2.64	$C_{17}H_{34}O_2$
n-Hexadecanoic acid	256	34.675	4.03	$C_{16}H_{32}O_2$
Hexadecanoic acid	284	35.47	0.37	$C_{18}H_{36}O_2$
10-Methylundecan-4-olide	198	36.025	0.16	$C_{12}H_{22}O_2$
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	294	36.52	0.78	$C_{19}H_{34}O_2$
8,11,14-Docosatrienoic acid, methyl ester	348	36.625	1.39	$C_{23}H_{40}O_2$
Phytol	296	36.83	3.29	$C_{20}H_{40}O$
Methyl stearate	298	37.095	0.35	$C_{19}H_{38}O_2$

9,12-Octadecadienoic acid (Z,Z)	280	37.255	0.46	$C_{18}H_{32}O_2$
Dichloroacetic acid, tridec-2-ynyl ester	306	37.375	1.20	$C_{15}H_{24}Cl_2O_2$
Octadecanoic acid	284	37.815	0.50	$C_{18}H_{36}O_2$
Benzyl .beta.-d-glucoside	270	40.575	2.57	$C_{13}H_{18}O_6$
Octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	446	41.615	0.20	$C_{28}H_{46}O_4$
3,7,11,15-Tetramethylhexadecane-1,2,3-triol triacetate / Byrsonic acid	456	41.83	0.15	$C_{26}H_{48}O_6$
Palmitoyl chloride	274	42.625	0.47	$C_{16}H_{31}ClO$
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	330	42.86	1.99	$C_{19}H_{38}O_4$
Methyl 20-methyl-heneicosanoate	354	43.005	0.29	$C_{23}H_{46}O_2$
(E)-3,3'-Dimethoxy-4,4'-dihydroxystilbene	272	44.25	0.36	$C_{16}H_{16}O_4$
Oleoyl chloride	300	44.555	0.84	$C_{18}H_{33}ClO$
Octadecanoic acid, 2,3-dihydroxypropyl ester	358	44.81	0.32	$C_{21}H_{42}O_4$
Tetracosanoic acid, methyl ester	382	44.895	0.17	$C_{25}H_{50}O_2$
Cyclohexane, octadecyl-	336	45.255	0.17	$C_{24}H_{48}$
13-Docosenamide, (Z)-	337	45.475	2.11	$C_{22}H_{43}NO$
(Z)-5-(Pentadec-8-en-1-yl)benzene-1,3-diol	318	45.7	0.23	$C_{21}H_{34}O_2$
1,3-Benzenediol, 5-pentadecyl-	320	45.79	0.17	$C_{21}H_{36}O_2$
Methyl 2-hydroxy-tetracosanoate	398	46.48	0.27	$C_{25}H_{50}O_3$
gamma.-Tocopherol	416	49.345	0.24	$C_{28}H_{48}O_2$
Cholesta-4,6-dien-3-ol, (3.beta.)-	384	49.795	1.44	$C_{27}H_{44}O$
alpha.-Tocopherol-.beta.-D-mannoside	592	50.885	1.21	$C_{35}H_{60}O_7$

Table 3.2: List of compounds identified for methanol extract of *H. excelsa* by GC-MS.

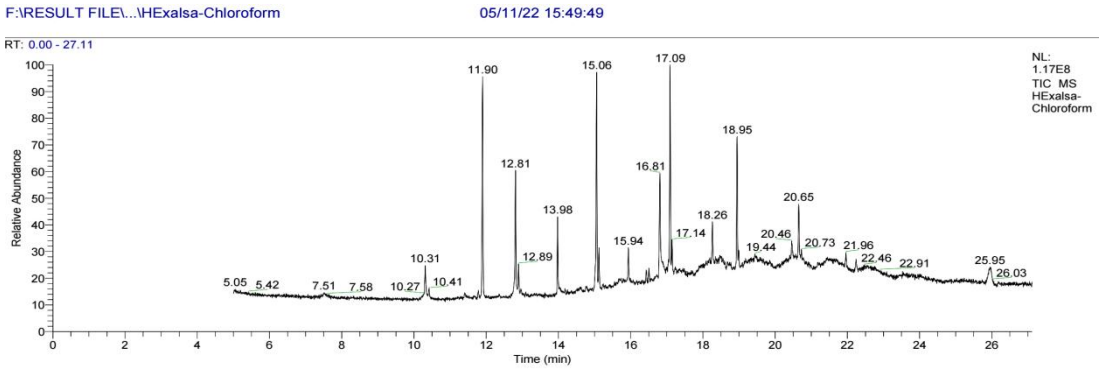

Figure 3.2: A chromatogram of the chloroform extract of *H. excelsa*.

Compound	Molecular weight (g/mol)	Retention Time (Minute)	% Area	Molecular formula
3-Trifluoroacetoxytridecane	296	7.51	1.35	$C_{15}H_{27}F_3O_2$
E-14-Hexadecenal	238	10.31	2.33	$C_{15}H_{27}F_3O_2$
Heptadecane, 2,6-dimethyl-	268	10.41	1.49	$C_{19}H_{40}$
2,4-Di-tert-butylphenol	206	11.9	9.02	$C_{14}H_{22}O$
Hexadecen-1-ol, trans-9-	240	12.81	5.67	$C_{16}H_{32}O$
Tetradecane, 2,6,10-trimethyl-	240	12.89	2.42	$C_{17}H_{36}$
3-Trifluoroacetoxypentadecane	324	12.96	1.67	$C_{17}H_{31}F_3O_2$
Dodecyl acrylate	240	13.98	4.09	$C_{15}H_{28}O_2$
exo-Tetracyclo[5.1.0.0(2,4).0(3,5)]octan-6-ol, 8-bromo-8-chloro-	234	14.78	1.86	C_8H_8BrClO
9-Eicosene, (E)-	280	15.06	9.12	$C_{20}H_{40}$
Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	347	15.94	2.98	$C_{19}H_{25}NO_5$
n-Hexadecanoic acid	256	16.81	5.58	$C_{16}H_{32}O_2$
Heptacos-1-ene	378	17.09	9.30	$C_{27}H_{54}$
Octadecane, 6-methyl-	268	17.14	3.35	$C_{19}H_{40}$
Tetracyclo[5.1.0.02,4.03,5]octan	204	18.19	2.60	$C_9H_{10}Cl_2O$

e, 8,8-dichloro-exo-6-methoxy-				
Phytol	296	18.26	3.81	$C_{20}H_{40}O$
Nonacos-1-ene	406	18.95	6.88	$C_{29}H_{58}$
Tetracyclo[5.1.0.02,4.03,5]octan e, 8,8-dichloro-exo-6-methoxy-	204	20.41	2.79	$C_9H_{10}Cl_2O$
[1,1'-Biphenyl]-2,3'-diol, 3,4',5,6'-tetrakis(1,1- dimethylethyl)-	410	20.46	3.16	$C_{28}H_{42}O_2$
Nonacos-1-ene	406	20.65	4.65	$C_{29}H_{58}$
2,4,6,8,10- Tetradecapentanoic acid, 9a- (acetyloxy)- 1a, 1b, 4,4a, 5,7a, 7b, 8,9a- decahydro- 4a, 7b- dihydroxy- 3- (hydroxymethy l)- 1, 1,6,8- tetramethyl- 5- oxo- 1H- cy clopropa[3,4] benz[1,2- e] azulen- 9yl ester, [1aR- (1aa, 1ba, 4aa, 7aa, 7ba, 8a, 9a, 9aa)]	606	20.73	2.98	$C_{36}H_{46}O_8$
9-(2',2'- Dimethylpropanoilhydrazono)- 3,6-dichloro-2,7-bis-[2- (diethylamino)-ethoxy]fluorene	576	21.96	2.79	$C_{30}H_{42}Cl_2N_4O_3$
Pentadec-7-ene, 7-bromomethyl-	302	22.24	2.98	$C_{16}H_{31}Br$
1,1-Dichloro-2-methyl-3-(4,4- diformyl-1,3-butadien-1- yl)cyclopropane	232	23.52	2.60	$C_{10}H_{10}Cl_2O_2$
1,1-Dichloro-2-methyl-3-(4,4- diformyl-1,3-butadien-1- yl)cyclopropane	232	24.01	1.95	$C_{10}H_{10}Cl_2O_2$
4,4-Difluororetinol (all-trans)		25.95	2.51	$C_{20}H_{28}F_2O$

Table 3.3: List of compounds identified for chloroform extract of *H. exelsa* by GC-MS.

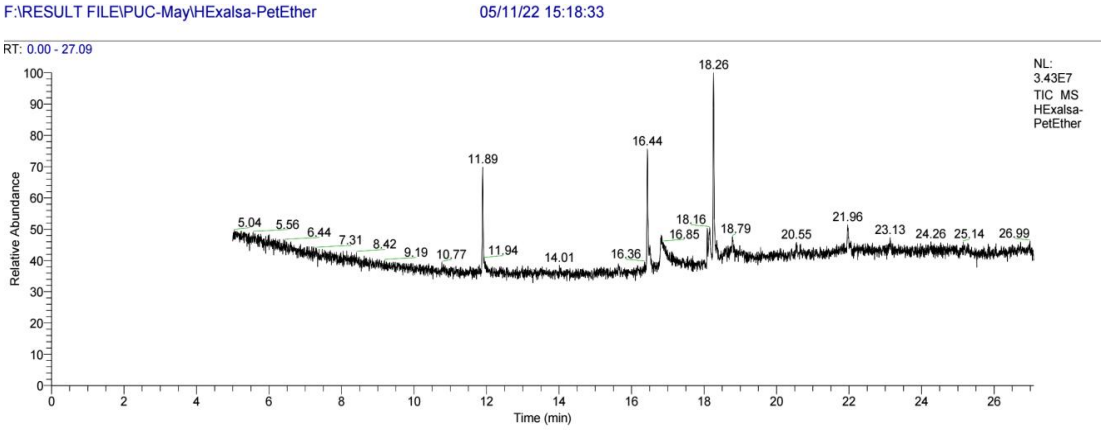

Figure 3.3 : A chromatogram of the petroleum ether extract of *H. excelsa*.

Compound	Molecular weight (g/mol)	Retention Time (Minute)	% Area	Molecular formula
2,4-Di-tert-butylphenol	206	11.89	12.92	$C_{14}H_{22}O$
Pentadecanoic acid, 14-methyl-, methyl ester	270	16.44	14.02	$C_{17}H_{34}O_2$
9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	212	16.85	8.49	$C_{11}H_{16}O_4$
cis,cis,cis-7,10,13-Hexadecatrienal	234	18.16	9.41	$C_{16}H_{26}O$
Phytol	296	18.26	18.45	$C_{20}H_{40}O$
9-Tetradecen-1-ol, acetate, (E)-	254	18.79	9.04	$C_{16}H_{30}O_2$
2-Piperidinone, N-[4-bromo-n-butyl]-	233	18.99	9.23	$C_9H_{16}BrNO$
9-octadecenoic acid, 2,2,2-trifluoroethyl ester	364	20.55	8.86	$C_{20}H_{35}F_3O_2$
Phthalic acid, 5-methylhex-2-yl heptadecyl ester	502	21.96	9.59	$C_{32}H_{54}O_4$

Table 3.4: List of compounds identified for petroleum ether extract of *H. exelsa* by GC-MS.

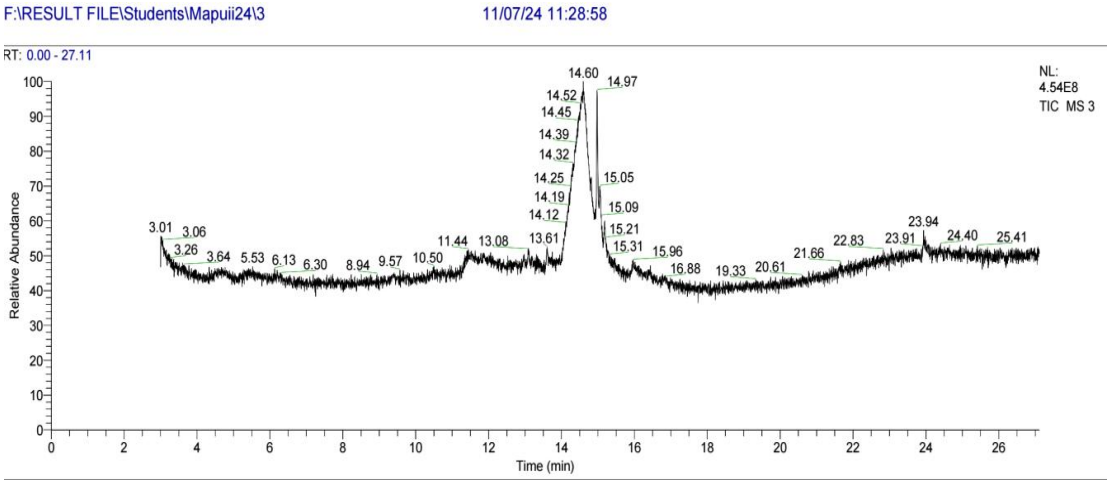

Figure 3.4 : A chromatogram of the aqueous extract of *H. excelsa*.

Compound	Molecular weight (g/mol)	Retention Time (Minute)	% area	Molecular formula
Hydroxyacetic acid, hydrazide	90	3.31	4.36	$C_2H_6N_2O_2$
Hydroxyacetic acid, hydrazide	90	3.61	4.36	$C_2H_6N_2O_2$
d-Glycero-d-ido-heptose	210	11.35	4.36	$C_7H_{14}O_7$
Isosorbide Dinitrate	236	11.41	4.63	$C_6H_8N_2O_8$
Isosorbide Dinitrate	236	11.44	4.63	$C_6H_8N_2O_8$
1-acetoxy-p-menth-3-one	212	13.10	4.63	$C_{12}H_{20}O_3$
[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	322	13.61	4.72	$C_{21}H_{38}O_2$
3-O-Methyl-d-glucose	194	14.32	6.76	$C_7H_{14}O_6$
3-O-Methyl-d-glucose	194	14.60	8.90	$C_7H_{14}O_6$
5-Hydroxy-4,4,6-trimethyl-7-oxabicyclo[4.1.0]heptan-2-one	170	14.97	8.72	$C_9H_{14}O_3$
Octan-2-one, 3,6-dimethyl-	156	15.05	6.23	$C_{10}H_{20}O$
2-Methyl-d-glucose	194	15.18	5.34	$C_7H_{14}O_6$
L-Glucose	180	15.34	4.45	$C_6H_{12}O_6$
Desulphosinigrin	279	15.96	4.27	$C_{10}H_{17}NO_6S$
Silicic acid, diethyl	296	22.83	4.45	$C_{10}H_{28}O_4Si_3$

bis(trimethylsilyl) ester				
Silicic acid, diethyl bis(trimethylsilyl) ester	296	23.04	4.63	$C_{10}H_{28}O_4Si_3$
Thymol, TBDMS derivative	264	23.94	5.16	$C_{16}H_{28}OSi$
Silicic acid, diethyl bis(trimethylsilyl) ester	296	24.40	4.80	$C_{10}H_{28}O_4Si_3$
Silicic acid, diethyl bis(trimethylsilyl) ester	296	27.07	4.63	$C_{10}H_{28}O_4Si_3$

Table 3.5: List of compounds identified for aqueous extract of *H. exelsa* by GC-MS.

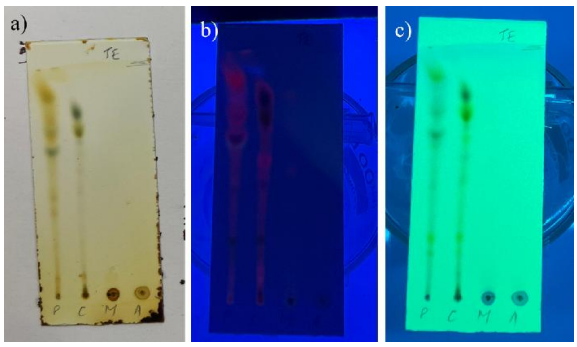

Figure 3.5: Chemical characterization of petroleum ether and chloroform extracts of *Helicia excelsa*. a) TLC plate with toluene and ethyl acetate (8:2) as a mobile phase after iodine chamber visualization; b) TLC profile at 366 nm; c) TLC profile at 254 nm.

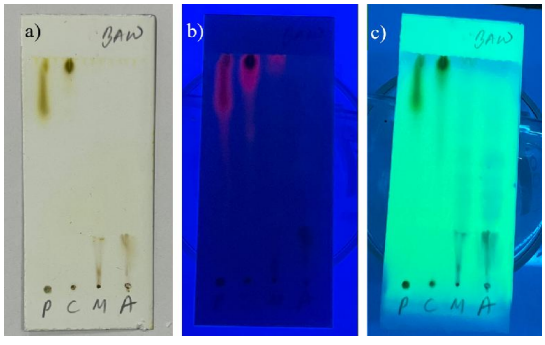

Figure3.6: Chemical characterization of methanol and aqueous extracts of *Helicia excelsa*. a) TLC plate with *n*-butanol, acetic acid and water (4:1:5) as a mobile phase after iodine chamber visualization; b) TLC profile at 366 nm; c) TLC profile at 254 nm.

CHAPTER - 4

DETERMINATION OF ANTIOXIDANT ACTIVITY OF *HELICIA EXCELSA*

INTRODUCTION

Antioxidants are molecules which occur naturally or synthetic that suppress oxidation of other molecules thereby preventing and reducing damages induced by free radicals such as molecules, atoms, or ions with free electrons. These free radicals are very unstable and can easily react with other molecules (Gulcin, 2025). These free radicals, such as reactive oxygen species (ROS), are produced by certain oxidation processes in the human body. When these free radical in association with essential molecules such as DNA, protein and lipids, they modified or inhibit the normal functions of these molecules, resulting to the development of various chronic diseases such as diabetes, cancer, cardiovascular disorders, hypertension, and so on (Chandimali *et al*., 2025). The broadened awareness of harmful effect of oxidative stress on human health captures the attention of many to the discovery of phyto-antioxidants due to the presence of various natural compounds such as alkaloids, tannins, polyphenols, flavonoids, phenols (Lobo *et al*., 2010).

The principal mechanism behind antioxidant activity of phytocompounds is because of their potential to scavenge free radicals, chelate metal ions, and suppress enzyme activity that induce oxidative stress. Various classes of these phytocompounds, also known as secondary metabolites of plants, are indicated to possess the ability to donate high electron which allow them to carry out their property as antioxidant. They are also demonstrated to enhance the endogenous antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase, which thus help in the reduction of oxidative stress (Cao *et al*., 2011). For instance, ascorbic acid, a natural antioxidant, is not present in people who have a vitamin C deficiency disease called scurvy, which develops when collagen synthesis fails to operate properly. In this condition, ascorbic acid plays the most crucial role to sustain the collagen synthesis by maintaining the enzymes responsible for stabilizing

collagen chains such as procollagen-proline dioxygenase (prolyl hydroxylase) and procollagen-lysine dioxygenase (lysyl hydroxylase) activities by reducing iron in the ferrous (Fe^{2+}) state at their active sites as these enzymes used ascorbic acid as their co-factor (Njus *et al*., 2020). While gallic acid, at low doses, acts as antioxidant that benefits human health in cardiovascular, cancers and mitochondrial dysfunctions, however, it functions as pro-oxidant at high doses and induce adverse effect in tissues (Abarikwu et *al*., 2020). Quercetin, a derivative of flavonol, subclass of flavonoid, is well-known for its benefit to human health as it possesses stabilizing effect of ROS, and inhibiting property against inflammatory enzymes such as lipoxygenase (LOX) and cyclooxygenase (COX). Additionally, it is known to sustain cardiovascular health, protect neurological functions, and exhibiting potent anticancer, antihistamine, antidiabetic, and anti-obesity activities (Chiang *et al*., 2023; Homayoonfal *et al.*, 2023).

Meanwhile, synthetic phenolic antioxidant like butylated hydroxytoluene (BHT) is also prevalently used in commercialized pharmaceutical and food industry. When in association with other antioxidant such as butylated hydroxyanisole (BHA), it is effectively used to prevent oils and lipid-rich food products from oxidation (Yehye, 2015). However, these synthetic antioxidants highlighted potential risks of toxicity and carcinogenicity, phyto-antioxidants are generally considered more acceptable and safer for long-term use (Shahidi Ambigaipalan, 2015). Moreover, due to exhibiting strong free radical scavenging properties, phytochemicals have been extensively investigated for their true therapeutic potential (Prior *et al*., 2005).

Among several methods developed for antioxidant activity assay, the assessment of antioxidant from the family Proteaceae was primarily executed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) and Total Phenolic Content (TPC) assays. These techniques are favoured due to their directness, affordability and immediate execution. Various extraction solvents, plant parts, numerous geological locations and protocols have been used to carry out the antioxidant activity for different plants of Proteaceae. For instance, the aqueous ethanol extract of *Oreocallis gandiflora* leaves from Ecuador expressed a radical scavenging activity with IC_{50} value of 6.69 ± 1.39 μg/ml, in the meantime the

absolute ethanol had an IC_{50} of 292.37 ± 9.37 µg/ml (Jhang *et al*., 2023). On the other hand, among proteaceae species studied, *Roupala paulensis* showed TPC with 24.27 ± 0.76 g Gallic Acid Equivalent (GAE)/100 g of the extract, while it has a relatively low DPPH radical scavenging activity compared to the trunk of *Helicia terminalis* exhibited the IC_{50} value of 156.9 mg/ml using the DPPH assay (Vinueza *et al*., 2018; Giang *et al*., 2019). Moreover, some species like *Akea sericea* is also known to surpass common super-fruit namely Kakadu plum in having extremely high content of ellagic acid (Luis *et al*., 2011). Additionally, studies on another species of *Helicia, H. nilagirica* bark from Mizoram, India also showed a potent antioxidant activity by expressing TPC with 62.75 mg GAE/g, total flavonoid content (TFC) with 56 mg quercetin equivalent (QE)/g and IC_{50} of 3.92 and 6.86 µg/ml for DPPH and Hydroxyl radical scavenging, respectively, suggesting strong free-radical scavenging ability (PC *et al*., 2014). Since approximately only 30% of Proteaceae species have been explored for phytochemical profiling, and even fewer species have been investigated in deeper level (Jhang *et al*., 2023), in-depth study is then required to fully understand their antioxidant property, specifically within the less unexplored genus *Helicia.*

MATERIALS AND METHODS

Chemicals and reagents:

Diphenyl-1- picrylhydrazyl (DPPH), quercetin, gallic acid, and ascorbic acid were purchased from SIGMA – ALDRICH, USA. Methanol was obtained from Oxford Lab Fine Chem LLP, Maharashtra, India. Ferric chloride was purchafrom Avantor Performance Materials India Limited, Maharashtra, India. Ammonium molybdate, Trichloroacetic acid (TCA), sodium carbonate, sodium hydroxide were procured from Merck Life Science Private Limited, Mumbai, India. Sodium nitrate, sodium phosphate and aluminum chloride were obtained from HiMedia Laboratories Private Limited, Mumbai, India. Potassium ferricyanide was obtained from LobaChemie Private Limited, Mumbai, India. Folin-ciocalteu's reagent was obtained from SD Finechem Limited, Mumbai, India. Sulfuric acid was obtained from Thermo Fisher scientific India Private Limited, Telangana, India.

Quantitative estimation of antioxidant content of the plant extracts:

The antioxidant activity of the extracts were qualitatively determined by using standard protocols mentioned below:

A) Total phenolic content (TPC):

The total phenolic content of *H. excelsa* extracts (HEM, HEC, HEP and HEC) were executed using slightly modified Folin-Ciocalteu assay based on standard protocol of Singleton & Rossi (1965). A standard solution, gallic acid was prepared in a serial concentrations viz. 10, 20, 40, 60, 80 and 100 µg/ml. The plant sample solutions were also prepared in triplicate at a concentration of 100 µg/ml with their respective extract solvent. To each test tube containing different concentration of the gallic acid and plant extracts, 5 ml of 10-fold diluted Folin-Ciocalteu reagent and 4 ml of 0.7 M sodium carbonate were added. To complete the reaction, the mixtures were then allowed to incubate for 1 hour at room temperature (25-28ºC). After incubation, the absorbance of each solution was taken at 765 nm using a UV-Visible spectrophotometer (LABTRONICS LT-2700). The absorbance values of the gallic acid were then used to construct a standard calibration curve. Therefore, from the graph value of the standard, the total phenolic content of the plant extracts were

determined. The total phenolic content of the extract was expressed as milligrams of gallic acid equivalent per gram of dried extract (mg GAE/g).

B) Total flavonoid content (TFC):

The total flavonoid content of the plant extracts was determined by following the standard protocol of Zhishen *et al*. (1999) with some changes. Quercetin - the standard antioxidant solution was prepared in six different concentrations ranging from 10, 20, 40, 60, 80, and 100 µg/ml and the concentration of the plant samples were prepared at 100 µg/ml and a triplicate was maintained for each sample. Each prepared solution was sequentially mixed with 2 ml distilled water, 3 ml of 5% sodium nitrite solution, 0.3 ml of 10% aluminum chloride solution, and 2 ml of sodium hydroxide and adjusted the final volume to 10 ml with distilled water. After incubating the reaction mixtures at room temperature for 1 hour, the absorbance was measured at 510 nm using a UV-Visible spectrophotometer (LABTRONICS LT-2700). A calibration curve generated using the absorbance values of the standard quercetin at different concentrations, the flavonoid content of the plant samples were calculated. The flavonoid content of the samples was then expressed as milligrams of quercetin equivalent per gram of dried extract (mg QE/g).

C) Total antioxidant capacity (TAC):

The total antioxidant capacity of the extracts was assessed using the phosphomolybdenum method with ascorbic acid as the standard following the standard protocol developed by Prieto *et al*. (1999) with slight modifications. A series of concentrations (10, 20, 40, 60, 80, 100 µg/ml) for the standard and a triplicate of 100 µg/ml for each plant sample were prepared in distilled water. To each test tube, 3 ml of reagent solution - consisting of sulfuric acid, sodium phosphate and ammonium molybdate - was added. The mixtures of the solution were then incubated at 95ºC for 90 minutes. After allowing the mixtures to cool down at room temperature, the absorbance was taken 695 nm using a UV-Vis spectrophotometer (LABTRONICS LT-2700). The absorbance values of different concentrations of ascorbic acid was used to plot the calibration curve and the linear regression of the standard curve was then used to calculate the content of the total

antioxidant of the plant samples. The total antioxidant capacity was represented as milligram of ascorbic acid equivalent (AAE) per gram of the dried extract.

Electron Transfer (ET) - based antioxidant assays:

The antioxidant capacity of the plant extracts to reduce an oxidizing agent through electron donation was assessed by two methods using standard protocols:

A) Ferric Reducing Antioxidant Power (FRAP):

The ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) reduction potential of the plant extracts was determined by using ascorbic acid as a standard reference following a standard protocol of Oyaizu (1986) with minor adjustments. A serial concentrations of the plant samples, as well as the standard were separately prepared as 10, 20, 40, 60, 80, and 100 µg/ ml, while maintaining a triplicate for each concentration. To each test tube, 2.5 ml of 6.6 pH phosphate buffer and 2.5 ml of 1% potassium ferricyanide were added and the mixtures were incubated at 50ºC for 30 minutes. After the reaction was complete, 2.5 ml of 10% trichloroacetic acid (TCA) was added to all the tubes and centrifuged the mixture at 3000 rpm for 10 minutes. 2.5 ml of supernatant was pipetted out from each tube and diluted with 2.5 ml distilled water and then finally added freshly prepared 0.5 ml of 0.1% ferric chloride. A blank solution was also set up by following the same procedure but in the absence of the extracts or ascorbic acid. After 10 minutes incubation, the absorbance of each sample against the blank solution was measured at 700 nm using UV-Vis spectrophotometer (LABTRONICS LT-2700). The absorbance values of different concentrations of the plant samples and the standard were then used to construct the graph.

B) DPPH (2, 2 -diphenyl-1-picrylhydrazyl) free radical-scavenging activity assay:

The radical scavenging activity of the antioxidant in the plant samples was evaluated using DPPH - a stable free radical - that accepts an electron and/or hydrogen atom. This assay was based on Blois (1958) with some changes by using Butylated hydroxytoluene as a reference standard. The volume of different concentrations viz. 10, 20, 40, 60, 80 and 100 µg/ml prepared for the plant extracts and the standard were made to 3 ml with methanol. To each sample prepared and maintained in triplicate, 0.5 ml of 0.1 mM DPPH was added, while the blank solution

had 1 ml of DPPH without samples. After 30 minutes incubation at 37ºC, the absorbance was taken at 517 nm. The absorbance value was then converted into percentage inhibition activity by the following formula:

$(A_C - A_S) / A_C \times 100$

Where; A_C = Absorbance of control, A_S = Absorbance of sample

The activity of DPPH scavenging activity was represented as IC_{50} value which was calculated by using GraphPad Prism version 8.0.2 from the non-linear regression (curve fit) value.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of the mean (SEM) from three independent replicates (n = 3). GraphPad Prism software version 8.0.2 was used to perform ordinary One-way analysis of variance (ANOVA) for IC_{50} value of the plant extracts and two-way ANOVA for reducing capability of the plant extracts by following Tukey's multiple comparison test to assess significant differences between treatment groups. A p value <0.05 was considered statistically significant. The levels of statistical significance were denoted using asterisks as follows:

ns: not significant ($p > 0.05$), *: $p \leq 0.0332$, **: $p \leq 0.0021$, ***: $p \leq 0.0002$, ****: $p < 0.0001$.

RESULTS

Total Phenolic Content (TPC) of the plant extracts:

The absorbance value of a serial concentration of Gallic acid at 765 nm was shown in **Figure 4.1**. The total phenolic content of the extracts of *H. excelsa* calculated from the standard graph values were found to be 11.768 ± 0.078, 4.516 ± 0.085, 2.392 ± 0.068 and 11.711 ± 0.346 GAE mg/g of methanol, chloroform, petroleum ether and aqueous extracts of the plant, respectively.

Total flavonoid content (TFC) of the plant extracts:

The flavonoid content of the *H. excelsa* was calculated from the standard graph value plotted for the standard Quercetin as shown in **Figure 4.2**. The value of flavonoid for methanol, chloroform, petroleum ether and aqueous extracts of the plant, respectively, were found to be 69.48 ± 5.80, 64.27 ± 1.04, 47.6 ± 2.76 and 60.00 ± 7.64 QE mg/g, respectively.

Total antioxidant capacity (TAC) of the plant extracts:

The total antioxidant of the extracts of *H. excelsa* leaves was calculated from the linear graph value of the calibration curve prepared for the standard ascorbic acid as shown in **Figure 4.3**. The value was then evaluated as 100.901 ± 2.823, 11.395 ± 0.445, 8.802 ± 0.123, 26.364 ± 0.360 AAE mg/g of methanol, chloroform, petroleum ether and aqueous extracts of the plant, respectively.

Ferric reducing antioxidant power (FRAP) activity of the extracts of *H. excelsa*:

The reduction of Fe^{3+} to Fe^{2+} by the four extracts methanol, chloroform, petroleum ether and aqueous extracts of the plant was assessed by measuring the absorbance given by different concentration viz. 10, 20, 40, 60, 80 and 100 µg/ml at 700 nm using ascorbic acid as reference standard. The plant extracts expressing concentration dependent reducing activity was shown in **Figure 4.4**. Among the extracts, methanol extract showed highest reducing activity at all concentrations which is almost equivalent to the standard ascorbic acid, followed by aqueous extract which shows slightly lower activity as compared with methanol extract. The

comparatively lower activity was possessed by chloroform extract, and petroleum ether extracts was found to have the least activity.

Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the four extracts of *H. excelsa*:

The antioxidant property of *H. excelsa* was assessed using DPPH radical scavenging activity. The extracts, as well as the standard ascorbic acid were prepared in a serial concentrations of 10, 20, 40, 60, 80 and 100 µg/ml, in which the scavenging activity was obtained to increase with an increase in concentration **(Figure 4.5)**. The percentage scavenging activity of the extracts was plotted against log-doses to calculate the IC_{50}. Of all the extracts, methanol extract showed the highest antioxidant activity by giving IC_{50} of 5.677 ± 0.363, which does not show statistical significance at $p>0.05$ when comparing with BHT (5.677±0.363). However, the other extracts chloroform, petroleum ether and aqueous extracts were found to be statistically significant from each other, including BHT and methanol extract as shown in **Figure 4.6**.

DISCUSSION

Synthetic antioxidants such as BHT, BHA, Pert-butylhydroquinone (TBHQ), ethoxyquin and propyl gallate were once used worldwide as preservatives across foods, feeds, cosmetics and industrial products due to their effectiveness and stability (Xiao *et al*., 2024, Xu *et al.*, 2021). However, scientific evidence accumulated their genotoxic, cytotoxic and carcinogenic effects that led to the rise in public health concerns and regulatory measures. Meanwhile, studies claimed that high-dose exposure to BHA, BHT, TBHQ and propyl gallate can lead to DNA damage, oxidative stress and organotoxicity. Moreover, TBHQ has been associated with stomach tumors in rodents model which possibly cause endocrine disruption (Esazadeh *et al*., 2024). As a result, these antioxidants are either banned or limited their use in some countries that encourages many industries to shift to natural alternatives from plants and herbs like rosemary extracts, tocopherols and acerola which will provide the customers safety trends and minimal processed with simple and transparent ingredients.

Medicinal plants are the rich reservoir of secondary metabolites that exhibit strong antioxidant activity. These antioxidants are complex chemical groups such as phenols, flavonoids, carbohydrates, and several more. For instance, *Phyllanthus emblica (*Indian gooseberry or amla) fruit, one of the richest nutritional fruits, is recognized for its high content of phenolic and flavonoid with enormously strong free-radical scavenging activity in DPPH (86.69% inhibition) and FRAP assays, and is also rich in p- coumaric, chlorogenic, and ferulic acids which make them excellent candidate for neutraceutical product development (Wanyo *et al*., 2024). Interestingly, in this study, it was observed that methanol achieved inhibition activity of 97.22 ± 0.69% at the highest concentration i.e. 100 µg/ml that outstandingly surpasses both *P. emblica* and even the standard BHT (96.54±0.22). This conspicuous efficacy remarks the potent antioxidant activity of *H. excelsa* compared to not only the amla, but also other high potent antioxidant plants such as *Monodora myristica* (Moukette *et al*., 2015), *Lepisanthes alata* (Anggraini *et al*., 2019) and *Elsholtzia densa* (Khan *et al*., 2012).

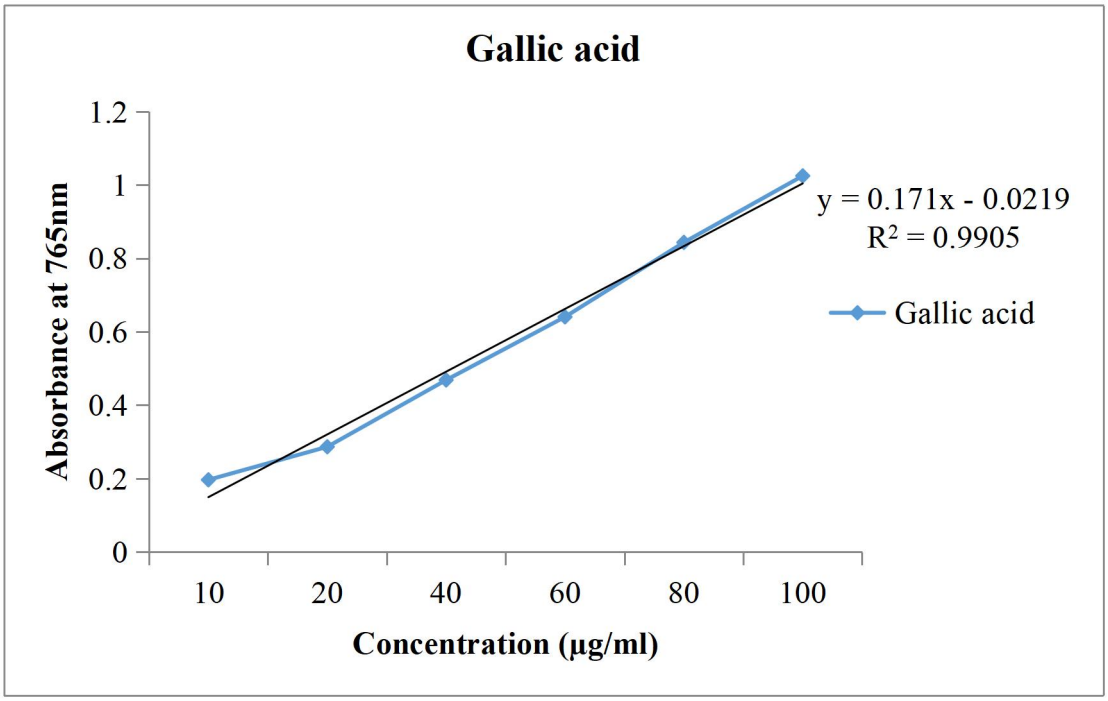

Figure 4.1: Standard calibration curve of gallic acid for total phenol assay. Black line represents the linear graph.

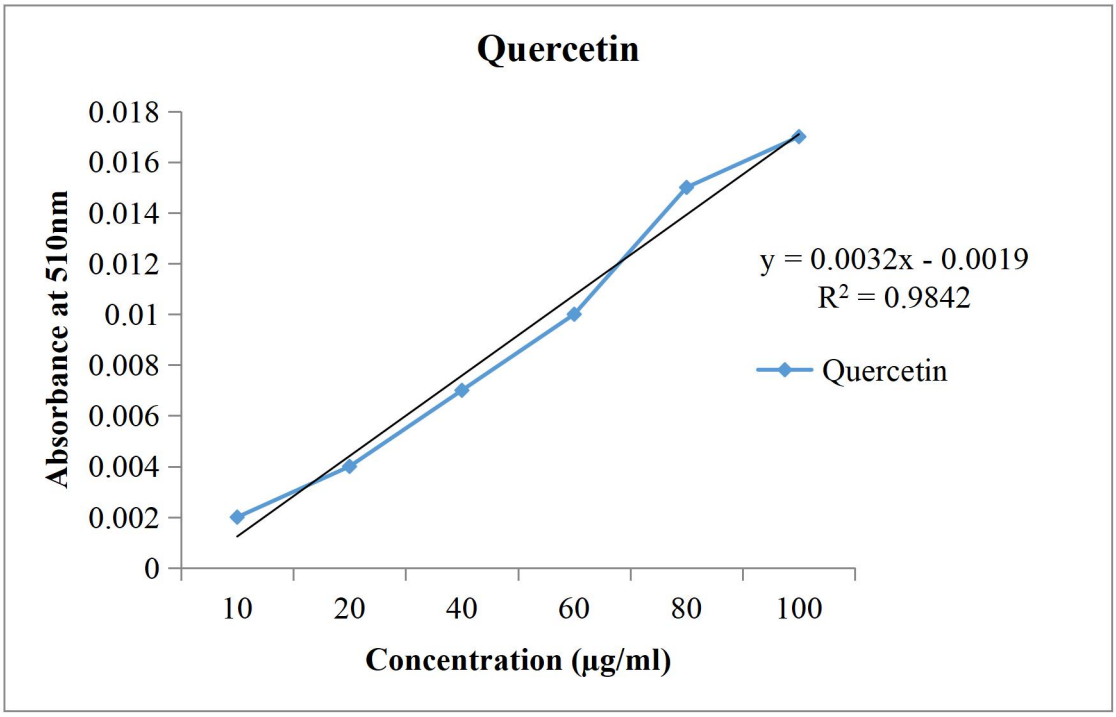

Figure 4.2: Standard calibration curve of quercetin for total flavonoid assay. Linear black line represents the linear graph.

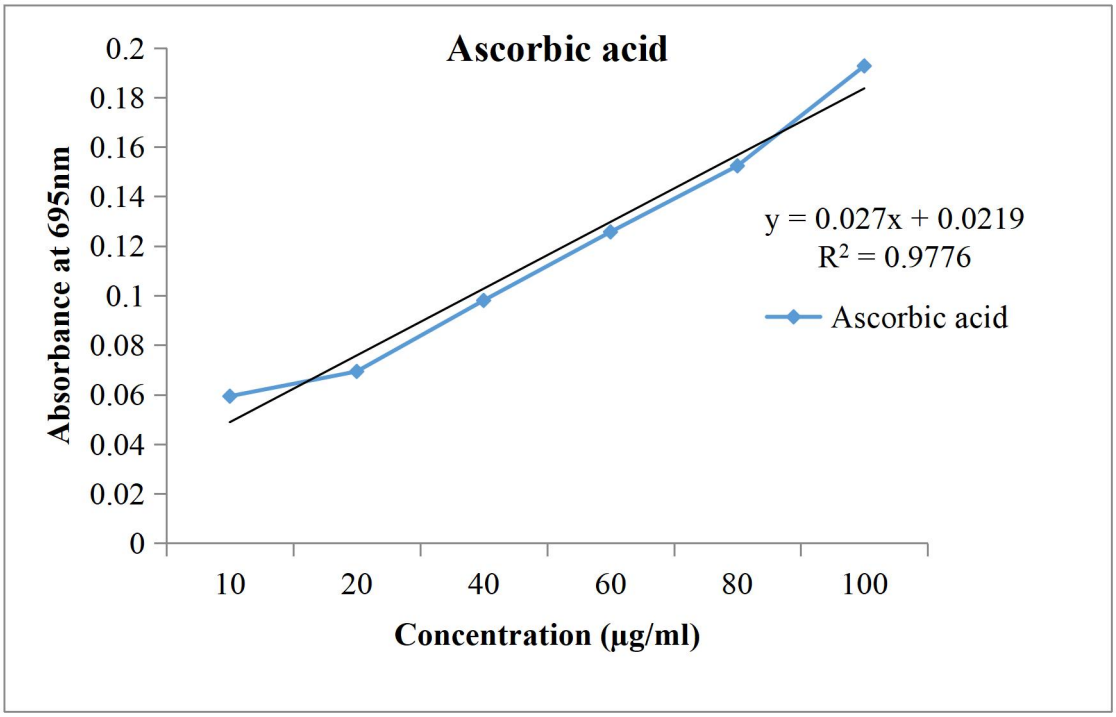

Figure 4.3: Standard calibration curve of ascorbic acid for total antioxidant assay. Linear black line represents the linear graph.

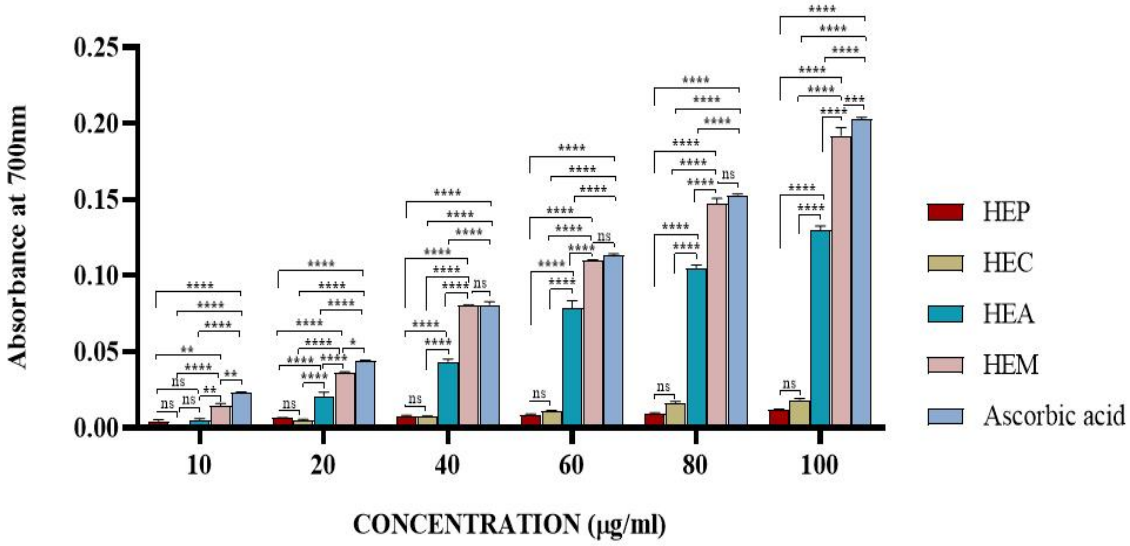

Figure 4.4: Potassium ferri-cyanide-reducing activity of ascorbic acid and extracts of *H. excelsa* (HEP, HEC, HEM and HEA). Different asterisks indicate statistical significant differences at different levels of significance ($p<0.05$).

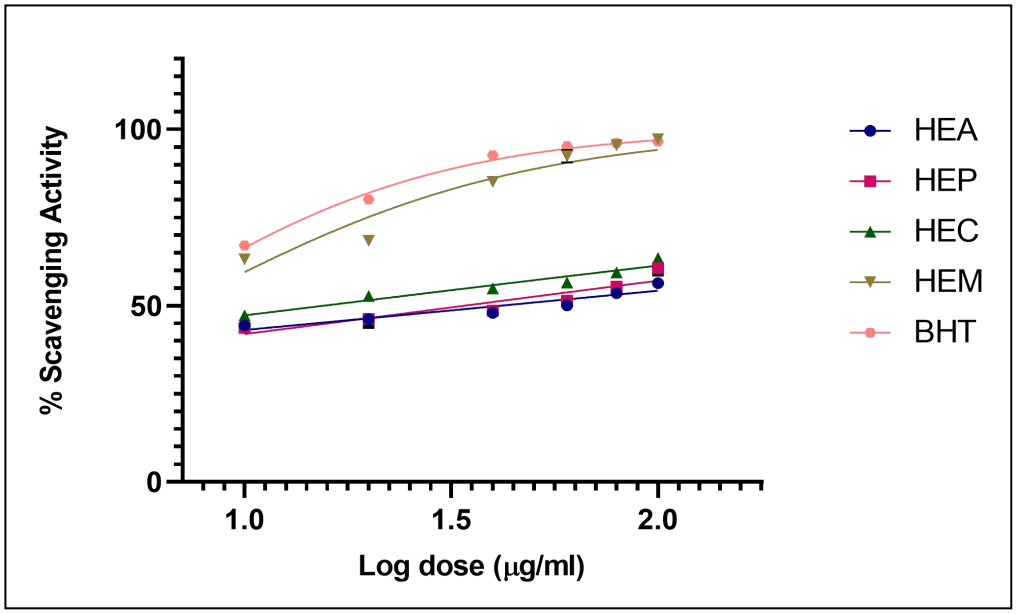

Figure 4.5: A graph showing the percentage DPPH scavenging activity of butylated hydroxytoluene (BHT) and extracts of *H. excelsa* (HEM, HEC, HEP, HEA).

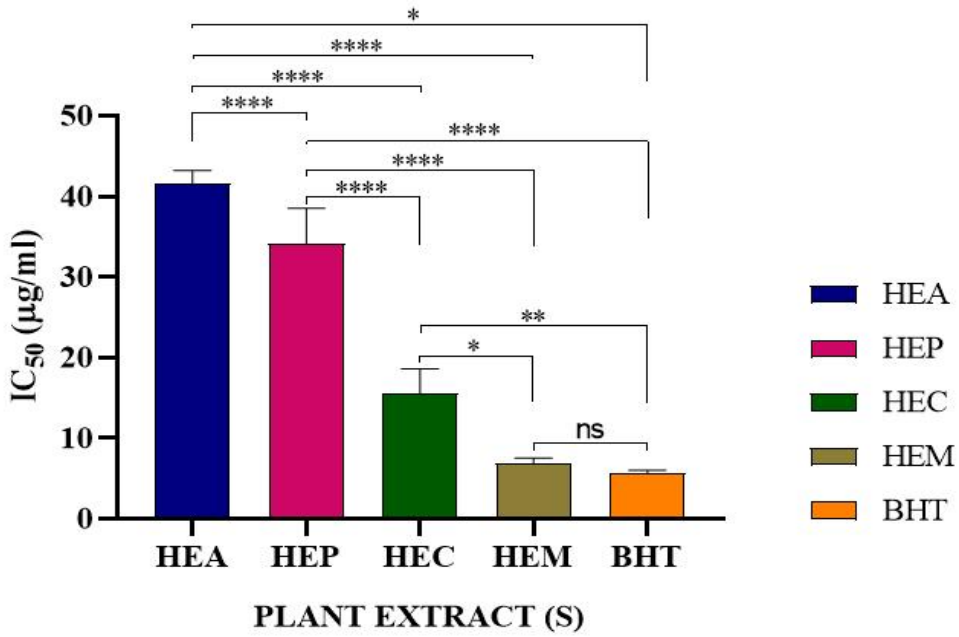

Figure 4.6: IC_{50} of DPPH free radical scavenging activity of butylated hydroxytoluene (BHT) and extracts of *H. excelsa* (HEM, HEC, HEP, HEA). Different asterisks indicate statistical significant differences ($p<0.05$).

CHAPTER - 5

ASSESSMENT OF ANTIMICROBIAL ACTIVITY OF *HELICIA EXCELSA*

INTRODUCTION

The discovery of antimicrobial agents is one of the greatest milestones in conventional medicine, and its wide application extensively decreases mortality and disease rates caused by infectious diseases. Antimicrobial drugs such as antifungal, antimicrobial, antiviral, and antiparasitic have helped revolutionizing modern healthcare systems and its significant contribution to extending human lifespan is highly remarkable (Ventola, 2015). However, overuse and misapplication of these drugs had accelerated the evolution and worldwide prevalence of antimicrobial resistance (AMR), which occurs when antibiotics loss its ability to kill the target microorganisms such as bacteria and fungi, causing an escalating threat to public health that may nullify years of accomplishments in healthcare industries.

WHO announced AMR as one of the top ten global health threats. If present patterns still continued, it is estimated that by 2025, complications due to AMR could cause 10 million deaths annually (Kariuki, 2024). Apart from resistance, the application of regular antibiotic drugs are found to be associated with various adverse effects including allergies, cardiovascular problems, gastrointestinal disorders, and many others that can give rise to disrupting the immune system and overall health (Sanchez *et al*., 2016). In addition to that, continued dependency on antibiotics can further lead to secondary infections and development of resistant strains, which are substantially more complicated than the original infection, and sometimes, impossible to treat. Such cases, which are classified under a serious threat, have been reported in Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), Methicillin-resistant *Staphylococcus aureus* (MRSA), Multidrug-resistant *Pseudomonas aeruginosa* and *Clostridium difficile*-associated diarrhea (Redfield, 2019; Leffler & Lamont, 2015). As per India TB report 2021, within Mizoram itself,

out of 1749 TB cases, 6.4% were turned out to be infected by MDR-TB (Sailo & Kumar, 2025).

The fungal strains of *Fusarium* species are the soil fungi which casually caused keratomycosis, that can lead to blindness, and skin infections. The most prevalent causal strain among them is *F. solani*, followed by *F. oxysporum* that adapted resistance to most of the azole antifungal classes while exhibiting high minimum inhibitory concentrations (Tortorano *et al*., 2008). Plant pathogenic fungi like *Pyricularia oryzae,* a causal agent of rice and wheat blast diseases also adversely affects global rice production by exhibiting resistance to a group of the site-specific agricultural fungicides such as Quinone-outside Inhibitors (QoI), sterol 14α-demethylation inhibitors (DMI), which are mainly associated with target site alteration and mutation (Vicentini *et al*., 2022). Fluconazole, however, is still considered ideal for standard antifungal agent for both *in vivo* and *in vitro* research due to its high solubility in water, bioavailability, low toxicity, low protein binding, broad fungal spectrum and low resistance rate (Rex *et al*., 2001; Anand *et al*., 2022; Eksi, 2013). On the other hand, bacterial strains like *Bacillus subtilis, Klebsiella pneumoniae, Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium* which are responsible for causing various diseases including healthcare-associated infections, pneumonia, urinary tract infections, food poisoning, skin, gastroenteritis and many others showing high to medium resistance to broad bacterial spectrum like cephalosporins, azithromycin, carbapenem, clindamycin, chloramphenicol, penicillin and ampcillin depending on the region. In the meantime, ciprofloxacin is still favoured in experimental settings due to having high potent and rapid bactericidal action with MIC ranging from 0.015-1 μg/mL against pathogenic gram positive and gram negative bacteria, excellent *in vivo* efficacy, long half life and low protein binding when comparing with other antibacterial classes such as norfloxacin, gentamicin, cefotaxime (Yassin and Ahmad, 2012; Gach *et al*., 2024; You and Shi, 2024; Veeraraghavan and Walia, 2019; Sohani *et al*., 2025).

Although these antibiotics, particularly ciprofloxacin, which has always been opted for primary treatment for life threatening infections caused by bacteria, initially revealed strong inhibition compared to other drug classes, it still poses a resistant threat to many regions around the world. For instance, as reported from developing countries like Iran and Bangladesh, *Escherichia coli* isolates are now resistant to ciprofloxacin at rapid rates of ~65 and ~89%, respectively. The same pattern of resistance has also been reported in *Salmonella spp.* and *Pseudomonas aeruginosa* from different parts of the world (Neyestani *et al*., 2023; Rehman *et al*., 2019; Chang *et al*., 2021). Meanwhile, fluconazole resistance has also been rising among *Candida* spp., especially a vaginal *C. albicans* isolates which showed resistance rising from 22.8 to 35.7% over a period of 10 years (Sobel, 2023). Simultaneously, *Cryptococcus* spp. also showed resistance to the same antifungal drug at a speed of 12.1 to 24.1% during 1988–2017 (Bongomin *et al*., 2018).

Due to this alarming antimicrobial resistance exacerbates, the search for antimicrobial alternatives from medicinal plants have emerged as they are often regarded as safer, more effective and sustainable than the conventional antimicrobial agents. Interestingly, medicinal plants are a rich reservoir of secondary metabolites such as alkaloids, tannins, flavonoids, terpenoids, and many others which contribute to their significant antimicrobial properties that serve them as a promising candidates for antimicrobial drug development. Their potential is further validated by their long history of application in traditional medicine and by demonstrating effective inhibitory activity against wide range of pathogenic bacteria and fungi, as well as drug-resistant strains (Cowan, 1999). Fortunately, the use of medicinal plants remains a fundamental component of primary healthcare in regions where modern medical facilities are yet limited. With regard to that, Mizoram, one of the northeastern states of India is a region where ethnomedicinal knowledge has been preserved across generations and continues to be actively practiced. Several studies on plants native to this region have reported to exhibit outstanding antimicrobial efficacy, highlighting their true potential as alternative antimicrobial agent (Lalfakzuala *et al*., 2007).

Therefore, this thesis also aims to explore the antimicrobial efficacy of selected medicinal plant, *H. excelsa,* traditionally used in Mizoram and assesses their effectiveness through *in vitro* assays. Moreover, this study would hopefully contribute to the global search for novel, plant-derived antimicrobial agents while outlining and safeguarding the valuable ethnomedicinal tradition of Mizoram.

MATERIALS AND METHODS

Chemicals:

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Chemicals Private Limited, Bangalore, India. Potato dextrose agar, nutrient agar, nutrient broth, ciprofloxacin and fluconazole were obtained from HiMedia Laboratories Private Limited, Mumbai, Maharashtra, India.

Microorganisms used:

Bacillus cereus (ATCC 13061)*, Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 10145), *Baccilus subtilis* (ATCC 11774), *Staphylococcus aureus* (ATCC 11632), *Staphylococcus aureus* (ATCC 700698), *Salmonella typhimurium* (ATCC 51812), *Micrococcus luteus* (ATCC 10240), and *Fusarium keratoplasticum* (ATCC 36031) were obtained from Microbiologics, Saint Cloud, Minnesota, USA. *Fusarium solani* (MTCC 350) and *Pyricularia oryzae* (MTCC 1477) from Institute of Microbial Technology (IMTECH), Chandigrah, India, while *Fusarium oxysporum* (MK209108) was locally isolated from ginger soft rot tissue and identified at Research and Instumentation Centre, Pachhunga University College and submitted to GenBank.

Assessment of Antibacterial activity of *H. excelsa* by well diffusion method:

The *in vitro* antibacterial activity of *H. excelsa* extracts (HEM, HEC, HEP and HEA) was carried out using well diffusion method following the protocol developed by Devillers et al. (1989) with slight modifications. The bacterial strains used were *Bacillus cereus, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Baccilus subtilis*, *Staphylococcus aureus*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Micrococcus luteus* representing gram positive and gram negative bacteria. Each extract was diluted to a series of concentrations viz. 500, 250, 125 and 62.5 mg/ml. Ciprofloxacin served as the standard drug, and dimethyl sulfoxide (DMSO) was used as the control. Each agar plate was carefully inoculated by evenly spreading the test bacterial strain using L-spreader. Using a sterile 6 mm cork borer, six wells were aseptically made in each plate which were designed for four concentrations of the plant extract, a standard antimicrobial agent

and a control solution. Afterwards, 50 µl of the extract solutions, 2 µl of the standard and 5 µl of the control were impregnated into their respective wells. The plates were then incubated at 28 ± 2 °C for 24 hours.

After incubation, the diameter of the inhibition zones was measured using a dial caliper. The final inhibition zone for each extract was calculated by subtracting the diameter of the well i.e. 6 mm from the total measured diameter. Results were represented as millimeter (mm) as an indication of antibacterial potency.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *H. excelsa*:

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the *H. excelsa* leaf extracts were assessed using a resazurin-based ten-fold dilution assay in a 96-well microtiter plate, following the standard method of Elshikh et al. (2016) with some changes. 150 µl of a stock solution of the extracts i.e. 62.5 mg/ml for HEM and HEC, 250 mg/ml for HEA was loaded to the first well of each column, followed by 150 µl of nutrient broth media containing resazurin dye. A ten-fold serial microdilution was achieved across the wells up to the 10^{th}. As each row of the plate corresponded to a different bacterial strain, a 6 µl of the bacterial inoculum was added to each well, while 11^{th} and 12^{th} were reserved for positive control (bacteria without extract) and negative control (media only), respectively. The plate was incubated at 37 ± 2°C for 24 hours. Subsequently, the wells that did not show any visible color change of resazurin dye from blue (no growth) to pink (bacterial growth) were carefully observed. The lowest concentration attaining no color change was then recorded as MIC.

To evaluate the MBC, the wells showing no color change (no bacterial growth) up to some showing bacterial growth (wells showing pink color) were further streaked onto nutrient agar plates by using inoculating loop. Plates were then incubated under the same condition. The lowest concentration of the extract that devoid of any visible bacterial growth on the agar surface was then recorded as MBC.

Determination of anti-fungal activity of *H. excelsa*:

The antifungal potential of *H. excelsa* extracts (HEM, HEC, HEP and HEA) against four pathogenic fungal strains, namely *Fusarium keratoplasticum*, *Fusarium oxysporum*, *Fusarium solani* and *Pyricularia oryzae*, was determined using the modified poisoned-food technique based on the standard protocol (Mohana and Raveesha, 2007). Plant extracts prepared in a concentration series of 1%, 0.5%, 0.25%, and 0.125% (w/v) were mixed with 20 ml molten, sterilized potato dextrose agar (PDA). The mixtures were directly poured in to sterile plates and allowed them to cool down for about 30 minutes. Plates containing 0.01% fluconazole (v/v) served as the standard control, and plates having only PDA was maintained as the negative control. Mycelial plugs (~6 mm in diameter) from actively growing margins of 5-7 days old fungal cultures were aseptically inoculated at the center of each treatment plate. The plates were incubated continuously at 28 ± 2 °C and the radial expansion of fungal colonies was measured for seven days on a daily basis using a dial caliper.

The percentage inhibition of fungal growth resulting from treatment was calculated using the formula (Singh & Tripathi, 1999):

% inhibition = dc – dt/dc x 100

Where dc = Average mycelial growth in control,

dt = Average mycelial growth in treatment.

STATISTICAL ANALYSIS

All experimental data were expressed as the mean ± standard error of the mean (SEM) from three independent replicates (n=3). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to assess significant differences between treatment groups. Analyses were conducted using GraphPad Prism software version 8.0.1. A p-value of ≤ 0.05 was considered statistically significant. The levels of statistical significance were denoted using asterisks as follows:

ns: not significant ($p > 0.05$), *: $p \leq 0.0332$, **: $p \leq 0.0021$, ***: $p \leq 0.0002$, ****: $p < 0.0001$.

RESULTS

Assessment of antimicrobial activity of *H. excelsa*:

The antibacterial activity of the four different extracts of *H. excelsa* (HEM, HEC, HEP and HEA) prepared in four different concentrations viz. 500, 250, 125 and 62.5 mg/ml was performed on eight bacterial strains namely *Bacillus cereus, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Baccilus subtilis*, *Staphylococcus aureus*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Micrococcus luteus*. The methanol extract exhibited concentration-dependent antibacterial activity against all the tested bacterial strains, with the highest zone of inhibition observed against *B. cereus* was displayed in **Figure 5.1** and **Figure 5.2**. The chloroform extract also showed dose-dependent inhibition activity for all the concentrations against all bacterial strains, except *S. typhimurium* which did not response to the concentration-based treatment and no activity against *M. luteus* (**Figure 5.3** and **Figure 5.4**). The aqueous extract expressed its activity against *B. cereus, P. aeruginosa* and *M. luteus* at all concentrations, however, *B. subtilis* was inhibited only up to 250 mg/ml concentration, while the lowest concentration (62.5 mg/ml) was found ineffective against *K. pneumoniae*, *S. aureus* 1, *S. auresus* 2 and *S. typhimurium* (**Figure 5.5** and **Figure 5.6**). Meanwhile, the petroleum extract did not exhibit bacterial growth inhibition against any of the tested strains (**Figure 5.7**).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *H. excelsa*:

MIC performed for HEM, HEC and HEA by resazurin-based ten-fold dilution assay in a 96-well plate were shown in **Figures 5.8**, **5.10**, and **5.12**, respectively, and the MBC by streak plate method on nutrient agar were displayed in **Figures 5.9**, **5.11**, and **5.12**. The concentration of MIC and MBC of the plant extracts against the tested microorganisms were presented in **Tables 5.1, 5.2,** and **5.3**. The extracts exhibited notable inhibitory activity against each bacterial strain. The methanol extract exhibited its lowest MIC at 1.95 mg/ml against *B. cereus* and *S. aureus* 1 with MBC at 62.5 and 3.91 mg/ml, respectively, followed by 3.91 mg/ml against *S. typhimurium* and *M. luteus* with 7.81 and 15.63 mg/ml MBC, respectively. MIC at 7.813 mg/ml

against *B. subtilis, K. pneumoniae* and, *P. aeruginosa* were having MBC at 15.63, 7.813, and 15.63 mg/ml, respectively, and the highest MIC was observed against *S. aureus* 2 at 15.63 mg/ml with MBC having the same concentration. The chloroform extract had its lowest MIC (0.98 mg/ml) against *B. cereus* with MBC at 31.25 mg/ml. MIC at 1.95 mg/ml was demonstrated against *B. subtilis*, *K. pneumoniae*, and *S. typhimurium* with MBC at 3.91, 7.81, and 3.91 mg/ml, respectively. The lowest MIC in aqueous extract was expressed at 7.81 mg/ml against *B. subtilis*, *K. pneumoniae*, *B. cereus*, and *S. typhimurium* while showing their MBC at 15.63, 31.25, 15.63, and 31.25 mg/ml, respectively. MIC of 15.63 mg/ml was expressed against *S. aureus* 1, *S. aureus* 2, and *P. aeruginosa* with MBC at 15.63 mg/ml in both *Staphylococcus* strains, and 31.25 mg/ml in *P. aeruginosa.* The highest MIC and MBC both at 125 mg/ml of aqueous extract of the plant was also observed against *M. luteus*. However, since the petroleum extract of the plant did not show activity using well-diffusion method, the MIC and MBC were not performed.

Antifungal activity:

The antifungal activity of *H. excelsa* extracts (HEM, HEC, HEP and HEA) prepared in four different concentrations viz. 1, 0.5, 0.25, and 0.125% was assessed based on poisoned food method. Upon the treatment of methanol extract, dose-dependant activity against all the tested fungal strains was shown in **Figure 5.14** and **Figure 5.15**. Among the fungal strains, across all the concentrations, the highest mycelial growth inhibition was exhibited against *P. oryzae*, in which, even the lowest dose (0.125%) surpassed the inhibition activity of the standard drug with highly significant difference between them. The second highest antifungal activity was observed in the fungal strain of *F. oxysporum*, followed by *F. solani*, and the lowest inhibition activity of the extract was observed against *F. keratoplasticum* with as low as 3.19±0.51%. The chloroform extract showed its antifungal property with dose-dependant activity against the tested fungal strains, except *F. oxysporum*, which revealed a very similar activity for 0.5 and 0.25% with no statistical difference between them (**Figure 5.16**, **Figure 5.17**). The highest inhibition activity was observed on the highest concentration against *F. oxysporum*, followed by upon the same dose of treatment against *F. keratoplasticum*. Meanwhile, the lowest activity

was expressed at 0.125% against *F. oxysporum* with the growth inhibition percentage of 16.95±0.45%. Upon the treatment of petroleum ether extract, concentration dependent activity against all the pathogenic fungal strains was demonstrated in the **Figure 5.18** and **Figure 5.19**. The highest antifungal activity over the entire concentration range was indicated against *F. keratoplasticum* while the comparable inhibition activity was expressed against fungal strains of *F. oxysporum* and *P. oryzae*. The lowest activity was observed against *F. solani*. The aqueous extract also exhibited dose-dependant activity against all the tested fungal strains was seen in the **Figure 5.20** and **Figure 5.21**. Among the fungal mycelia, *P. oryzae* was found to be the most susceptible upon the treatment through all concentrations. The mycelium growth inhibition percentage against the other fungal strains was in the order of *F. oxysporum>F. solani>F. keratoplasticum*, with no significant variation, at the same doses, in between *F. keratoplasticum* and *F. solani*.

DISCUSSION

Since the discovery of antibiotics like penicillin and streptomycin, it has revolutionized medical system in the 20th century as these drugs reduced the rate of fatal diseases such as pneumonia, tuberculosis and sepsis (Aminov, 2010). Antifungal drug like fluconazole has also achieved a remarkable transformation in the history of the management of a disease like mycoses (Perfect, 2017). However, the overuse and misuse in humans and agricultural practices of these drugs that has been significantly depleting their effectiveness over the years, which finally leads to one among the top ten global health threats as they develop resistance in diseases that were previously treatable by these drugs. Hence, the urgent search for the alternative antimicrobial drugs from medicinal plants have been prioritized by many researchers across the world. The bioactive secondary metabolites are known to help plants against microbial infections and other external agents, accordingly, the compounds produce by plants are therefore extensively used in pharmaceutical companies as they exhibit strong antimicrobial activity (Cowan, 1999; Harvey *et al*., 2015).

Among several other plants exhibiting enormous antimicrobial activities, plants from proteaceae family also indicating its antimicrobial efficacy against some pathogenic microorganisms. For instance, *Macadamia integrifolia* fruit exhibited its MIC at as low as 5.3 µg/ml against *E. coli, Roupala brasiliensis* stem at ~15.6 µg/ml against *S. aureus, Grevillea avellana* aerial at ~64 µg/ml against *P. aeruginosa.* These activities are linked to the compounds possessed by the plants across proteaceae such as Bis-5-alkylresorcinols, alkaloids and phenols - which are known for their contribution to antimicrobial activity of the plants (Zhang *et al*., 2023; Gadea *et al*., 2022). Moreover, *Hakea salicifolia* and *H. cericeae* expressed their inhibition efficacy against resistant strains such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Staphylococcus aureus* (VRSA) at the concentration ranging from 7.5 - 62 µg/ml, while *Protea caffra* and *Lomatia hirsuta* successfully inhibited the growth of *E. faecalis* and *C. albicans*, respectively (Madureira, 2012; Zhang *et al*., 2023). Additionally, a phytochemical profiling of *H. nilagirica* from Mizoram verified the presence of good amount of flavonoid and phenols, which suggest a strong possibility of such activity even though direct antimicrobial activity

studies are not found (Zoremsiami & Jagetia, 2018; PC *et al*., 2014). Moreover, although the antibacterial effect of the plants from Proteaceae family has been explored in few pathogenic bacteria, the investigation on antifungal activity remains lacking. Therefore, in order to leave a mark for future research on the entire genus of *Helicia*, this study includes a thorough analysis of the antifungal properties of the selected plant, which represents the first evaluation of antifungal agents from the whole genus against some pathogenic fungal strains.

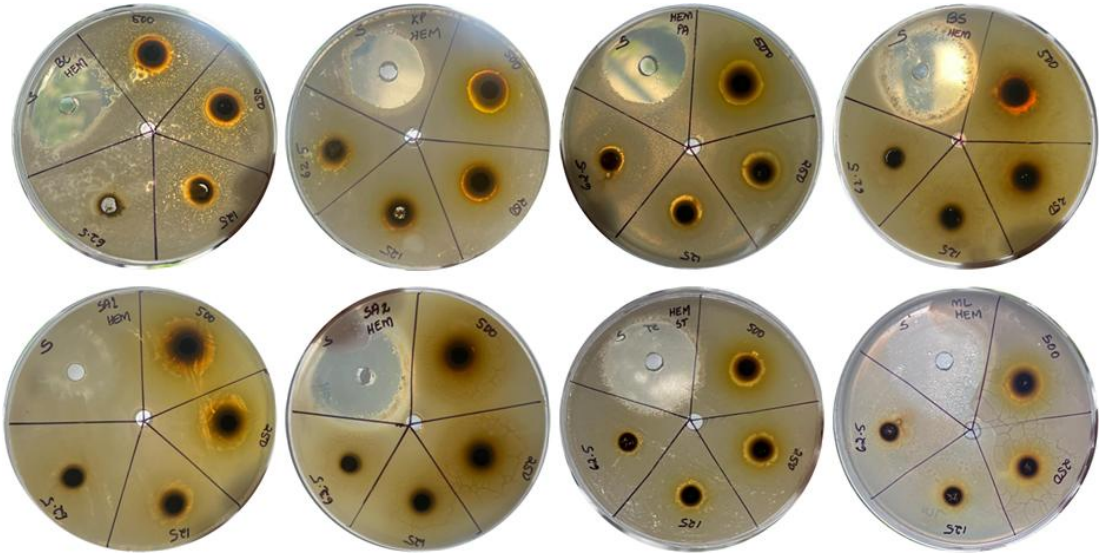

Figure 5.1: Plates showing the assessment of antibacterial activity by agar well diffusion method for four different concentrations of *H. excelsa* methanolic extract, a standard drug and negative control against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

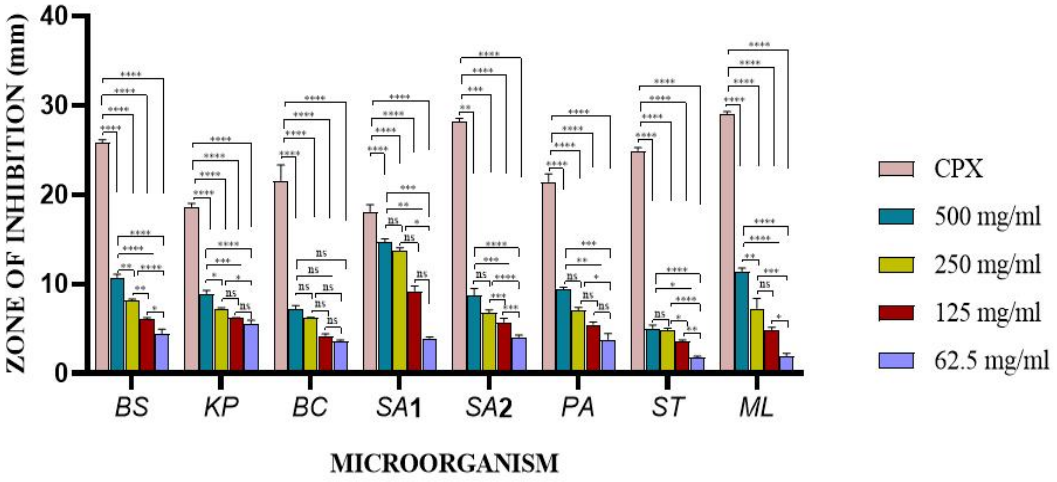

Figure 5.2: A graphical representation of the formation of zone of inhibition by four different concentrations of *H. excelsa* methanolic extract and a standard drug, ciprofloxacin (CPX) against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

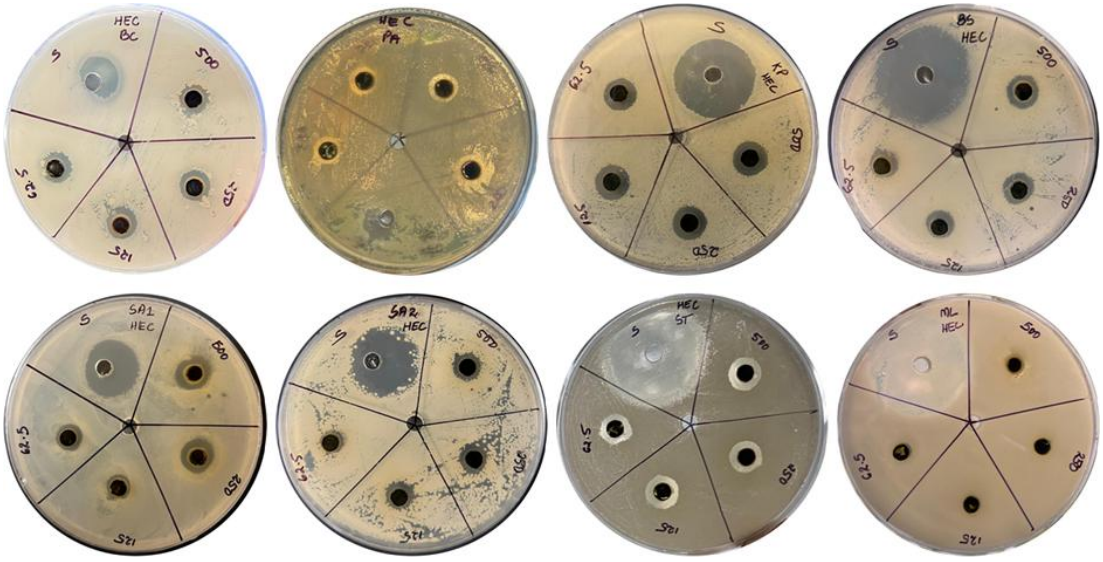

Figure 5.3: Plates showing the assessment of antibacterial activity by agar well diffusion method for four different concentrations of *H. excelsa* chloroform extract, a standard drug and, negative control against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

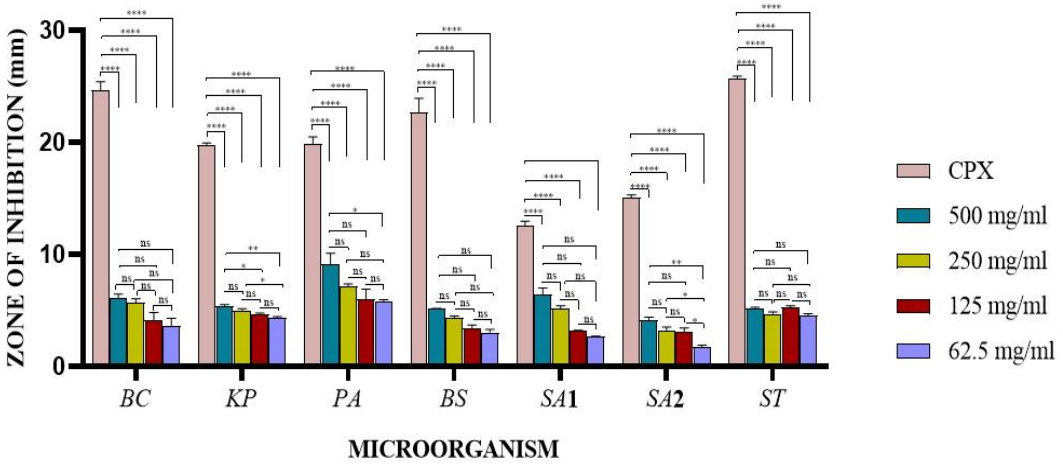

Figure 5.4: A graphical representation of the formation of zone of inhibition by four different concentrations of *H. excelsa* chloroform extract and a standard drug, ciprofloxacin (CPX) against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

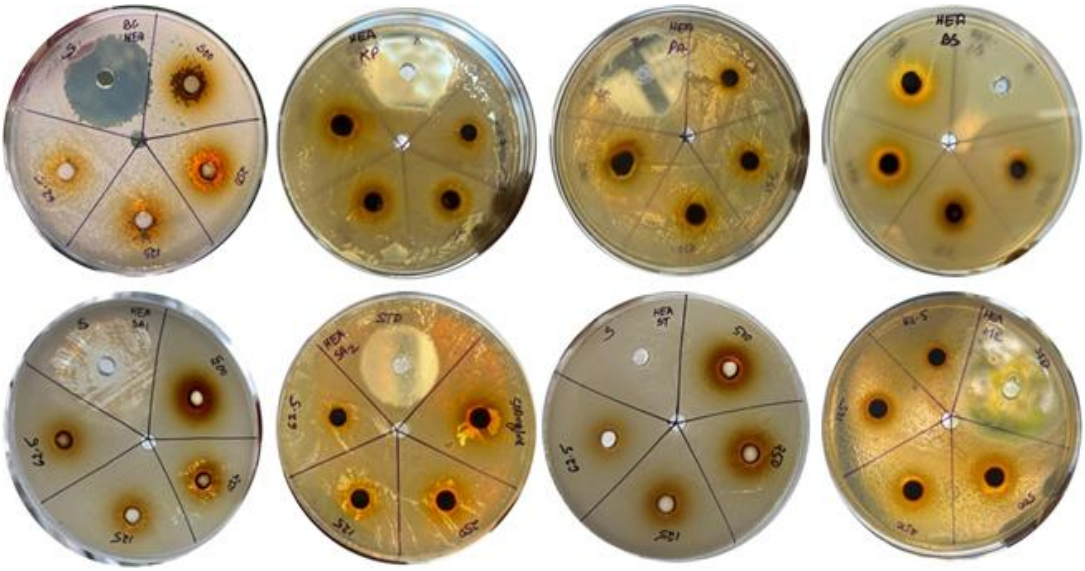

Figure 5.5: Plates showing the assessment of antibacterial activity by agar well diffusion method for four different concentrations of *H. excelsa* aqueous extract, a standard drug and negative control against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

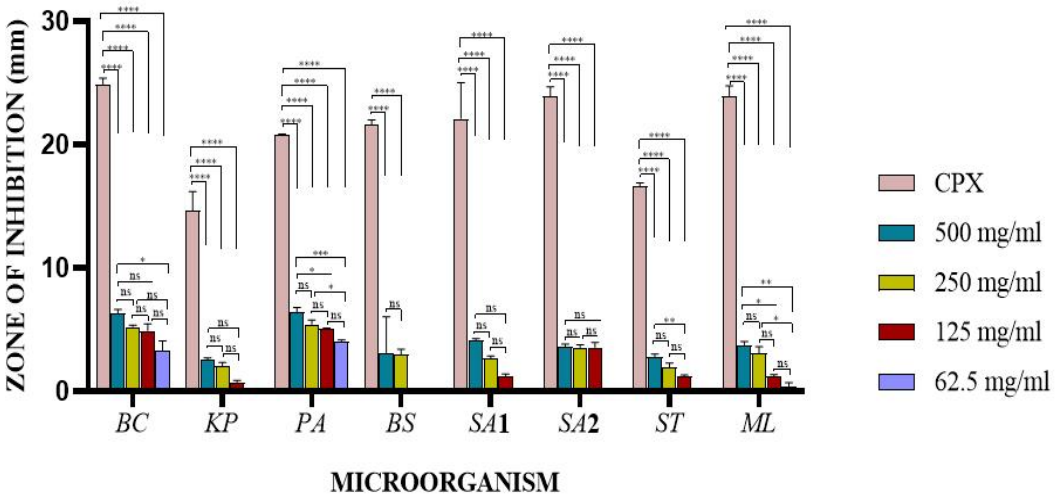

Figure 5.6: A graphical representation of the formation of zone of inhibition by different concentrations prepared for *H. excelsa* aqueous extract and a standard drug, ciprofloxacin (CPX), against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus.*

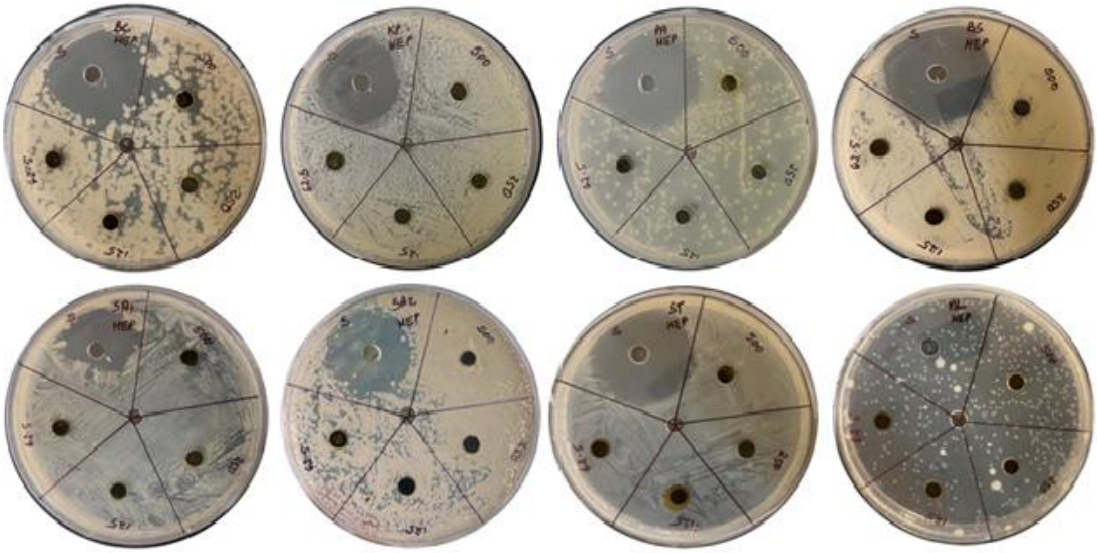

Figure 5.7: Plates showing the assessment of antibacterial activity by agar well diffusion method for four different concentrations of *H. excelsa* petroleum ether extract, a standard drug and negative control against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

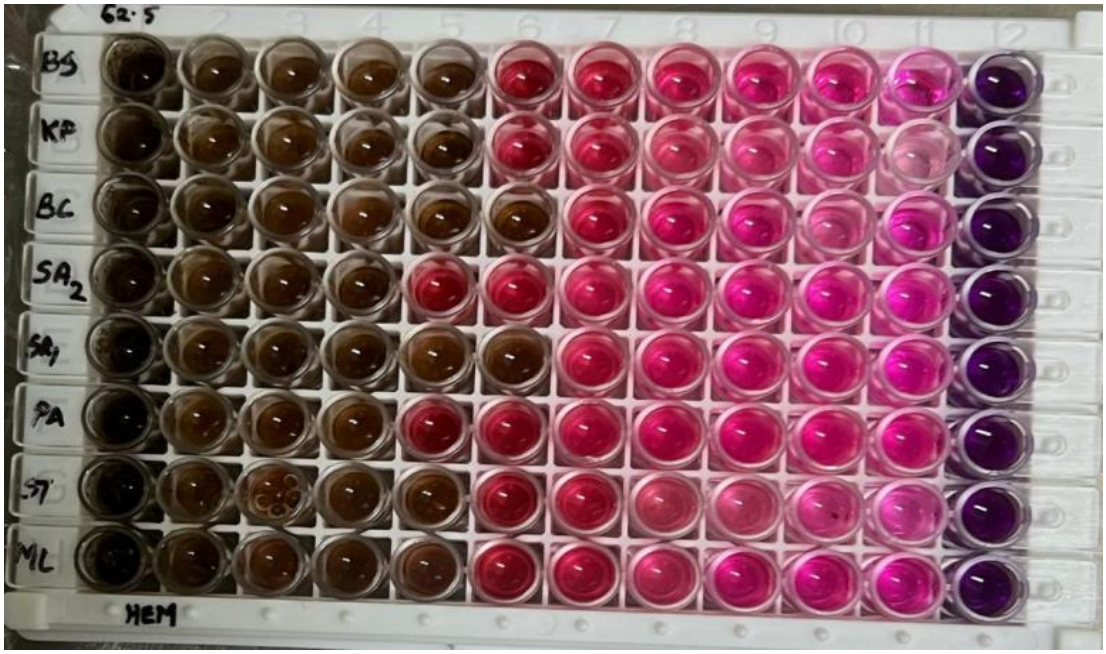

Figure 5.8: A plate showing minimum inhibitory concentration (MIC) of *H. excelsa* methanolic extract assessed using resazurin as an indicator against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

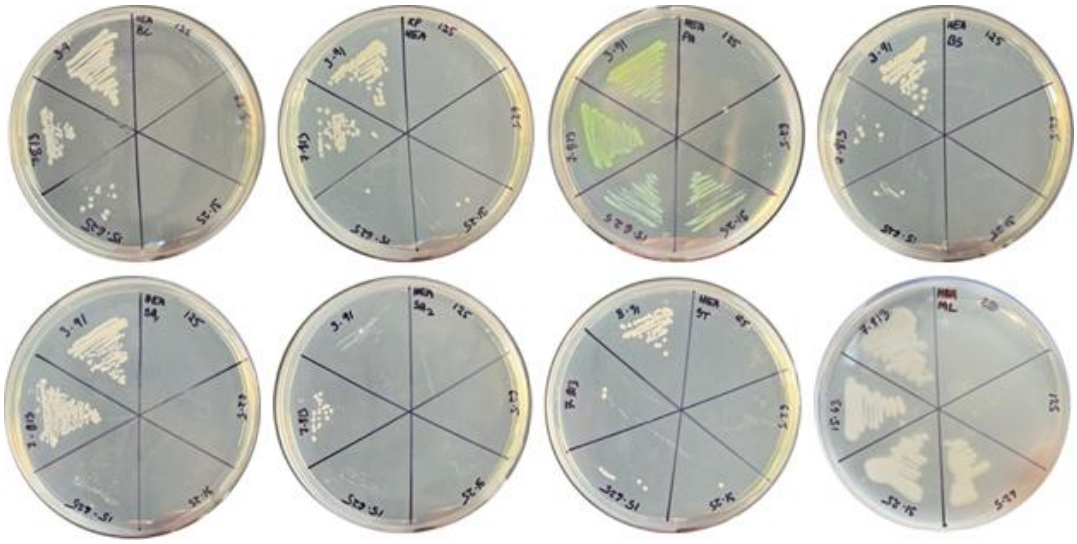

Figure 5.9: Culture plates showing minimum bactericidal concentration (MBC) of *H. excelsa* methanolic extract assessed using agar streaking method against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

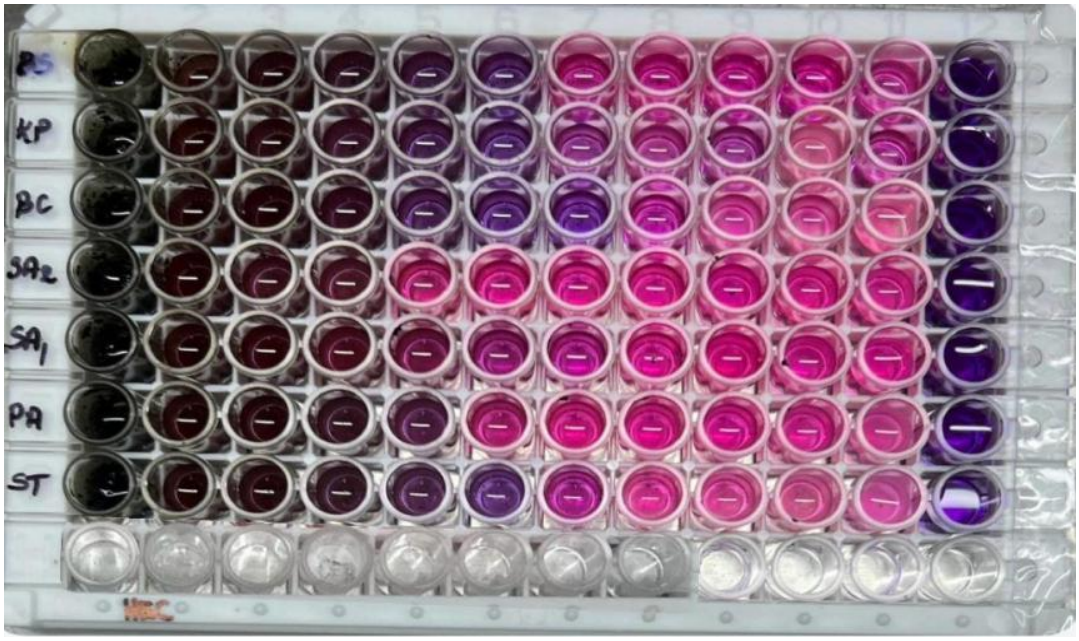

Figure 5.10: A plate showing minimum inhibitory concentration (MIC) of *H. excelsa* chloroform extract assessed using resazurin as an indicator against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

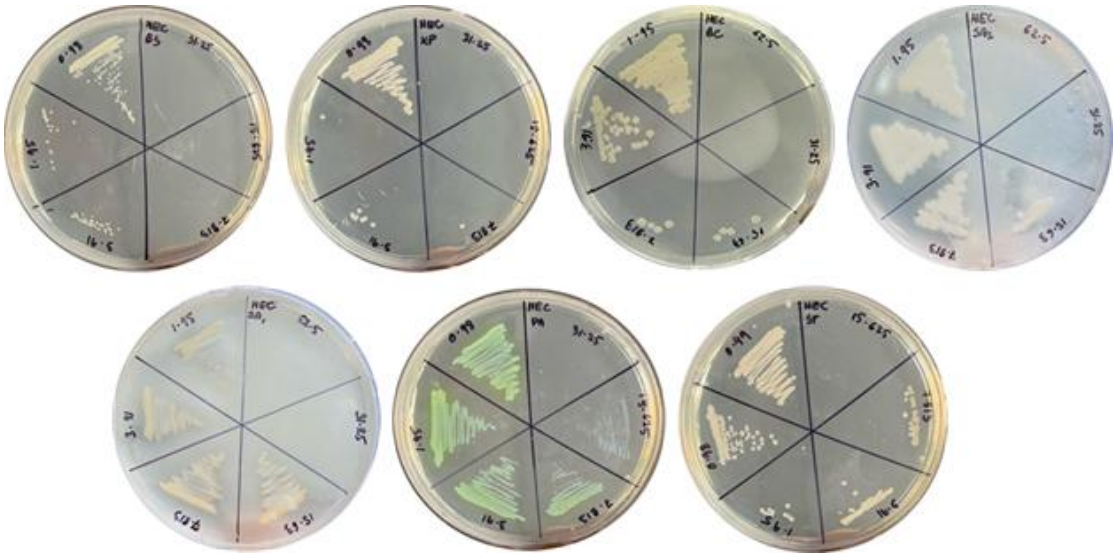

Figure 5.11: Culture plates showing minimum bactericidal concentration (MBC) of *H. excelsa* chloroform extract assessed using agar streaking method against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

Figure 5.12: A plate showing minimum inhibitory concentration (MIC) of *H. excelsa* aqueous extract assessed using resazurin as an indicator against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

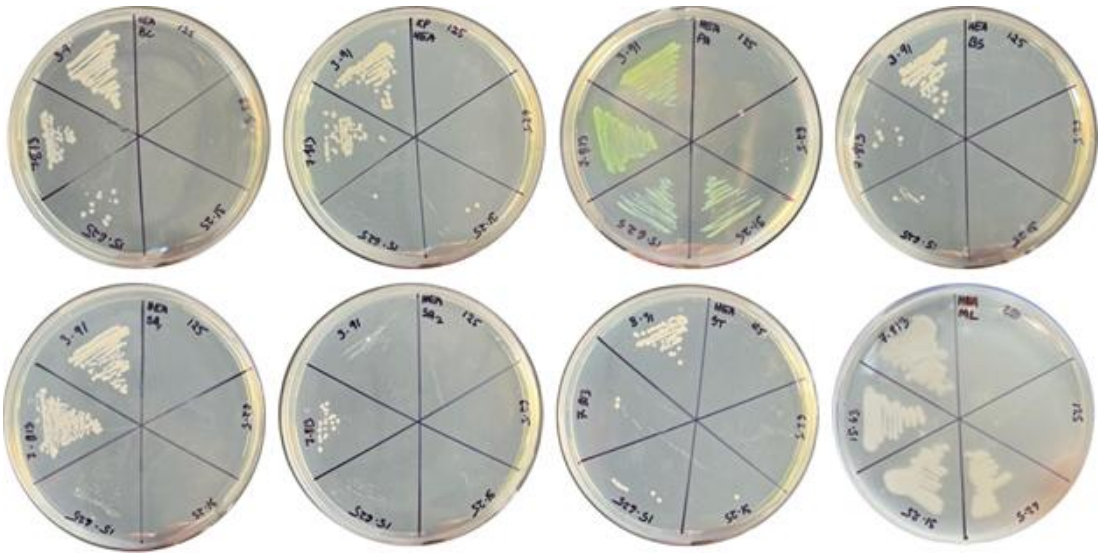

Figure 5.13: Culture plates showing minimum bactericidal concentration (MBC) of *H. excelsa* aqueous extract assessed using agar streaking method against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

MICROORGANISM	MIC (mg/ml)	MBC (mg/ml)
Bacillus subtilis	7.81	15.63
Klebsiella pneumoniae	7.81	7.81
Bacillus cereus	1.95	62.5
Staphylococcus aureus 1	1.95	3.91
Staphylococcus aureus 2	15.63	15.63
Pseudomonas aeruginosa	7.81	15.63
Salmonella typhimurium	3.91	7.81
Micrococcus luteus	3.91	15.63

Table 5.1: Table showing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *H. excelsa* methanolic extract against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

MICROORGANISM	MIC (mg/ml)	MBC (mg/ml)
Bacillus subtilis	1.95	3.91
Klebsiella pneumoniae	1.95	7.81
Bacillus cereus	0.98	31.25
Staphylococcus aureus 1	7.81	31.25
Staphylococcus aureus 2	7.81	31.25
Pseudomonas aeruginosa	3.91	15.63
Salmonella typhimurium	1.95	3.91

Table 5.2: Table showing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *H. excelsa* chloroform extract against seven pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa* and *S. typhimurium*.

MICROORGANISM	MIC (mg/ml)	MBC (mg/ml)
Bacillus subtilis	7.81	15.63
Klebsiella pneumoniae	7.81	31.25
Bacillus cereus	7.81	15.63
Staphylococcus aureus 1	15.63	15.63
Staphylococcus aureus 2	15.63	15.63
Pseudomonas aeruginosa	15.63	31.25
Salmonella typhimurium	7.81	31.25
Micrococcus luteus	125	125

Table 5.3: Table showing minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *H. excelsa* aqueous extract against eight pathogenic bacterial strains - *B. subtilis*, *K. pneumoniae*, *B. cereus*, *S. aureus* strain 1, *S. aureus* strain 2, *P. aeruginosa*, *S. typhimurium* and *M. luteus*.

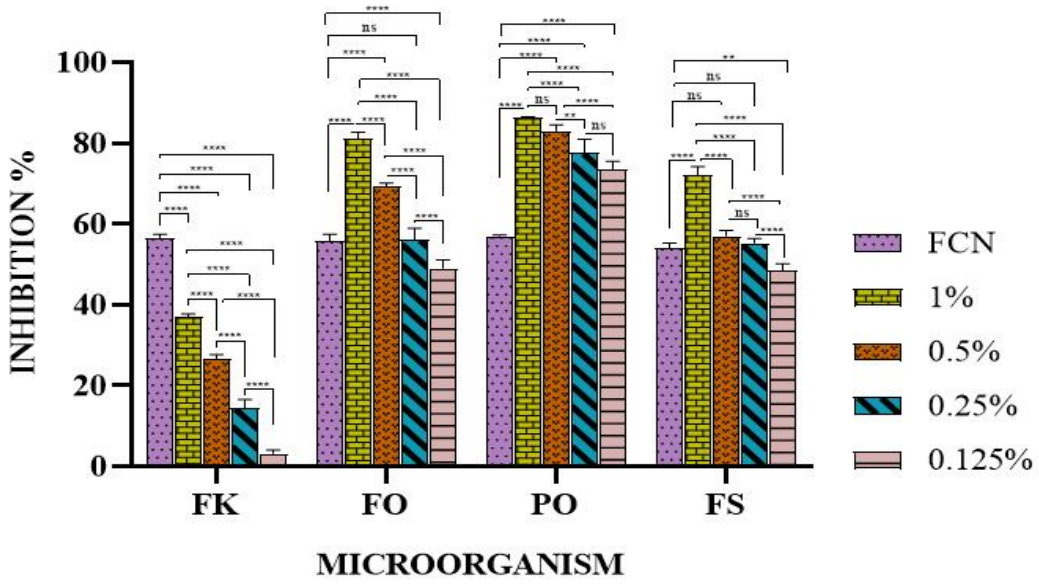

Figure 5.14: A graphical representation of the inhibition percentage of *H. excelsa* leaf methanolic extract and a standard drug - fluconazole (FCN) calculated from control (untreated) against four pathogenic fungal strains - *Fusarium keratoplasticum* (FK), *Fusarium oxysporum* (FO), *Pyricularia oryzae* (PO) and *Fusarium solani* (FS).

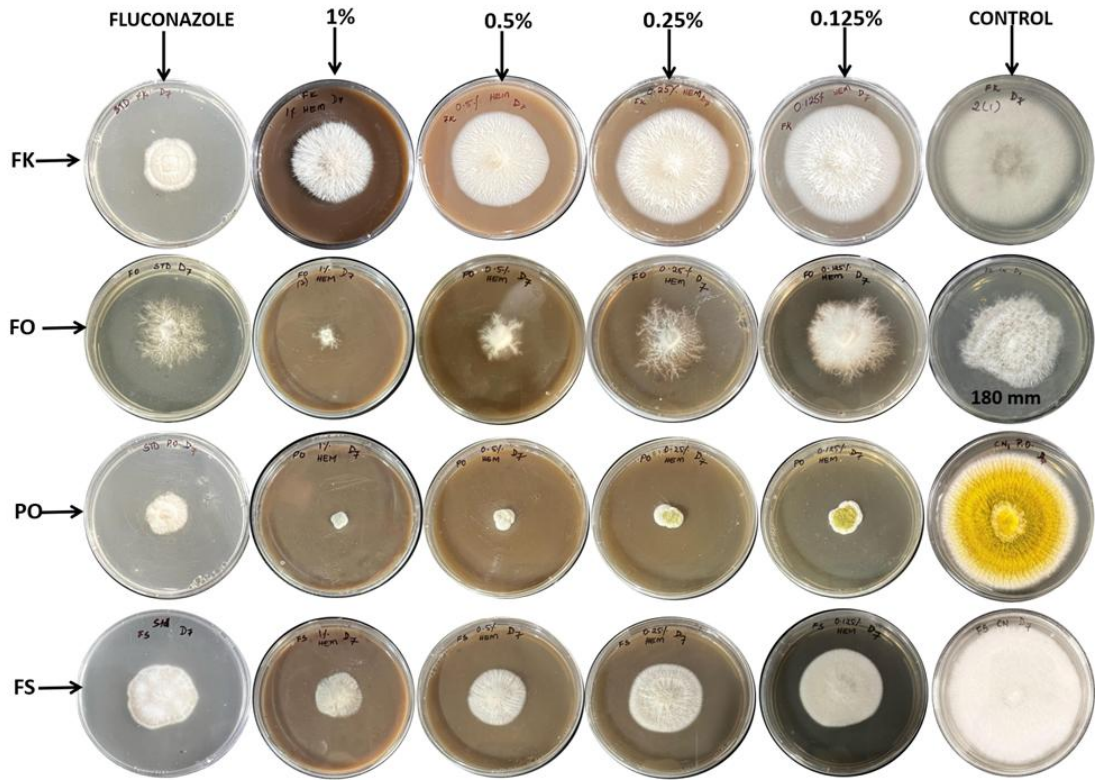

Figure 5.15: Culture plates showing inhibition activity of the methanolic extract of *H. excelsa* on fungal strains after seven days of incubation.

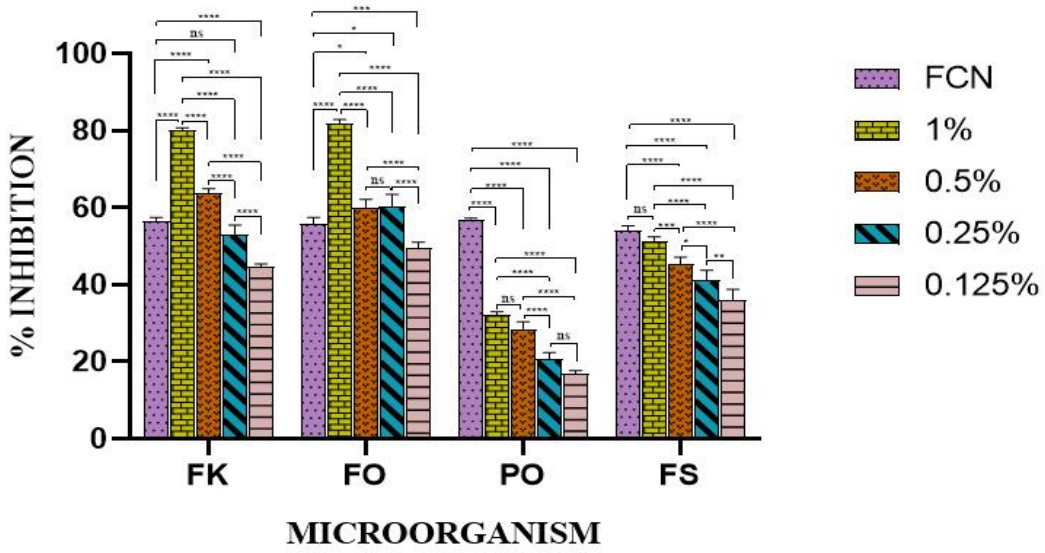

Figure 5.16: A graphical representation of the inhibition percentage of *H. excelsa* chloroform extract and a standard drug - fluconazole (FCN) calculated from control (untreated) against four pathogenic fungal strains - *Fusarium keratoplasticum* (FK), *Fusarium oxysporum* (FO), *Pyricularia oryzae* (PO) and *Fusarium solani* (FS).

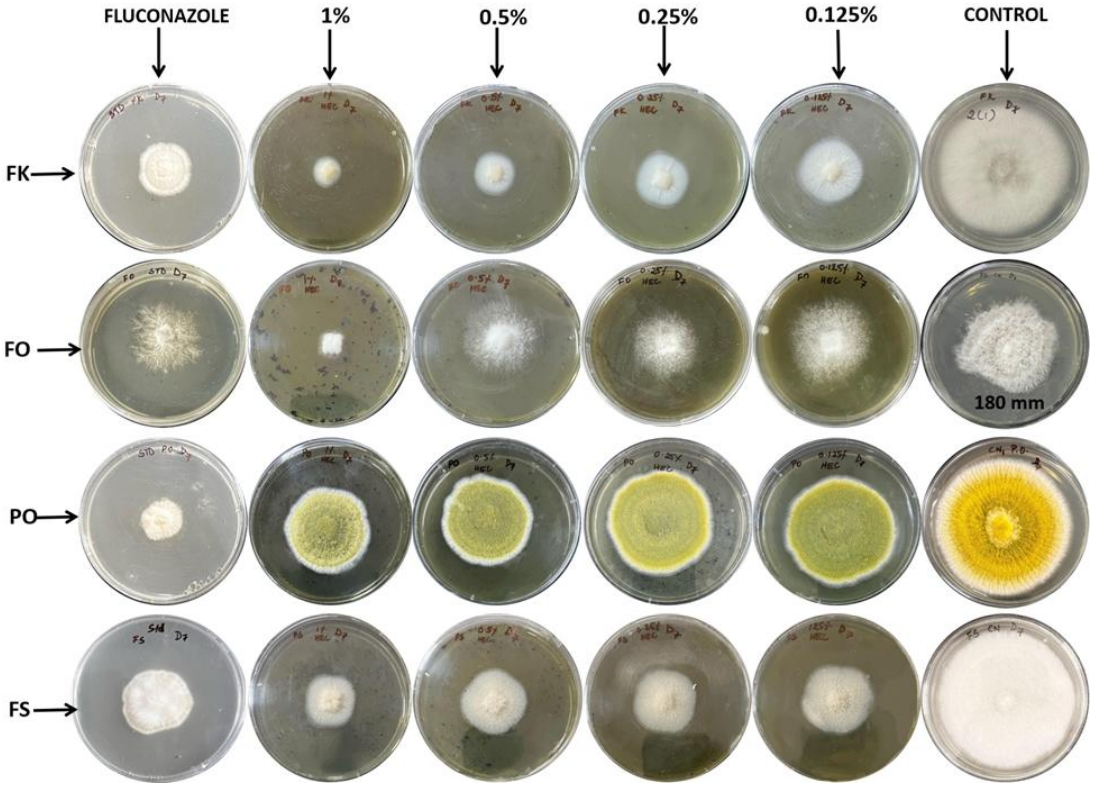

Figure 5.17: Culture plates showing inhibition activity of the chloroform extract of *H. excelsa* on fungal strains after seven days of incubation.

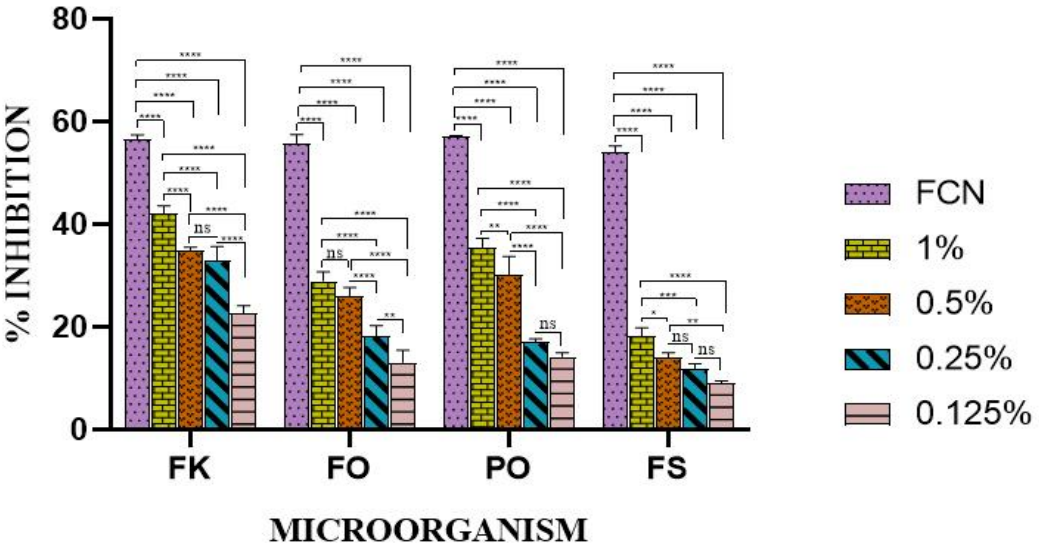

Figure 5.18: A graphical representation of the inhibition percentage of *H. excelsa* petroleum ether extract and a standard drug - fluconazole (FCN) calculated from control (untreated) against four pathogenic fungal strains - *Fusarium keratoplasticum* (FK), *Fusarium oxysporum* (FO), *Pyricularia oryzae* (PO) and *Fusarium solani* (FS).

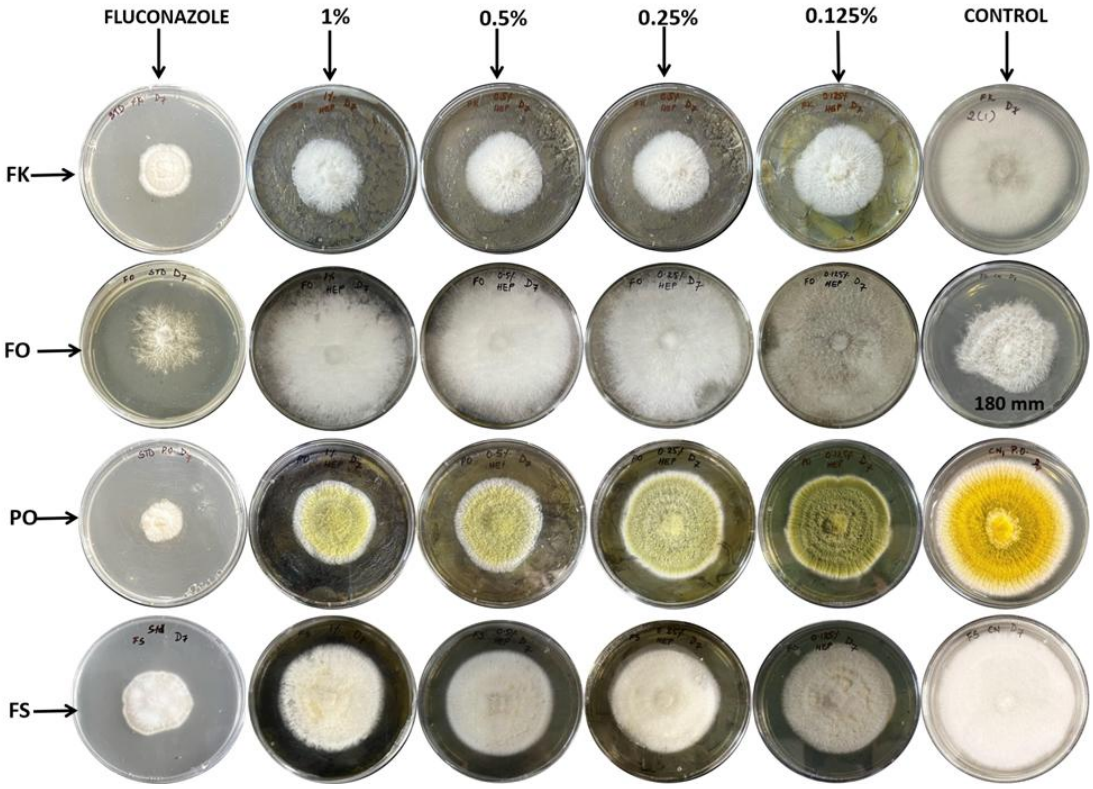

Figure 5.19: Culture plates showing inhibition activity of the petroleum ether extract of *H. excelsa* on fungal strains after seven days of incubation.

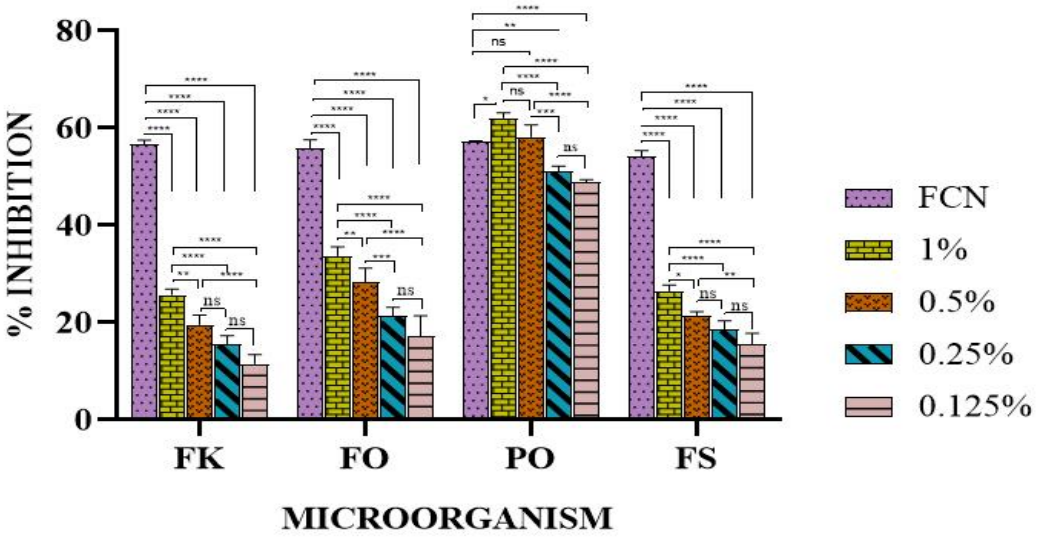

Figure 5.20: A graphical representation of the inhibition percentage of *H. excelsa* aqueous extract and a standard drug - fluconazole (FCN) calculated from control (untreated) against four pathogenic fungal strains - *Fusarium keratoplasticum* (FK), *Fusarium oxysporum* (FO), *Pyricularia oryzae* (PO) and *Fusarium solani* (FS).

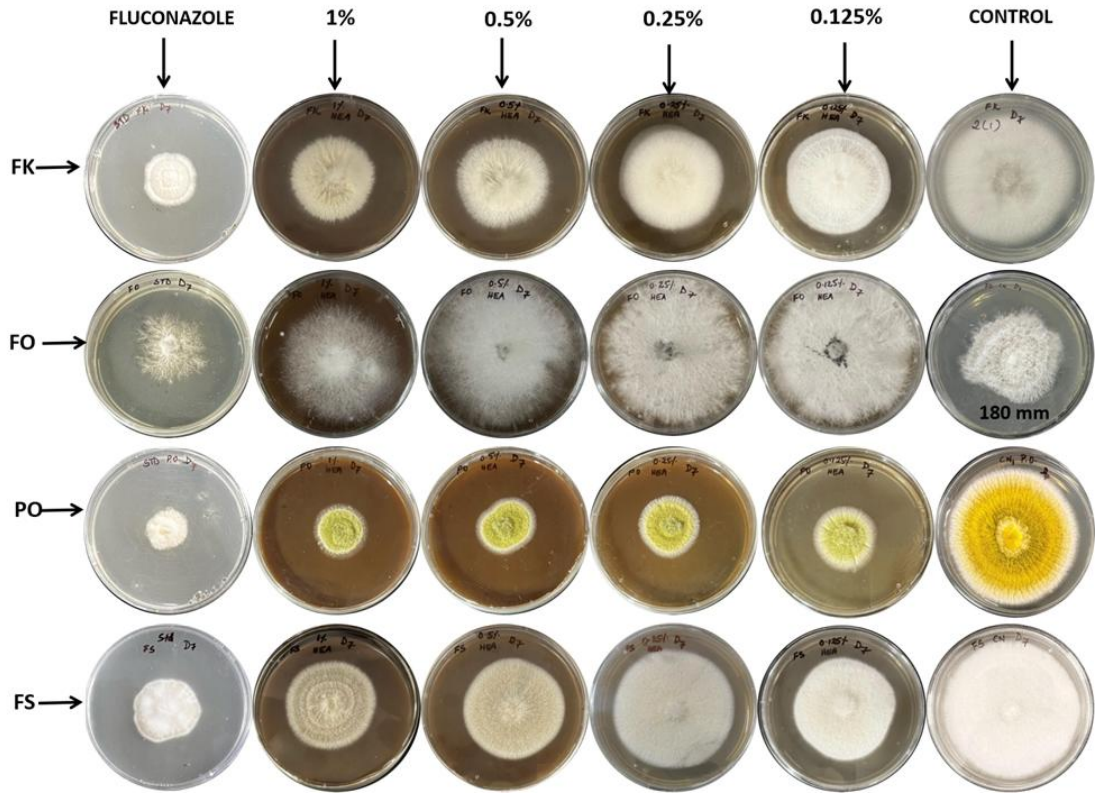

Figure 5.21: Culture plates showing inhibition activity of the aqueous extract of *H. excelsa* on fungal strains after seven days of incubation.

CHAPTER - 6

ASSESSMENT OF ANTHELMINTIC ACTIVITY OF *HELICIA EXCELSA*

INTRODUCTION

Parasitic infections caused by intestinal helminths, known as helminthiasis, and protozoan parasites persist a critical public health concern not only in areas with limited availability to sanitary practices and healthcare, but also in developed countries where parasitic infections cause majority of illness and disease. Each year, at least 30% of the global population are at risk of parasitic infection which leading approximately 450 million people to illness (Abdalal *et al*., 2024). The infection due to parasites are classified as one of the neglected tropical diseases (NTD), however, 1.5 billion individuals from the low-income regions of the world are still infected by parasites such as *Ascaris lumbricoides*, *Giardia lamblia*, *Trichuris trichiura*, and others (Mitra & Mawson, 2017). The parasites get to human hosts through different ways including the ingestion of spoiled and unclean food and water, vector-borne transmission, and physical contact with contaminated environments. The frequency of the infection is notably impacted by environmental factors such as temperature, altitude, soil type, pressure, rainfall, and humidity (Hotez *et al*., 2014; Dybing *et al*., 2013). For instance, infection of hookworms like *Necator americanus* and *Ancylostoma duodenale* are prevalent at the land surface temperature of 38-40°C, and at or below temperature of 5°C, infection by *A. lumbricoides* and *T. trichiura* dominates the other parasitic infections (Brooker *et al*., 2004). These infections often result in adverse outcomes including nutritional deficiency, anemia, cognitive impairments, growth retardation, and in chronic condition, disability and even death (Abdalal *et al*., 2024).

Apart from human, parasites infect other animals as well. In particular, tapeworm like *Raillietina echinobothrida* Megnin, 1881, an endoparasite belonging to Davaineidae, uses chicken (*Gallus gallus* Linnaeus, 1758) as its definitive host. Due to this host-parasite interaction, the fowls are severely affected through

intestinal blockage, cellular damage, anemia, malnutrition, secondary infections by virus and bacteria, and in some cases, leading to death (McDougald, 2003). These infections also impact adverse economic consequences in poultry farming as they cause reduction in productivity through retarding growth rate, reducing production levels and quality of meat and egg, decrease feed effectiveness, and lower laying performance (Permin & Hansen, 1998).

Since *R. echinobothrida* lacks proper digestive system as it lives in the small intestine of poultry, where food is already broken down into smaller molecules such as fatty acids, amino acids and monosaccharides by the host organism (Smyth & McManus, 1989), the tegumental enzymes like acid and alkaline phosphatases are crucial for the parasite to survive within the host, and in that case, its tegument functions as the major site for nutrients uptake from the host. These phosphatase enzymes then dissolve organic phosphates and fuel parasite's biochemical activity by releasing inorganic phosphates (Barrett & Rawlings, 2007).

Among numerous drugs for the treatment of variety of parasitic worms, albendazole from a drug class of benzimidazole is frequently used, which is still a primary anthelmintic drug administered on National Deworming Day in the major region in India. This broad-spectrum anthelmintic drug binds to β-tubulin in the cells of parasite, inhibiting the formation of microtubule that leads to impairment of nutrient uptake and depletion of energy which further leads to paralysis and eventually results in the death of the parasite (Lalchhandama, 2010; Fissiha & Kinde, 2021).

For more than two decades, due to the prolonged dependence and administration of synthetic anthelmintic drugs like albendazole, ivermectin, and fenbendazole for treating human and livestocks helminthiasis, the resistance has been recognized and its frequency has become a global phenomenon (Kaplan & Vidyashankar, 2012). For instance, the first report that suggested a decline in the efficacy of ivermectin in Ghana against *Onchocerca volvulus,* a causal parasite of river blindness. Some cases of decline in activity of benzimidazoles and pyrantel were also reported against human hookworms. The development of resistance to both

oxamniquine and praziquantel in the tropical blood fluke, *Schistosoma mansoni*. Moreover, lack of treatment response of two human hookworms, *Necator americanus* and *Ancylostoma duodenale* with mebendazole and pyrantel, respectively, were also documented, which was considered expressive for resistance development. At the same time, the isolates of *Haemonchus contortus*, a parasaitic nematode that mainly infects the true stomach of ruminants such as sheep, goat and cattle, failed to respond properly to any available anthelmintic drugs in some parts of South Africa and Brazil, that led to a critical point where sheep production is disrupted. Since only three classes of drugs under anthelmintic drugs such as benzimidazoles, imidazothiazoles, and macrocyclic lactones are available, with the increasing frequency of resistance worldwide, discovery of novel anthelmintic drugs is in high demand from medicinal plants having potent anthelmintic activity (Lalchhandama, 2010). Therefore, anthelmintic efficacy from different extracts of *H. excelsa* was implemented in this study.

Structural composition of *R. echinobothrida*

The adult worm has an elongated (~16-23 cm), whitish, segmented, proglottid bearing and dorso-ventrally flattened body which gradually tapers toward the posterior end. A bulbous pin-sized head structure at the very front of the worm called scolex is composed by four suckers around the proximal end which encircle a globular retractable structure called rostellum at the apical extremity of the scolex. *R. echinobothrida* has two concentric layers of hammer-shaped hooks called spines around the rostellum, while each sucker is equipped with 6-8 concentric rows of comparatively smaller hammer-shaped hooks. The hooks are responsible for firm attachment to the host tissue. The scolex is immediately followed by a stout and unsegmented region called neck.

The mature proglottid of *R. echinobothrida* has the outermost surface, called tegument, which is covered with a hair-like projections called microtriches that give a smooth-like appearance to the surface of the parasite. The internal layer next to tegument is the subtegument, thicker portion of the outer layer, which covers the innermost boundary of the tegument called basal lamina that has muscle layers called circular and longitudinal muscles lie beneath it. Underneath the muscle layers lies a

layer of parenchymal tissues that embedding the egg capsules, reproductive organs, which contain numerous ovaries called oncospheres. Along the side of the paremchyma, lateral nerve cords are located. At the posterior end of the proglottid, a distinct large vacuole called excretory canal, an excretory organ of cestode, is also present.

MATERIALS AND METHODS

Chemicals and reagents:

Ethanol was purchased from Oxford Lab Fine Chem LLP, Maharashtra, India. Hydrochloric acid, dimethylsulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, and distyrene plasticizer xylene (DPX) were procured from Merck Life Science Private Limited, Mumbai, India. Hematoxylin, eosin, glycine, xylene, p-nitrophenyl phosphate (pNPP), picric acid, citric acid, citrate buffer, and phosphate-buffered saline (PBS) were obtained from Himedia Laboratories Pvt. Ltd. Mumbai, India. Tetramethyl silane (TMS), and paraffin wax were purchased from SIGMA – ALDRICH, USA.

Recovery of gastrointestinal parasitic helminths from local fowls:

The intestines of the local fowls, *Gallus gallus* was collected from the slaughter house located at the main bazaar of Aizawl, Mizoram. The live gastrointestinal helminth parasite, *Raillietina echinobothrida* was dissected out and recovered from the chicken intestines. Only adult tapeworms were collected in a glass culture plate containing neutral phosphate-buffered saline (PBS) maintained at 37±1ºC in a microbiological incubator. The live parasites were then further subjected to the different doses of the treatments prepared from different extracts of *H. excelsa* leaves, including the standard (albendazole). The control was also maintained without treatment.

In vitro survival test of the treated parasites:

The efficacy of the plant extracts and the standard drug were assessed by treating the parasites in different concentrations viz. 20, 15, 10, 5 and 2 mg/ml according to Lalchhandama (2009) with slight modifications. The incubation medium for the plant extracts was prepared by dissolving the pre-weighed crude extracts in PBS complimented by 1% DMSO. Each treatment dose was maintained in three different culture plates. Each plate containing 5 adult model parasites represented one set of tapeworms. For albendazole, a reference standard, a manufactured dosage was directly used to determine its antiparasitic activity. Moreover, another set of tapeworms was also maintained in different plates

containing only an incubation medium (PBS with 1% DMSO). The survival time was then evaluated by dipping the parasites in lukewarm water (~45 ºC). The time when the parasites were remained static was taken as the time of death. The test performed in triplicates were divided into three groups for further anthelmintic activity tests. The parasites fixed in Bouin's solution for histological studies, 10% neutral buffered formalin (NBF) for scanning electron microscopical studies, and the last set of the treatment at -4 ºC for enzyme studies.

Histopathological assessment of *R. echinobothrida* treated with plant extracts:

Histological analysis of the parasites treated with different plant extracts and albendazole, including control was performed based on the following Jeremy et al. (2019) with some changes. The histological effect of each treatment group was evaluated by selecting the highest concentration i.e. 20 mg/ml in comparison with control group. The study was initiated with changing the parasites stored in Bouin's solution for 24 hours to 70% alcohol. The parasite samples cut at a certain length (~ 1 cm) were dehydrated by a series immersion in increasing ethanol concentration viz. 80, 90 and 100% for 1 hour each. The dehydrated tissues were then subjected to alcohol clearing agent, xylene, till they became transparent, succeeded by paraffin infiltration with wax:xylene (1:1) for about 40 minutes, and with molten paraffin wax for about 2 hours with 3 changes for 40 minutes each in an oven temperature constantly maintained at 58-60ºC. The making of paraffin-embedded tissue block was then started with placing a metal mold onto the ice pack, and embedding cassette was placed on top of a metal mold and poured melted paraffin wax in it. The wax-impregnated tissues were fixed in an upright position in embedding cassette filled with paraffin wax with the help of forceps. After cooling down, the block was removed from the cassette. Afterward, the block containing parasite tissues was sectioned transversely at 6 µM thickness using a microtome (MEDIMEAS MRM-ST). The sectioned tissues, called ribbons, were then attached to sterile 1% gelatin coated glass slides by immersing the wax-embedded tissue in a warm water and allowed to air dried for about 3 days to ensure tissue adherence, followed by deparaffinizing tissues by dipping them in xylene with 2 changes for 10 minutes each. The deparaffinized tissues were then dehydrated with serial decreasing alcohol

concentrations from 100, 90, 70% and distilled water for 10 minutes each. After that, the staining process was executed by immersing them in hematoxylin for about 1-2 minutes, followed by diluting the stain in running tap water for 10-15 minutes, dipping them again in 50 and 70% ethanol for 10 minutes each, and later in eosin for 1-2 minutes. The samples were dehydrated with a series of increasing concentration of ethanol viz. 70, 90 and 100% for 10 minutes each, followed by clearing the excess interference with xylene with 2 changes for 10 minutes each. The tissues stained with hematoxylin and eosin were then mounted with DPX, covered with cover slip and allowed to completely dried at room temperature. Finally, the parasite tissues were then observed under the microscope and assessed the effect of treatments in tissue morphology from the photographs.

Scanning electron microscopic (SEM) evaluation of the treated parasites:

Topographical studies of the parasites treated with different groups of the plant extracts and albendazole, and control without any treatment by scanning electron microscopy was carried out by following the standard method of Roy et al. (2008) with some modifications. The dead parasites from the highest concentration (20 mg/ml) of the treatment groups, including control were immediately fixed in 10% NBF for at least 24 hours. The fixed specimen were then washed with PBS for 30 minutes with two changes to remove the unwanted attachments on the surface of the parasites. Afterwards, they were subjected to dehydration with ascending concentrations of acetone viz. 30, 50, and 70% with two changes for 15 minutes each, and without delay, the process was continued with 80, 90, 95 and 100% with two changes each for 30 minutes. The dehydrated worms were then further processed with TMS in an eppendorf tube and left open at room temperature to attain complete dehydration. The dried samples were then carefully placed on a metal stub and coated with gold in a fine-coat ion sputter (MCI000 ION SPUTTER, HITACHI) for about 15-20 minutes. To complete the procedure, the specimen were finally viewed with SEM (TM4000Plus Tabletop Microscope, HITACHI) and assessed the anthelmintic activity of the treatments in contrast to the control.

Assessment of enzyme inhibition of the parasites:

The inhibiton activity of digestive enzymes of the cestode tegument, acid and alkaline phosphatase by the treatment groups, including control were assessed independently based on the standard protocols as mentioned below:

a) Acid phosphatase:

The alteration of acid phosphatase activity of the cestode tegument was estimated by following Andersch & Szcypinski (1947) with some adjustment. A measured amount of parasite tissues (100 mg) of different concentrations of the extracts and albendazole (20, 15, 10, 5 and 2 mg/ml), and control, stored in -4ºC, were homogenized in 1 ml of ice cold 50 mM citrate buffer adjusted to 5.3 pH. The homogenized tissues were then centrifuged twice at 10000 rpm for 20 minutes each using a centrifuge (Centrifuge 5430 R, Eppendorf) and the supernatant solution containing the enzyme extract was collected in a sterile eppendorf tube. The next step was proceeded with the addition of 0.5 ml of the enzyme extract into a warm 2.5 ml of pNPP substrate solution (EDTA + pNPP + citric acid) which was already incubated at 37ºC for 5 minutes, followed by the removal of 0.5 ml from the said mixture and immediately mixed with 4.5 ml of 0.085 N NaOH which served as a blank solution, while the remaining solution was further incubated for 10 minutes at 37 ºC. Then, 0.5 ml of the incubated mixture was mixed with another 4.5 ml of NaOH and used as the sample solution. The absorbance of each sample was then taken at 405 nm double beam UV-Vis spectrophotometer (LABTRONICS LT-2700) against the blank solution prepared exclusively for each sample. The enzyme activity of the sample was expressed as specific enzyme activity expressed per micromole substrate concentration released per milligram protein per hour (Unit/ µM/ mg/ h).

Where,

Unit = enzymatic activity, µM = micromolar concentration of the substrate,

mg = the amount of protein, h = hour

The percentage decrease of the AcPase activity was calculated by the formula:

$$\% \text{ decrease} = \frac{\text{control activity - treated activity}}{\text{Control activity}} \times 100$$

The percentage decrease was represented as percentage (%).

b) Alkaline phosphatase:

The inhibition of alkaline phosphatase activity of the cestode tegument of the plant extracts and albendazole was assessed based on the protocol of Laurent and Norberg (1960) with some modifications. Different concentrations viz. 20, 15, 10, 5 and 2 mg/ml prepared for the four plant extracts and albendazole, including control without treatment, stored in -4 ºC were taken out and weighed 100 mg each in their respective sterilized eppendorf tube tagged for all the treatments. The parasite tissues were crushed in 1 ml ice cold 50 mM glycine buffer (10.2 pH) with the help of micro pestle. The homogenized tissues were centrifuged at 10000 rpm for 20 minutes with two cycles. The clear supernatant solution containing the enzyme was collected in another eppendorf tube. 0.5 ml of the enzyme extract solution was then added to 2.5 ml of substrate solution prepared from $MgCl_2$ + pNPP + glycine. From this mixture, blank solution was prepared by pipetting out 0.5 ml and immediately mixed with a pre-warm 4.5 ml of 0.085 N NaOH solution, meanwhile, the remaining was incubated at 37ºC for 10 minutes. Thereafter, 0.5 ml of the incubated solution was taken and halted the reaction by mixing it with 4.5 ml NaOH solution, and this newly prepared solution was used as the sample. The absorbance of the sample against the blank solution was then taken at 405 nm using UV-Vis spectrophotometer (LABTRONICS LT-2700). The enzyme activity of the sample was expressed as specific enzyme activity expressed per micromole substrate concentration released per milligram protein per hour (Unit/ µM/ mg/ h).

Where,

Unit = enzymatic activity

µM = micromolar concentration of the substrate

mg = the amount of protein

h = hour

The percentage decrease of the AcPase activity was calculated by the formula:

$$\% \text{ decrease} = \frac{\text{control activity - treated activity}}{\text{Control activity}} \times 100$$

The percentage decrease was represented as percentage (%).

STATISTICAL ANALYSIS

All experimental data were expressed as the mean ± standard error of the mean (SEM) from three independent replicates ($n = 3$). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to assess significant differences between treatment groups. Analyses were conducted using GraphPad Prism software version 8.0.1. A p-value of ≤ 0.05 was considered statistically significant. The levels of statistical significance were denoted using asterisks as follows:

ns: not significant ($p > 0.05$), *: $p \leq 0.0332$, **: $p \leq 0.0021$, ***: $p \leq 0.0002$, ****: $p < 0.0001$.

RESULTS

Viability test of the treated parasites:

The life-span analysis of the worms upon the treatments of different concentrations of the plant extracts (HEM, HEC, HEP and HEA) and standard anthelmintic drug, albendazole, revealed significant difference against the control was shown in **Figure 6.1**. Remarkably, dose-dependent activity was seen in all the treatment groups. When the control group showed the highest survival time of 82.20 ± 7.22 hours, the lowest was observed in the exposure to the highest concentration (20 mg/ml) of albendazole with 4.89 ± 0.70 hours, while the rest of the doses viz., 15, 10, 5 and 2 mg/ml showed the time of death of the parasites at 9.67 ± 1.92, 16.03 ± 2.14, 20.513 ± 3.90 and 26.00 ± 4.19 hours, respectively. At the highest dosage among different concentrations of the extracts, the highest inhibition of survival of the parasites was revealed in the methanol extract with 16.32 ± 3.05 hours, followed by petroleum ether extract (24.03 ± 4.09 hours), aqueous extracts (24.88 ± 4.54 hours), and lastly, chloroform extract (26.53 ± 4.72 hours) with no statistical difference between them. On the other hand, the lowest inhibition, among the extracts, was observed at 2 mg/ml of petroleum ether extract with 52.77 ± 6.57 hours.

Histological studies of the the treated parasites:

The light micrograph of the transversely sectioned mature proglottid of the untreated parasite shown in the **Figure 6.2** revealed well-defined tegument and subtegument layers, large vacuole of lateral excretory canal, uniform layers of parenchyma cells, distinct shapes of numerous egg capsules containing a number of ovaries or oncospheres enclosed within them, clear nuclear cytoplasm beside oncosperes, clearly defined forms of two different layers of circular and longitudinal layers beneath the subtegument layer.

Treatment of *R. echinobothrida* with 20 mg/ml of the albendazole drug, as shown in **Figure 6.3** exposed a complete devoid of lateral excretory canal, and a magnified portion of the micrograph revealed filamentous and depletion of egg cells, irregular arrangement of longitudinal and circular muscles surrounding the parenchymal layer. However, the tegument and subtegument layers were observed to

be well-preserved. A mature proglottid of the parasite upon the treatment of methanol extract (**Figure 6.4**) exhibited irregular folds and shrinkage of the tegument and subtegumental layers and constriction of excretory canal. Moreover, it also revealed a reduction in egg cell, diffused parenchymal tissue, and disintegration of longitudinal muscle. As shown in the **Figure 6.5**, the chloroform extract also altered the morphology of parasite in a variety of ways such as removal of egg parenchyma leading to distorted configuration of the egg capsule, compression lateral excretory canal, evacuation of nuclear cytoplasm, and isolation of egg cell. In the cestode treated with petroleum ether extract as illustrated in the **Figure 6.6**, the tegument of a mature proglottid revealed the enlargement of the lateral excretory vacuole, assymmetrical folds in the tegumental layer, depletion of egg cells in various places resulting in shrinkage and clumping of the oncosphere at the central region, complete elimination of the parenchyma and muscle cells, and disappearance of nuclear muscle in every egg capsule. At last, regarding the cestode treated with aqueous extract shown in the **Figure 6.7**, a mature proglottid expressed a complete detachment of tegument and subtegument layers from the tissue with the reduction of lateral excretory canal in size, elimination of the parenchymal tissue and disassociation of the longitudinal muscle, minimization and isolation of egg cells, and an abnormal alignment of the egg capsules along the centre with absolute loss of nucleoplasm.

Assessment of the parasites using scanning electron microscopy (SEM):

The scanning electron micrographs of the untreated control of *R. echinobothrida* shown in the **Figure 6.8** and **Figure 6.9** displayed a scolex bearing well-arranged suckers and morphologically intact of protruding rostellum, and an enlarged portion of a sucker presented an entire set of hammer-shaped hooks. The tegument of mature proglottids also revealed a smooth surface without any deformities.

In the case of cestode treated with albendazole shown in the **Figure 6.10** and **Figure 6.11**, distortion of scolex with slight shrinkage of suckers with detached hooks, longitudinal folding of the proglottids, and tegumantal surface with irregular foldings at the tip of the proglottid were observed. The parasite treated with methanol

extract depicted in the **Figure 6.12** and **Figure 6.13**, it was assessed that the treatment affects the scolex with complete malformation of suckers and rostellum leading to total detachment of hooks, uneven fold and wrinkled-proglottids all over the topographical structure. As observed in the **Figure 6.14** and **Figure 6.15**, exposure of parasite to chloroform extract demonstrated intense shrinkage of the scolex with protruding suckers and crooked rostellum, complete loss of hooks in the suckers and disappearance of some in rostellum, occasional tegumental ridges in the anterior proglottids with disrupted and peeled tegument. When subjecting the parasite to petroleum ether extract of the plant shown in the **Figure 6.16** and **Figure 6.17**, it was visualized that extreme protuberance of rostellum and suckers with deeply folded neck, destruction of the sucker rim and hook defacement, and flattened teguments with continual folding. As for the treatment of the worm with aqueous extract as shown in the **Figure 6.18** and **Figure 6.19**, it was found that extensive degeneration of the scolex and rostellum, completely dislodged spines with pits and vacuolization on the surface, and formation of distinct corrugated folds and shrinkage on the teguments.

Determination of acid phosphatase (AcPase) activity of the treated parasite:

The acid phosphatase activities in *R. echinobothrida* maintained as untreated control, and treatments with different concentrations (20, 15, 10, 5 and 2 mg/ml) of albendazole and four different plant extracts were shown in **Figure 6.20**. The percentage decrease of enzyme activities of the parasites when subjected to the treatments was also presented in **Figure 6.21**. The control group exhibited the highest AcPase activity with 19.11 ± 1.35 unit/μM/mg/h. The treatment of cestodes with different doses of albendazole expressed the enzyme activity of 2.92 ± 0.39, 5.77 ± 0.15, 8.32 ± 0.26, 14.24 ± 0.20 and 16.71 ± 0.15 unit/μM/mg/h for 20, 15, 10, 5 and 2 mg/ml, respectively, with percentage decrease of enzyme activity of 84.71 ± 2.04, 69.80 ± 0.78, 56.47 ± 1.36, 25.88 ± 1.18 and 12.55 ± 0.78%, respectively. Among the treatments, across the concentration gradient from highest to lowest, methanol extract exhibited lowest activity of 0.60 ± 0.07, 1.57 ± 0.13, 2.40 ± 0.15, 5.02 ± 0.20 and 5.92 ± 0.07 unit/μM/mg/h with 96.86 ± 0.39, 91.76 ± 0.68, 87.45 ± 0.78, 73.73 ± 1.04 and 69.02 ± 0.39%. Meanwhile, chloroform and aqueous extracts

revealed almost identical dose-dependent activities of 3.30 ± 0.27, 7.57 ± 1.93, 8.24 ± 1.63, 10.27 ± 2.11, 14.09 ± 1.08 unit/μM/mg/h, and 6.00 ± 3.76, 7.42 ± 2.82, 9.14 ± 2.14, 10.87 ± 1.30, 13.34 ± 1.08 unit/μM/mg/h, respectively, and their percentage decrease from the control were 82.75 ± 1.41, 60.39 ± 10.08, 56.86 ± 8.55, 46.28 ± 11.04 and 26.27 ± 5.66% for chloroform extract, and 68.63 ± 19.69, 61.18 ± 14.74, 52.16 ± 11.21, 43.14 ± 6.80 and 30.20 ± 5.66% for aqueous extract with no significant differences across the two treatment groups at the same concentration. However, among the treatment groups, the only concentration-independent enzyme activity of cestodes was observed in the petroleum ether extract, which indicated the activity of 9.59 ± 0.33, 9.82 ± 0.45, 12.51 ± 1.59, 11.24 ± 0.39 and 13.04 ± 0.69 unit/μM/mg/h with the activity decreased by 49.80 ± 1.71, 48.63 ± 2.39, 34.51 ± 8.30, 41.18 ± 2.04 and 31.77 ± 3.59%. Overall, the lowest percentage decrease of enzyme activity was observed upon the treatment of petroleum ether extract, except the lowest dose (2 mg/ml), which is still greater than the treatment groups of aqueous and chloroform extracts at the same dose.

Determination of alkaline phosphatase (AlkPase) activity of the treated parasite:

The assessment of alkaline phosphatase activity of untreated group and treatment of *R. echinobothrida* with four different plant extracts of various concentrations viz. 20, 15, 10, 5 and 2 mg/ml were graphically represented in the **Figure 6.22**, and the percentage decrease of the AlkPase activity of the same treatments from untreated control was also shown in the **Figure 6.23**. The alkaline phosphatase activity was found to be the highest in the control group, that is 1344.42 ± 142.20 unit/μM/mg/h. Across varying concentrations from highest to lowest, the cestodes treated with the standard drug and the methanol extract of the plant exhibited closely similar dose-dependent enzyme activities of 31.47 ± 11.90, 106.12 ± 9.29, 139.84 ± 7.80, 158 ± 7.80, 194.70 ± 8.86 unit/μM/mg/h and 35.52 ± 1.62, 118.26 ± 11.92, 139.39 ± 9.74, 156.03 ± 9.09, 200.54 ± 7.02 unit/μM/mg/h, respectively, and the inhibition of AlkPase activity for the treatment of albendazole and methanol extract were calculated as 97.66 ± 0.88, 92.11 ± 0.69, 89.60 ± 0.58, 88.23 ± 0.58, 85.52 ± 0.66%, and 97.36 ± 0.12, 91.20 ± 0.89, 89.63 ± 0.72, 88.39 ± 0.68, 85.08 ± 0.52, respectively, without considerable variation across the two groups

at the same dose of treatment. A slightly higher enzyme activity of the cestodes with lower inhibition percentage was observed when treated with chloroform extract, viz. 293.17 ± 20.07, 310.25 ± 55.39, 347.57 ± 48.39, 345.77 ± 51.50 and 366.91 ± 58.68 unit/μM/mg/h with 78.19 ± 1.49, 76.92 ± 4.12, 74.18 ± 3.60, 74.28 ± 3.83 and 72.71 ± 4.36%. The worms on exposure to the petroleum ether extract revealed the enzyme activity of 133.54 ± 19.09, 137.59 ± 12.24, 310.70 ± 89.48 and 482.01 ± 145.64 unit/μM/mg/h with 90.07 ± 1.42, 89.77 ± 0.91, 76.89 ± 10.33, 77.12 ± 6.66 and 64.15 ± 10.83% reduction of the enzyme. Across all the concentrations, except the highest dose (20 mg/ml), of the treatment groups, the cestodes treated with aqueous extract showed a remarkable concentration-dependent with highest enzyme activity of 140.74 ± 32.53, 330.04 ± 94.94, 497.75 ± 152.44, 586.78 ± 153.48 and 859.26 ± 226.75 unit/μM/mg/h, and the reduction of the alkaline phosphatase enzyme calculated from the control was expressed as 89.53 ± 2.42, 75.45 ± 7.06, 62.98 ± 11.34, 56.35 ± 11.42 and 36.09 ± 16.87%.

DISCUSSION

Helminthiasis is one of the major health threats in human and animal and become an economic burden with the emergence of anthelmintic drugs globally. Due to this, scientists have nowhere else rather than returning to medicinal plants as sources of novel anthelmintic therapeutic agents (Manjusa & Pradeep, 2022). Plants from different families such as Fabaceae, Lamiaceae and Asteraceae are ethnomedicinally used as dewormers and numerous surveys have been conducted from various plant species of these families (Jato *et al*., 2022). Some plants from Mizoram, India were also evaluated for their anthelmintic properties of the above-mentioned families. For instance, the the treatment of *R. echinobothrida* with *Millettia pachycarpa* (Fabaceae) showed decreasing in concentration of trace metals such as calcium, magnesium, sodium and potassium, which are responsible for metabolic intermediates, respiration and whole parasites' physiology. Since the parasite-host interaction was altered, it was suggested that cause the death of the parasites (Lalchhandama *et al*., 2008). The study of anthelmintic effect on *R. echinobothrida* and *R. tetragona* through scanning electron microscopy upon treatment of *Acmella oleracea* (Asteraceae), *Schima wallichi* (Theaceae), *Ilex khasiana* (Aquifoliaceae), *Callicarpa arborea* (Lamiaceae), *Imperata cylindrica* (Poaceae), *Millettia pachycarpa* (Fabaceae), and *Acacia caesia* (Mimosaceae) all exhibited morphological changes such as shrunken teguments, severe alterations in the scolex including suckers and rotellum with loss of spines (Lalthanpuii *et al*., 2020; Lalthanpuii *et al*., 2024; Lalnunfela *et al*., 2020; Lalthanpuii & Lalchhandama, 2020; Roy *et al*., 2008; Lalchhandama, 2009).

This study also revealed that treatment of four different extracts of *H. excelsa* leaves on *R. echinobothrida* have shown different kinds of therapeutic effects on their anatomical structure including pit formation and irregular folding of the tegument with wrinked proglottids, distortion and intense shrinkage of the scolex with destruction of the suckers with crooked rostellum, and some to total loss of hammer-shaped hooks.

Anthelmintic drugs like albendazole are known to enter target parasites by either oral administration or by permeation through the external surface called tegument in cestodes. The tegument is metabolically active, structurally specialized junction to execute nutrient uptake from the host organism, secretion of glycoproteins for protective immunity against diseases, osmotic homeostasis and sensory reception. Thus, this passive transport across the tegument is the principal mechanism of dewormer drugs penetration into the worms which consequently induce destruction of the helminth's surface. Since anthelmintic drugs disrupt steady state of microtubule by binding specifically to the tubulins, it ultimately leads to cell lysis. As it inhibits glucose uptake which solely depends on polymerization of α- and β-tubulins, the occurrence of glucose depletion therefore leads the worms into starvation, followed by paralysis, and finally killed (Lalchhandama *et al*., 2008). The tegumental enzymes such as acid and alkaline phosphatase are known to be structurally very closed-knitted with teguments and subteguments of the cestodes, and the functions of the tegument are suggested to be functionally competent with the help of these enzymes (Dalton & Brindley, 2010). In this study, the two tegumental enzymes of *R. echinobthrida* extracted from the teguments of the parasites were studied. Between the two untreated groups for acid and alkaline phosphatases, the latter expressed a comparatively higher degree of activity. This finding supported the previous investigations conducted by Lalchhandama et al. (2008), that suggested among the two enzymes, AlkPase is the dominant enzyme. Moreover, the said research team also observed that the cestodes treated with albendazole, and methanol, ethanol and acetone extracts of *M. pachycarpa* exhibited significant inhibition in the two phosphatase enzyme activities.

The exploration of the inhibitory potential of *H. excelsa* leaves extracts and reference standard drug was also conducted in the tegumental enzymes of *R. echinobothrida.* It was found that the methanol, chloroform, petroleum ether and aqueous extracts of *H. excelsa*, including albendazole successfully inhibited the AcPase and AlkPase activities, at the same time, some extracts outstandingly surpassed the inhibition activity of the standard drug specifically in AcPase.

The further investigation of anthelmintic activity of the plant extracts by histological study of stained sections of the proglottid of the cestode demonstrated that various kinds of alterations including irregular folds and shrinkage of the tegument and subtegument, assymetrical organization and disintegration of the muscle tissues, isolation, loss, displacement and clumping of the egg cells, distortion, reduction and enlargement in size of the lateral excretory canal, elimination or dispersion of parenchymal tissues, and in some cases, loss of nucleoplasm. These observations were found to be substantiated by the findings which indicated that the reduction of the enzyme activities was correlated with histological degradation (Saha *et al*., 2023). The same cases were also expressed on the treatment of *A. oxyphylla* and *Alstonia scholaris* (Roy *et al*., 2007; Lalngaihmanawmi *et al*., 2025).

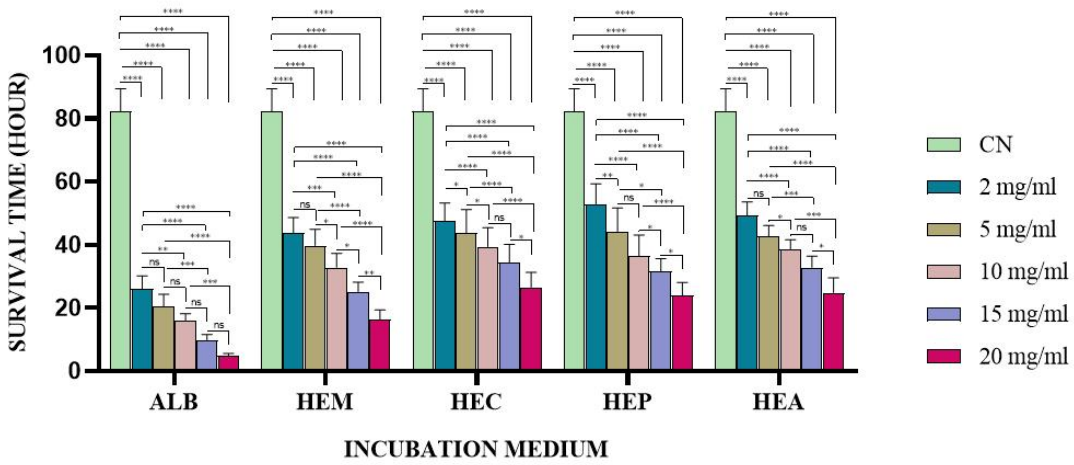

Figure 6.1: Time taken for different doses of *H. excelsa* extracts (HEM, HEC, HEP, HEA) to cause significant mortality on *R. echinobothrida* using albendazole as a reference anthelmintic drug.

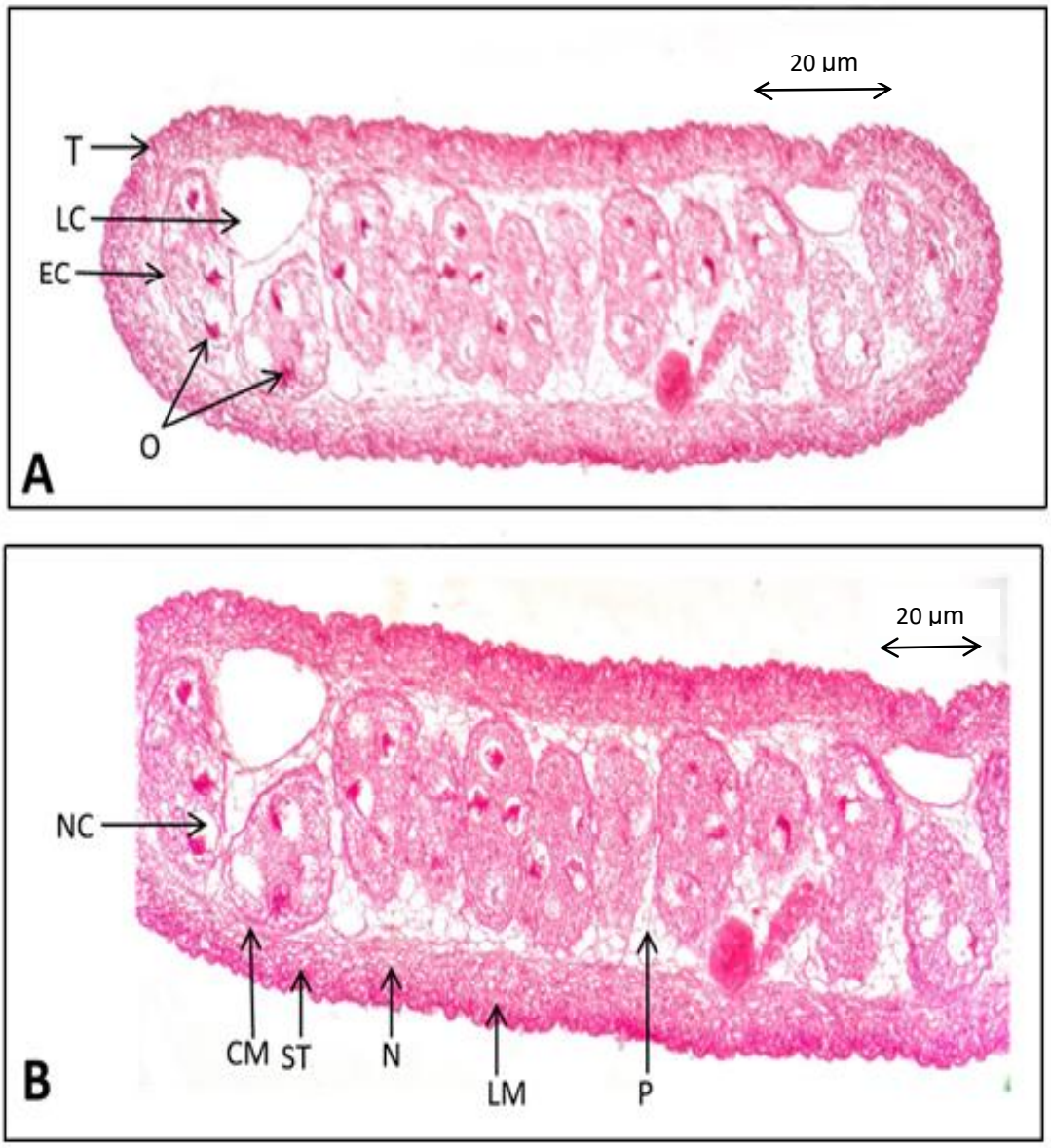

Figure 6.2: Light micrographs of histological section of untreated control of *R. echinobothrida.*

A: Mature proglottid showing a well-preserved tegument (T), a regular size lateral excretory canal (LC) and a number of eggs containing ovaries or oncosphere (O) in a large egg capsule (EC). x 20.

B: An enlarged portion of A displaying a fine subtegument (ST), nerve cords (N), longitudinal muscle (LM), circular muscle (CM) and a layer of parenchyma cells (P). NC - Nuclear cytoplasm x 40.

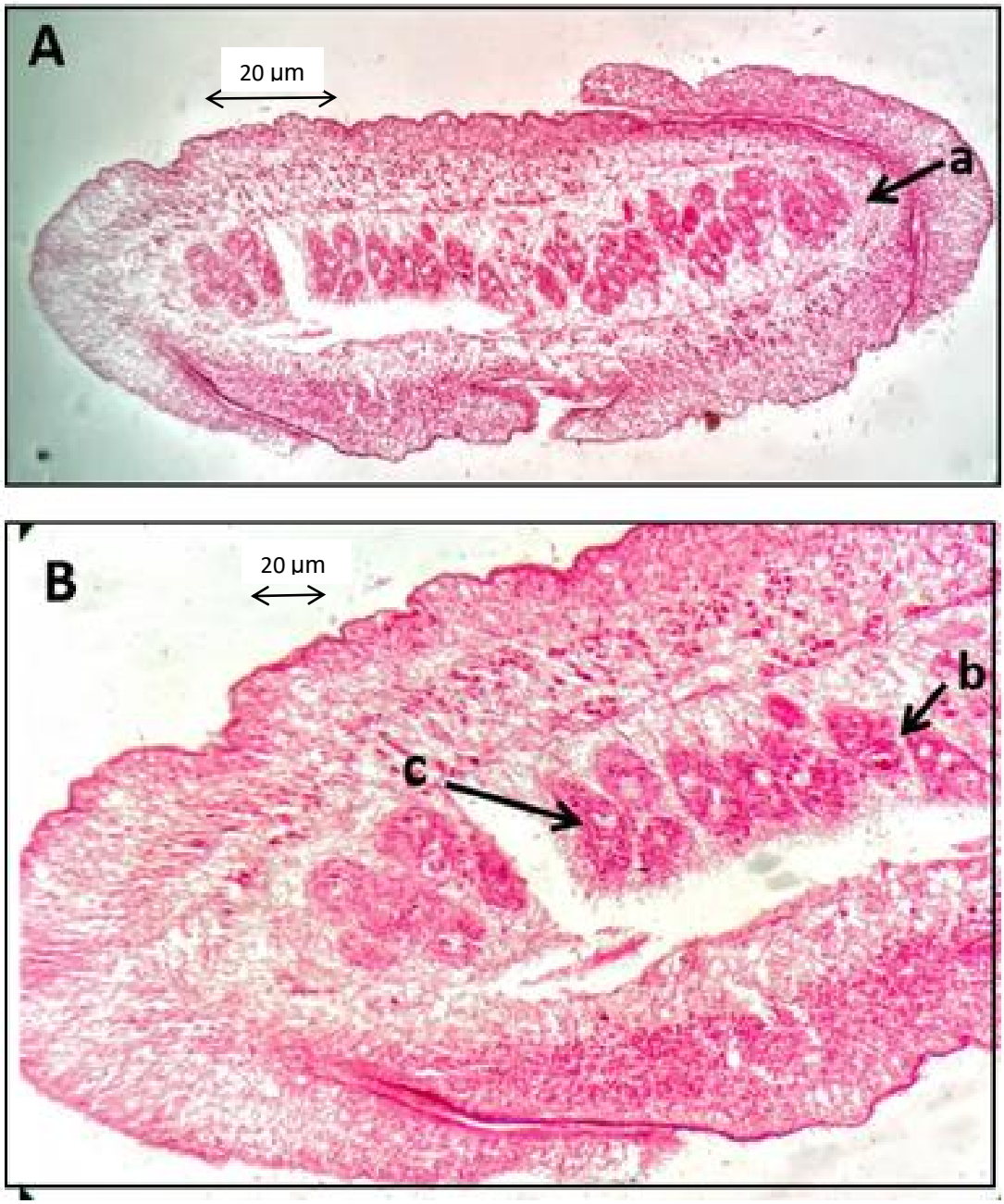

Figure 6.3: Light micrographs of histological section of *R. echinobothrida* treated with 20 mg/ml of the standard drug albendazole of *H. excelsa*.

A: The tegument of a mature proglottid showing a complete devoid of lateral excretory canal (a). x 20.

B: A magnified portion revealing filamentous (b) and loss of egg cells (c), irregular arrangement of longitudinal muscle and surrounding parenchyma. x 40.

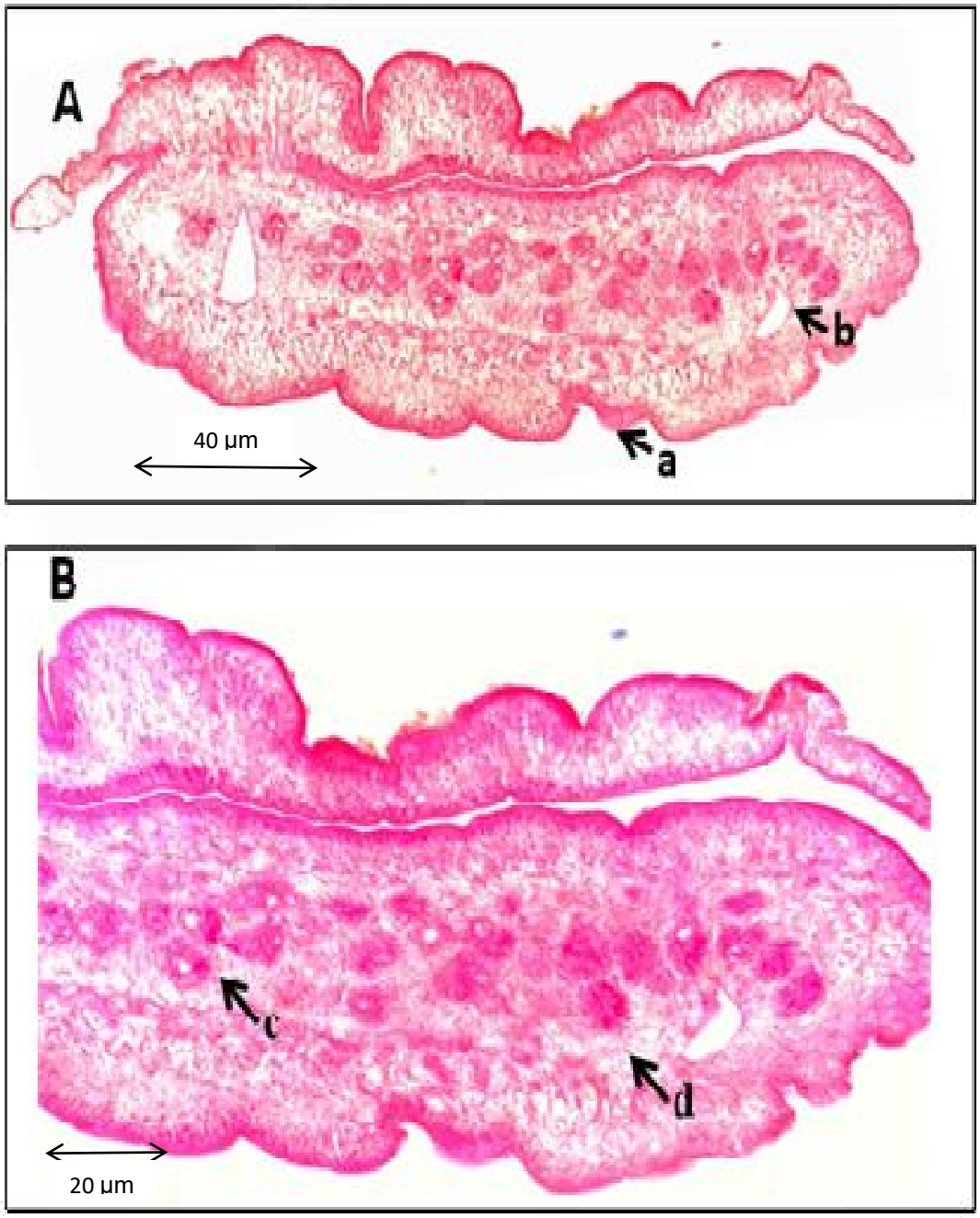

Figure 6.4: Light micrographs of histological section of *R. echinobothrida* treated with 20 mg/ml of the methanol extract of *H. excelsa*.

A: A mature proglottid exhibited irregular folds and shrinkage of the tegument and subtegumental layer (a) and constriction of lateral canal (b). x 20.

B: A magnified portion revealing egg cell reduction (c) and fuzzy egg parenchyma (d). Disintegration of longitudinal muscle is also visible. x 40

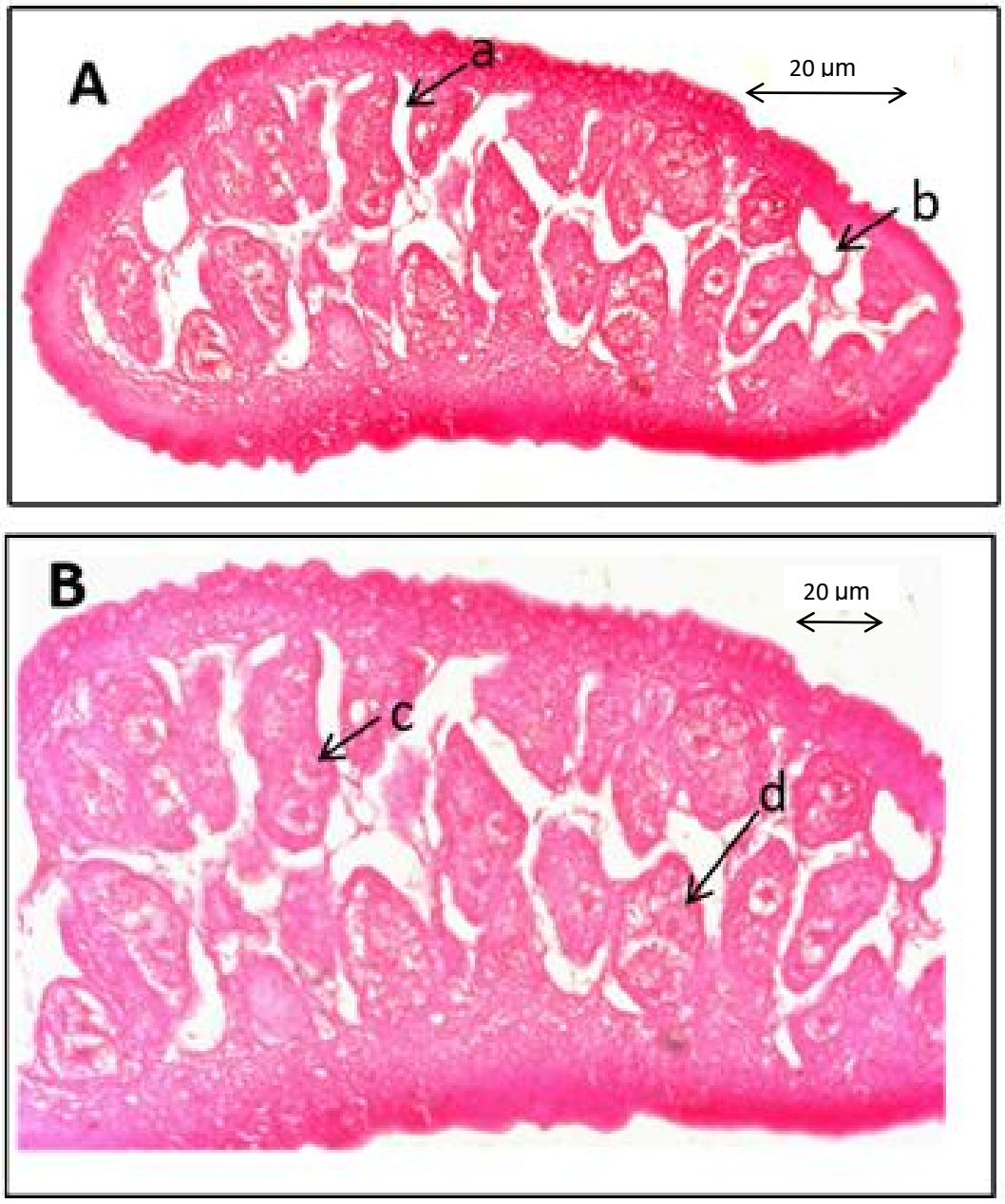

Figure 6.5: Light micrographs of histological section of *R. echinobothrida* treated with 20 mg/ml of the chloroform extract of *H. excelsa*.

A: The tegument of a mature proglottid showing removal of egg parenchyma (a) leading to aberrant arrangement of the egg capsule and narrowed lateral excretory canal (b). x 20.

B: An enlarged portion showcasing nuclear cytoplasm removal (c) and egg cell isolation (d). x 40.

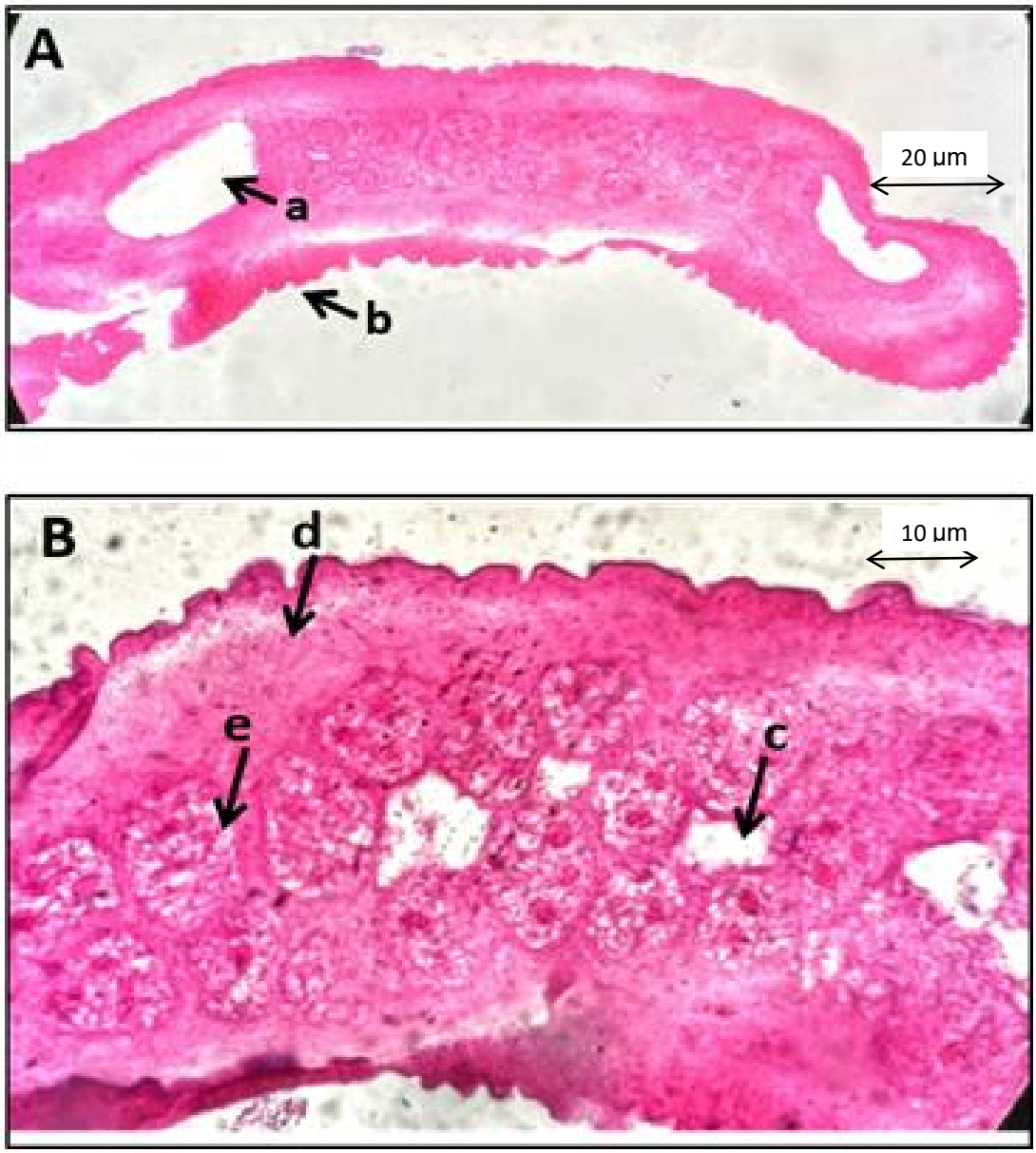

Figure 6.6: Light micrographs of histological section of *R. echinobothrida* treated with 20 mg/ml of the petroleum ether extract of *H. excelsa*.

A: The tegument of a mature proglottid revealing enlargement of the lateral excretory vacuole (a) and regular tegumental folds (b). x 20.

B: A magnified portion with loss of egg cells in various places (c) leading to shrinkage and clumping of the oncosphere at the centre, parenchyma and muscle cells completely removed (d) and disappearance of nuclear muscle in every egg capsule (e). x 40.

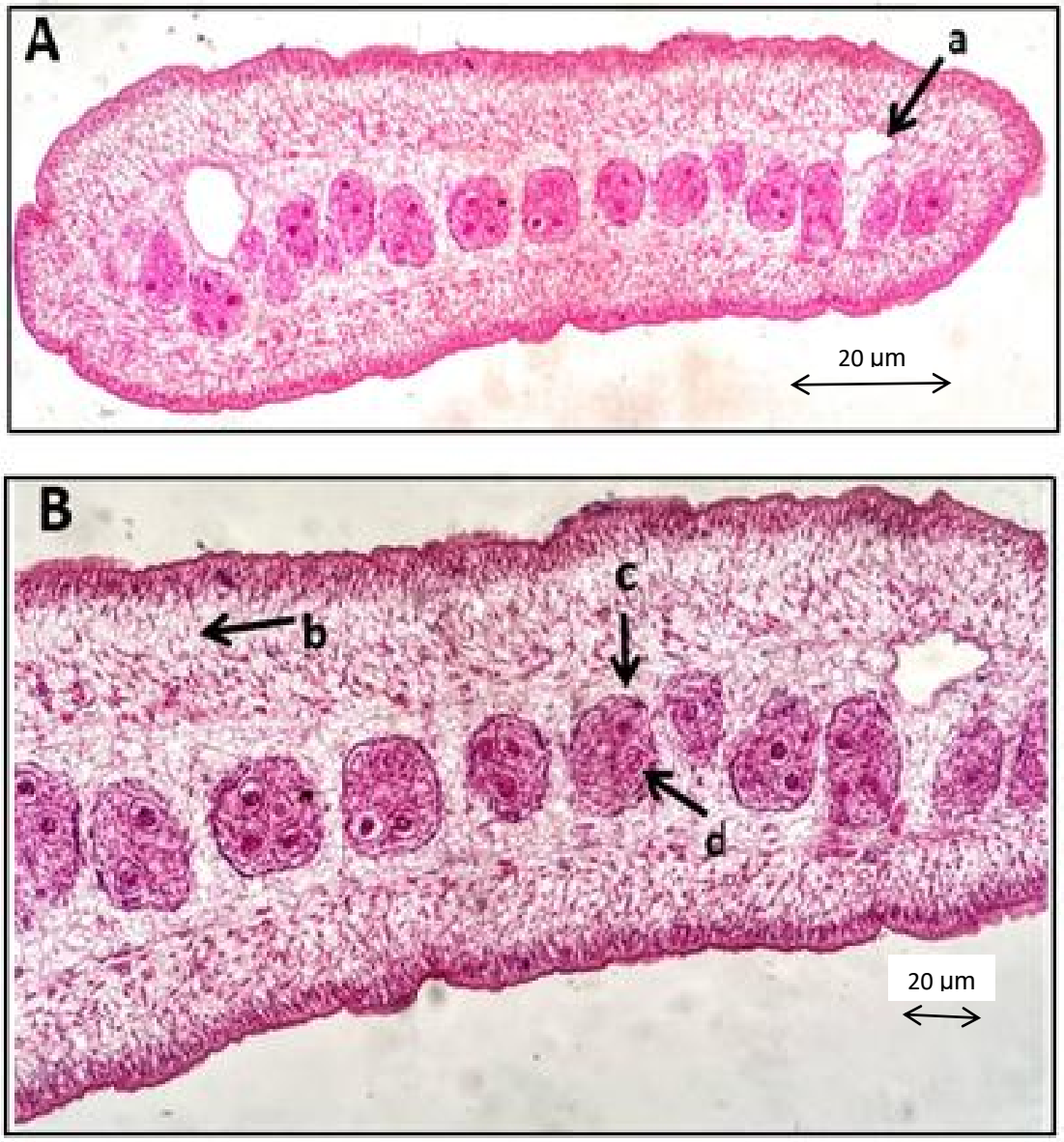

Figure 6.7: Light micrographs of histological section of *R. echinobothrida* treated with 20 mg/ml of the aqueous extract of *H. excelsa*.

A: The tegument of a mature proglottid expressing a complete disassociation of tegument and subtegumental from the tissue with reduction of lateral canal in size (a). x 20.

B: A magnified portion showing removal of parenchyma and disintegration of longitudinal muscle (b), reduction and isolation of egg cells (c), an unusual alignment of the egg capsules along the centre with complete loss of nucleoplasm (d). x 40.

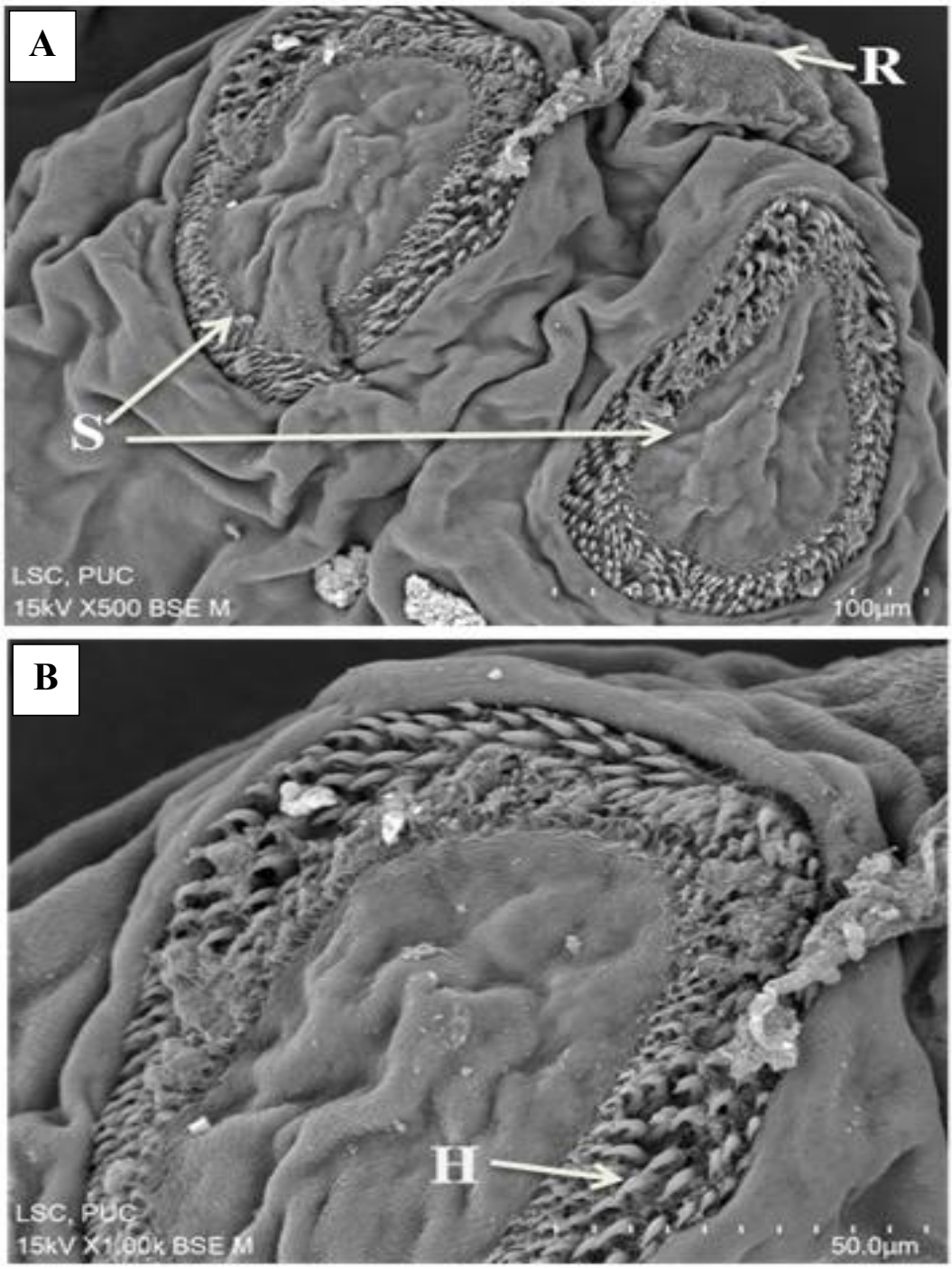

Figure 6.8: Scanning electron micrographs of the scolex of untreated control of *R. echinobothrida.*

A: A scolex bearing 2 well-arranged suckers (S) and rostellum (R).

B: An enlarged portion of sucker having a complete set of hammer-shaped hooks (H).

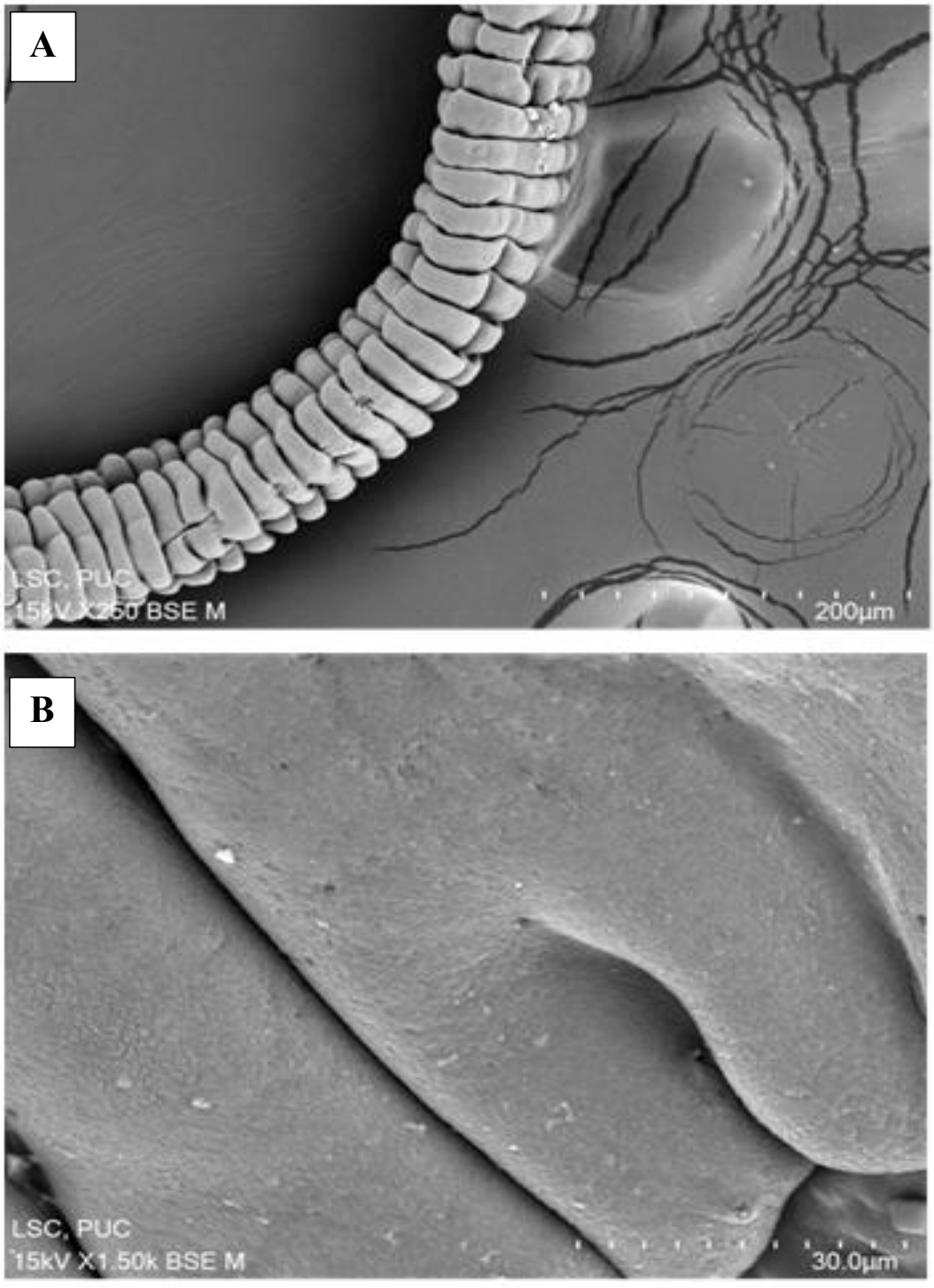

Figure 6.9: Scanning electron micrographs of the mature proglottid of untreated control of *R. echinobothrida.*

A: The tegument of mature proglottids.

B: A magnified portion of a smooth proglottid without any deformities.

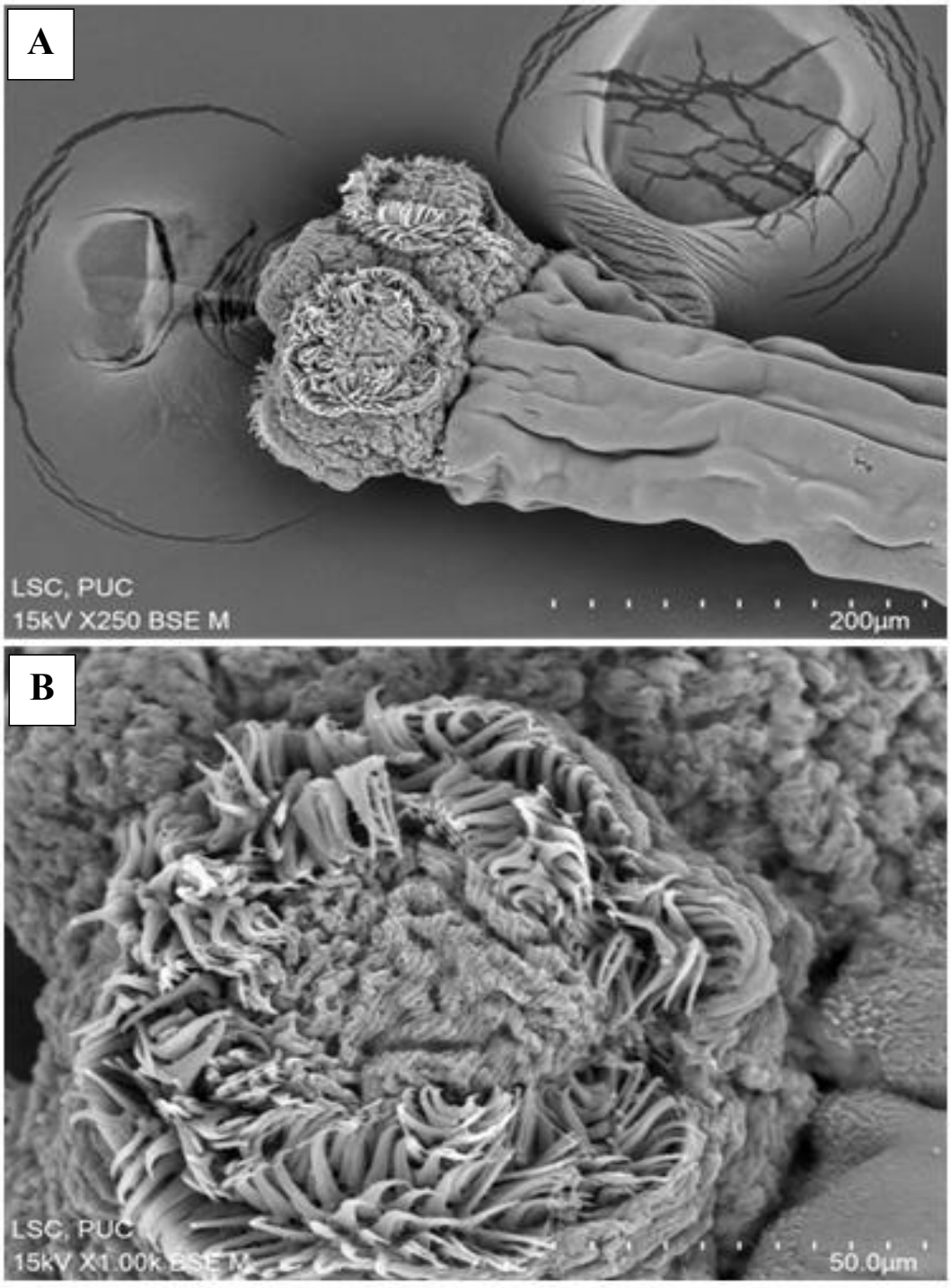

Figure 6.10: Scanning electron micrographs of the scolex of *R. echinobothrida* treated with 20 mg/ml of a standard drug albendazole.

A: Deformation of the scolex with slightly shrinkage of suckers.

B: An enlarged portion of the sucker showing detached hooks.

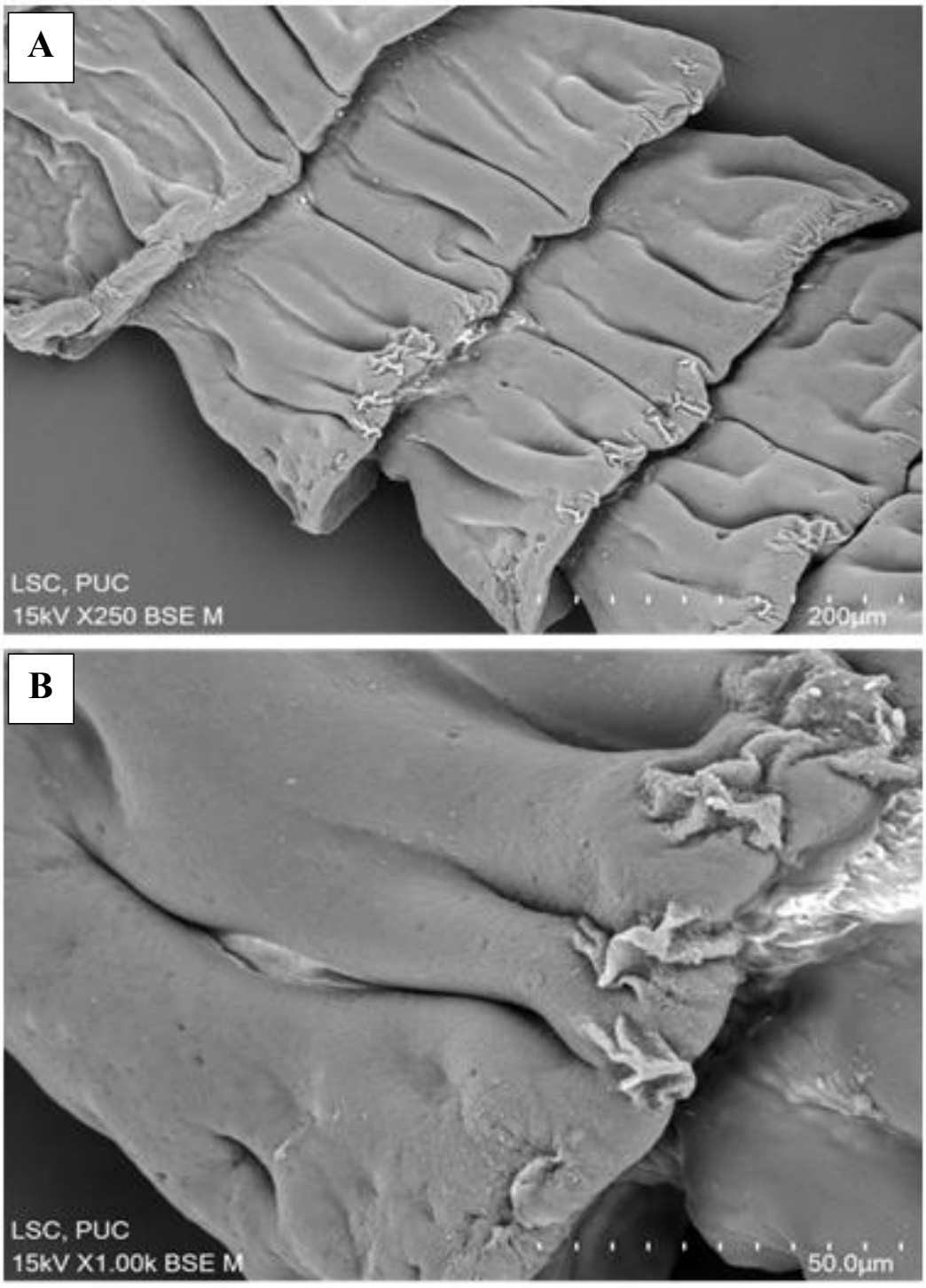

Figure 6.11: Scanning electron micrographs of mature proglottid of *R. echinobothrida* treated with 20 mg/ml of a standard drug albendazole.

A: Longitudinal folding of the proglottids.

B: Magnified tegumental surface having irregular foldings at the tip of the proglottid.

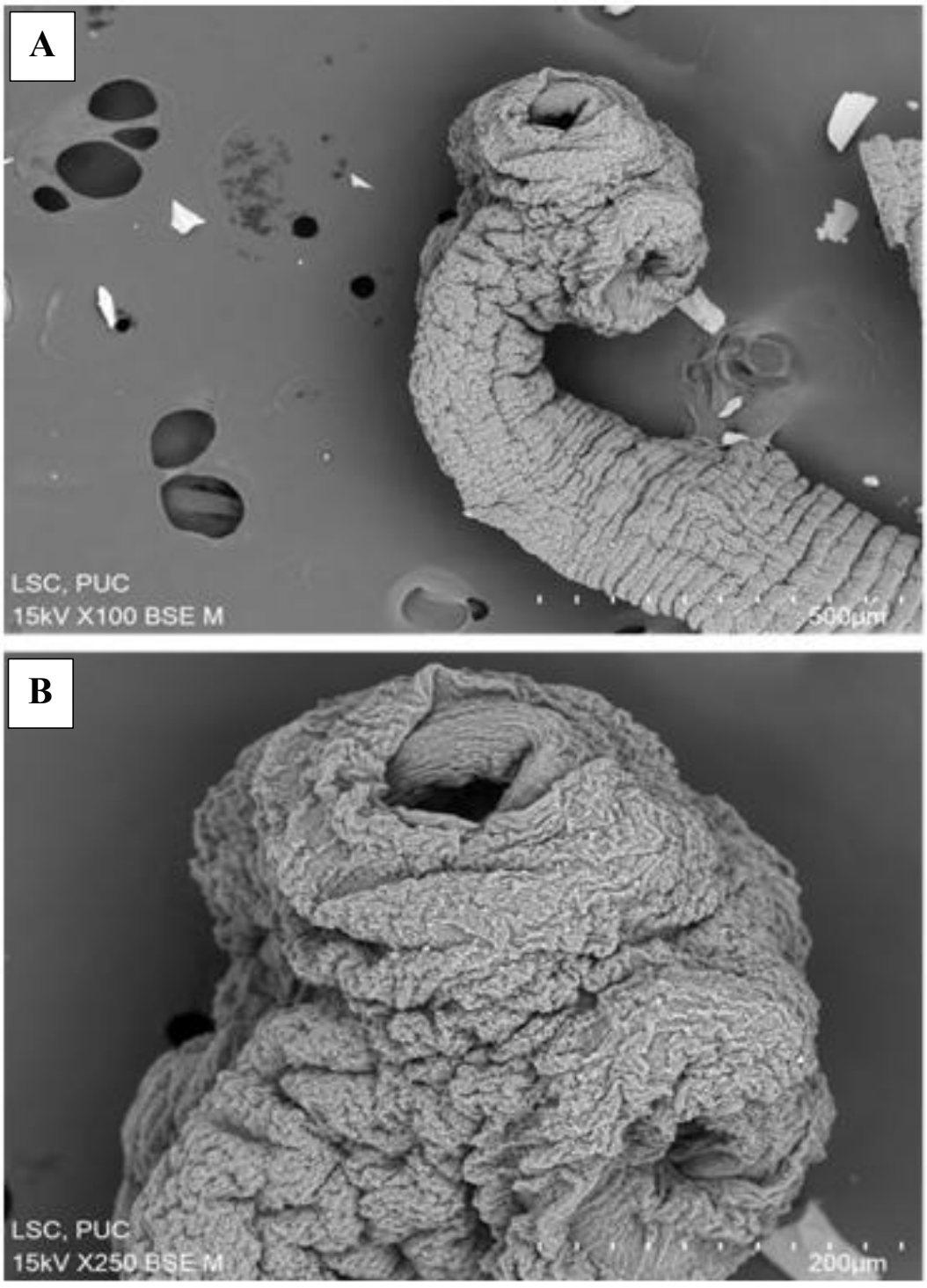

Figure 6.12: Scanning electron micrographs of the scolex of *R. echinobothrida* treated with 20 mg/ml of the methanol extract of *H. excelsa*.

A: A scolex posessing complete deformation of the sucker and rostellum.

B: Sucker deeply contorted entirely without hooks.

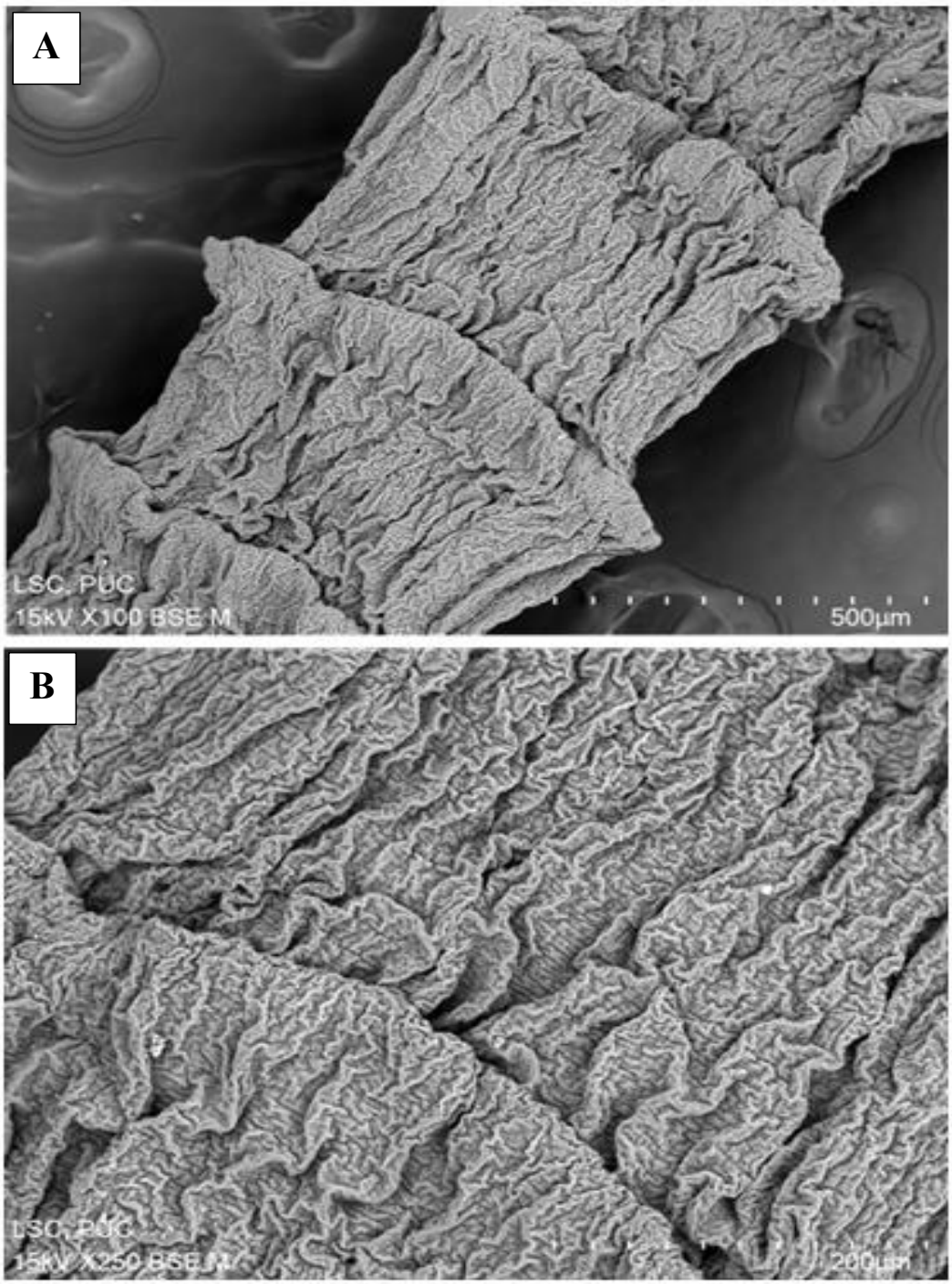

Figure 6.13: Scanning electron micrographs of mature proglottid of *R. echinobothrida* treated with 20 mg/ml of the methanol extract of *H. excelsa*.

A: Mature body segments with extreme shinkage and folding of the proglottids.

B: An enlarged portion of uneven folding and wrinkled-proglottids all over the surface topography.

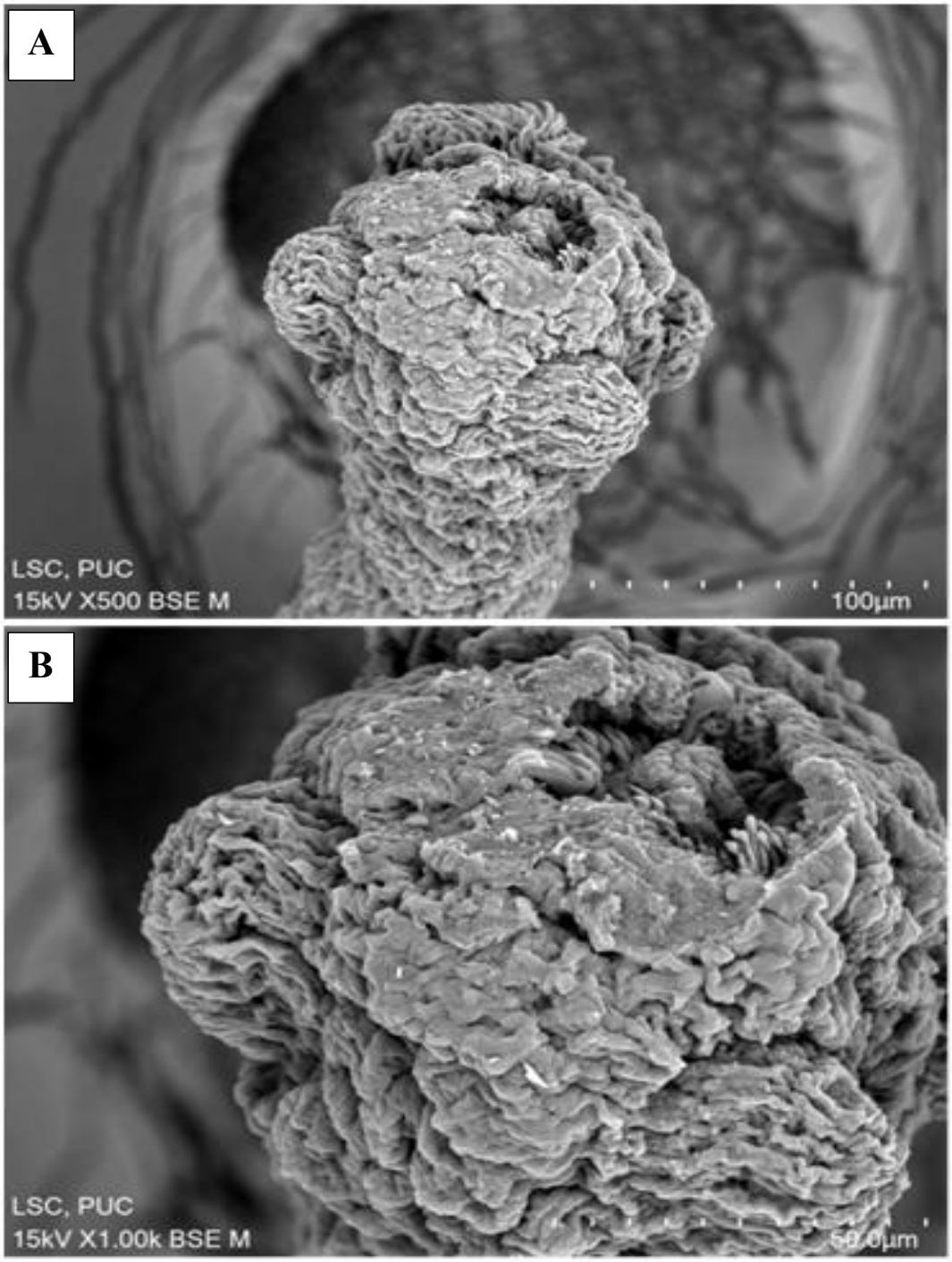

Figure 6.14: Scanning electron micrographs of the scolex of *R. echinobothrida* treated with 20 mg/ml of the chloroform extract of *H. excelsa*.

A: Shrinkage of the scolex with protruding suckers and crooked rostellum.

B: Enlarged scolex showing complete loss of hooks in the suckers and disappearance of some in rostellum.

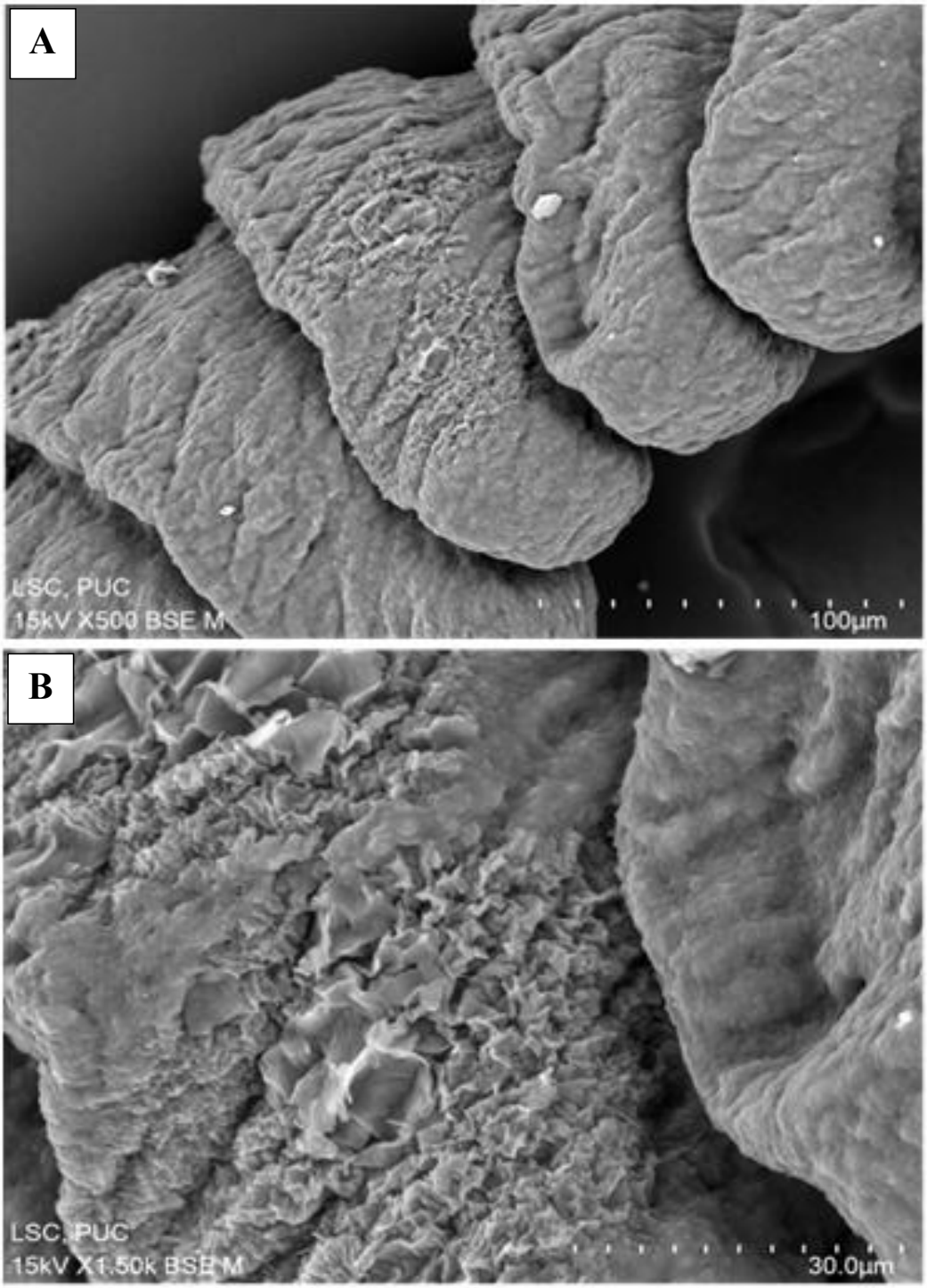

Figure 6.15: Scanning electron micrographs of mature proglottid of *R. echinobothrida* treated with 20 mg/ml of the chloroform extract of *H. excelsa*.

A: Anterior proglottids with intermittent tegumental folding.

B: An enlarged portion of cracking and peeling of the tegument.

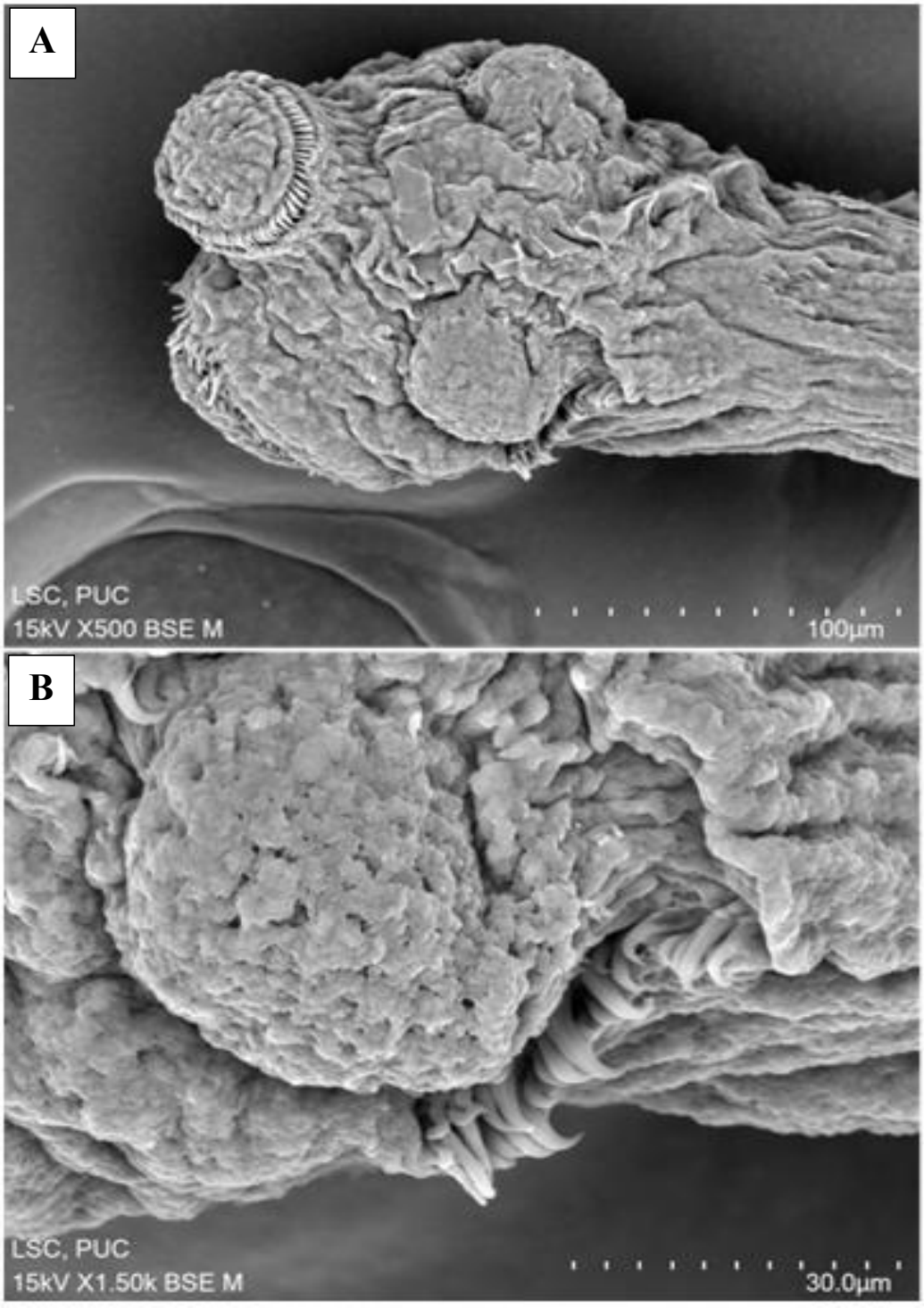

Figure 6.16: Scanning electron micrographs of the scolex of *R. echinobothrida* treated with 20 mg/ml of the petroleum ether extract of *H. excelsa*.

A: Extreme protuberance of rostellum and suckers with deeply folded neck.

B: An enlarged sucker with destruction of the sucker rim and hook defacement.

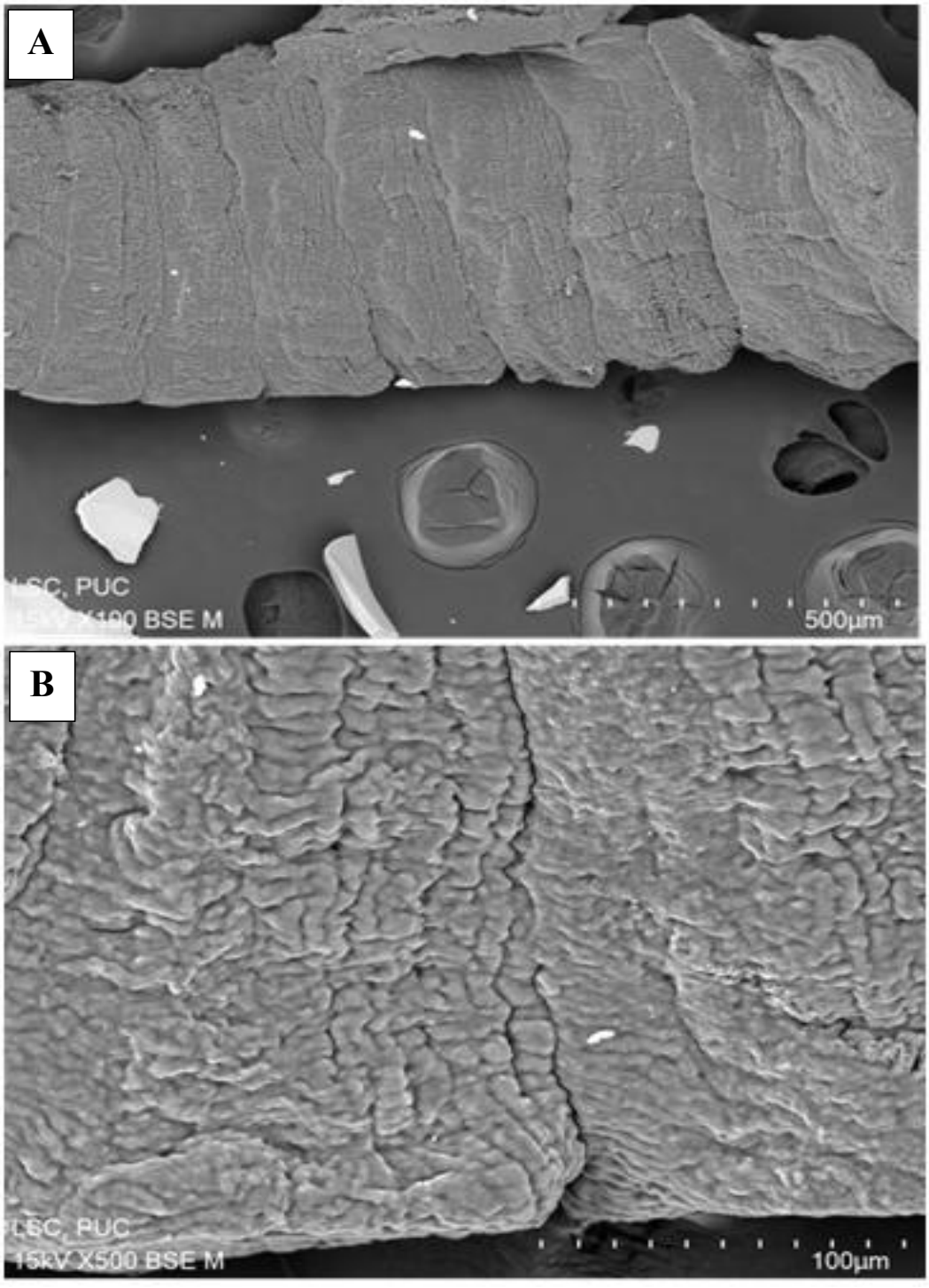

Figure 6.17: Scanning electron micrographs of mature proglottid of *R. echinobothrida* treated with 20 mg/ml of the petroleum ether extract of *H. excelsa*.

A: Flattened teguments with continual folding.

B: Magnified portion of the proglottid showing small and recurrent shrinkage.

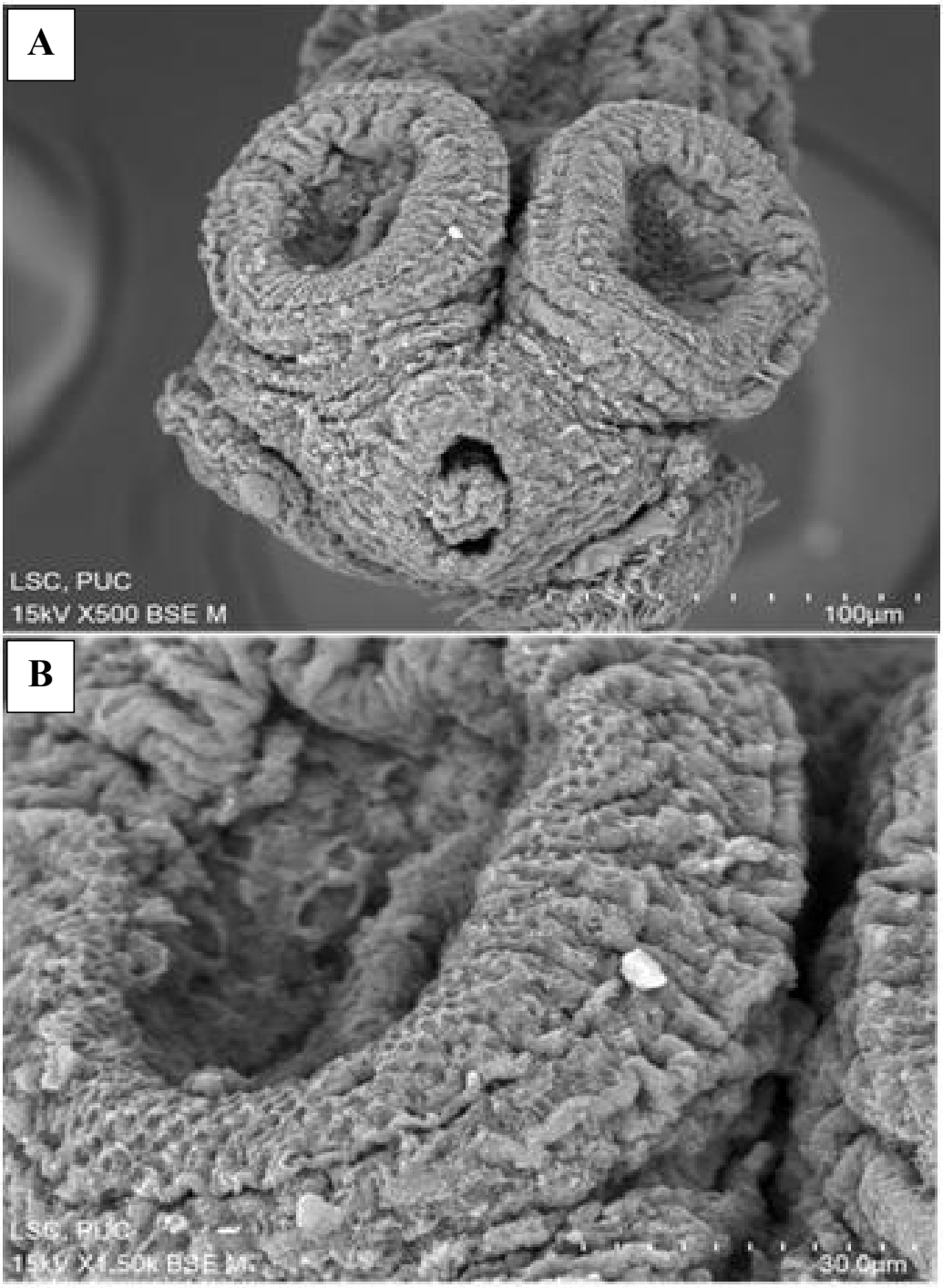

Figure 6.18: Scanning electron micrographs of the scolex of *R. echinobothrida* treated with 20 mg/ml of the aqueous extract of *H. excelsa*.

A: Extensive degeneration of the scolex and rostellum.

B: Enlarged sucker of completely dislodged spines with pits and vacuolization on the surface.

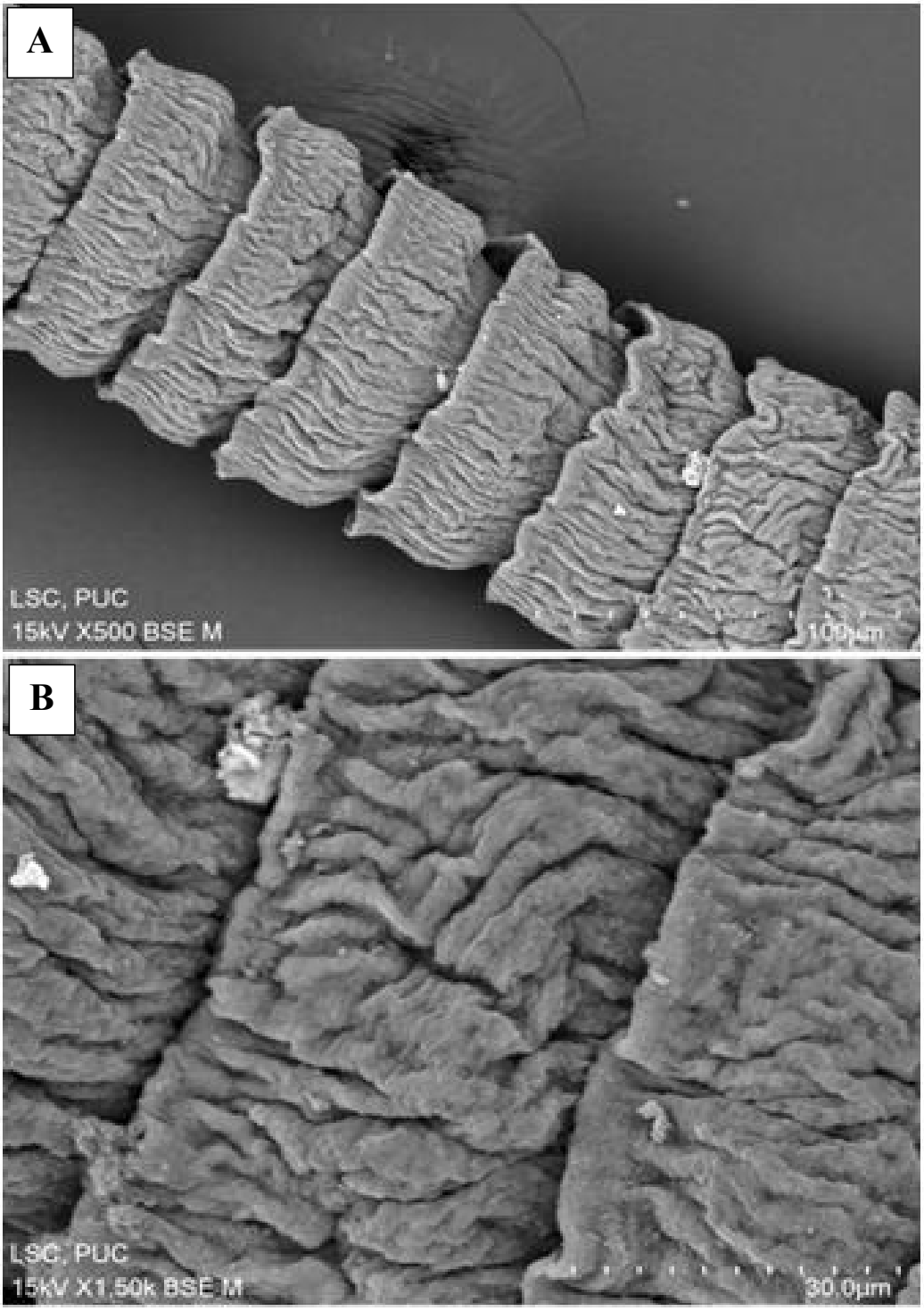

Figure 6.19: Scanning electron micrographs of mature proglottid of *R. echinobothrida* treated with 20 mg/ml of the aqueous extract of *H. excelsa*.

A: Formation of distinct corrugated folds and shrinkage on the teguments.

B: Magnified segment of the proglottids.

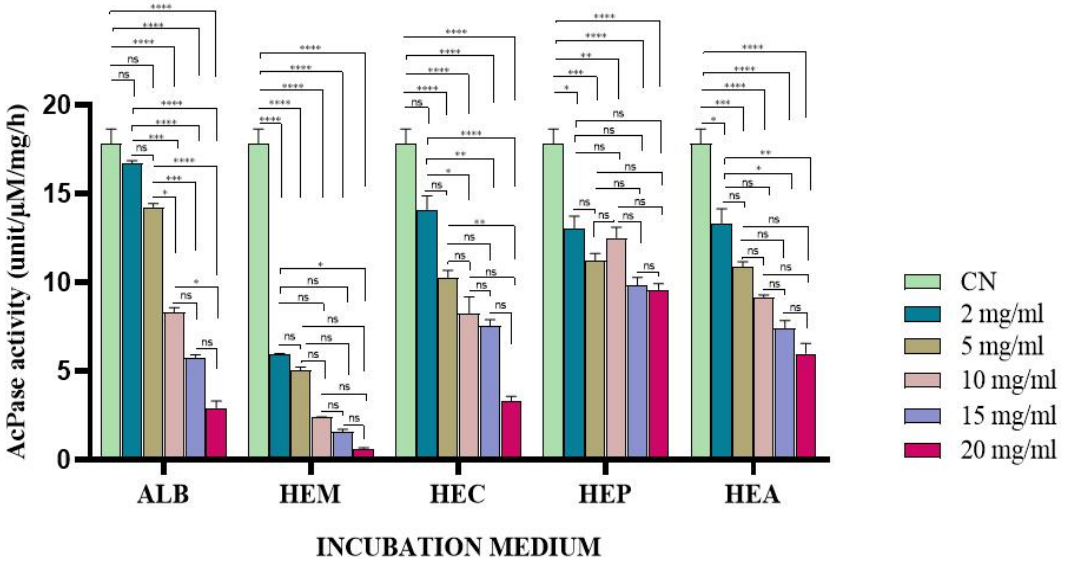

Figure 6.20: A graphical representation of the activity of acid phosphatase of tegumental enzyme of *R. echinobothrida* treated with different dose of the *H. excelsa* extracts (HEM, HEC, HEP, HEM) in reference with the standard drug i.e. albendazole.

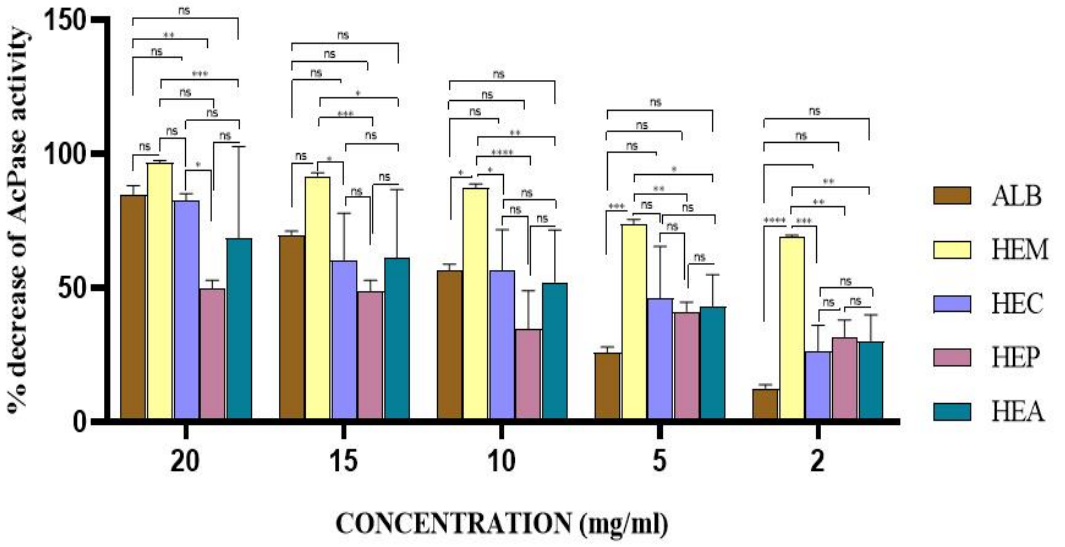

Figure 6.21: A graphical representation of percentage (%) decrease of acid phosphatase activity of tegumental enzyme of *R. echinobothrida* treated with different dose of the *H. excelsa* extracts (HEM, HEC, HEP, HEM) in reference with the standard drug i.e. albendazole.

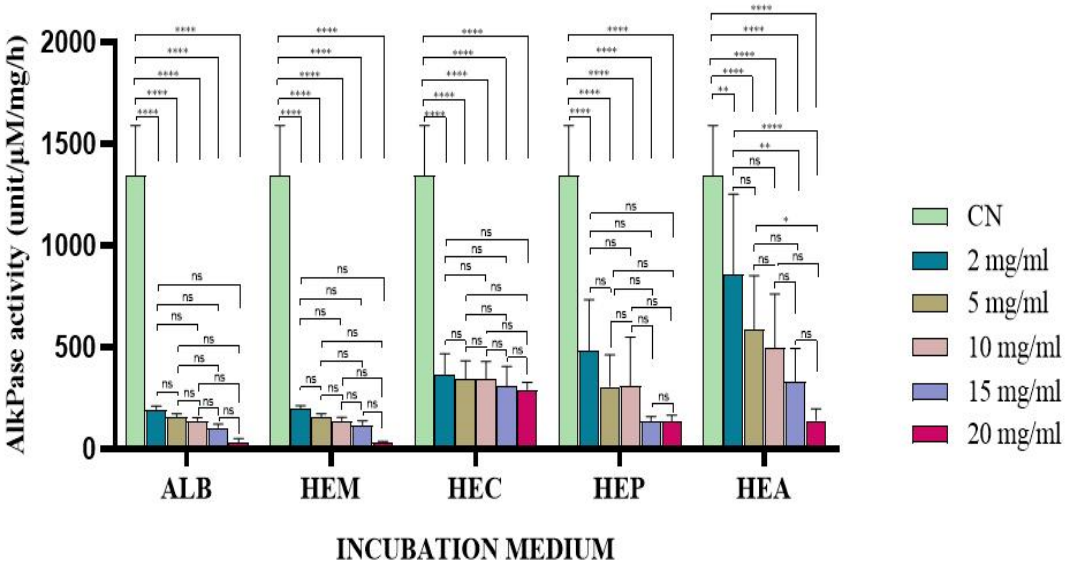

Figure 6.22: A graphical representation of the activity of alkaline phosphatase of the tegumental enzyme of *R. echinobothrida* treated with different dose of the *H. excelsa* extracts (HEM, HEC, HEP, HEM) in reference with the standard drug i.e. albendazole.

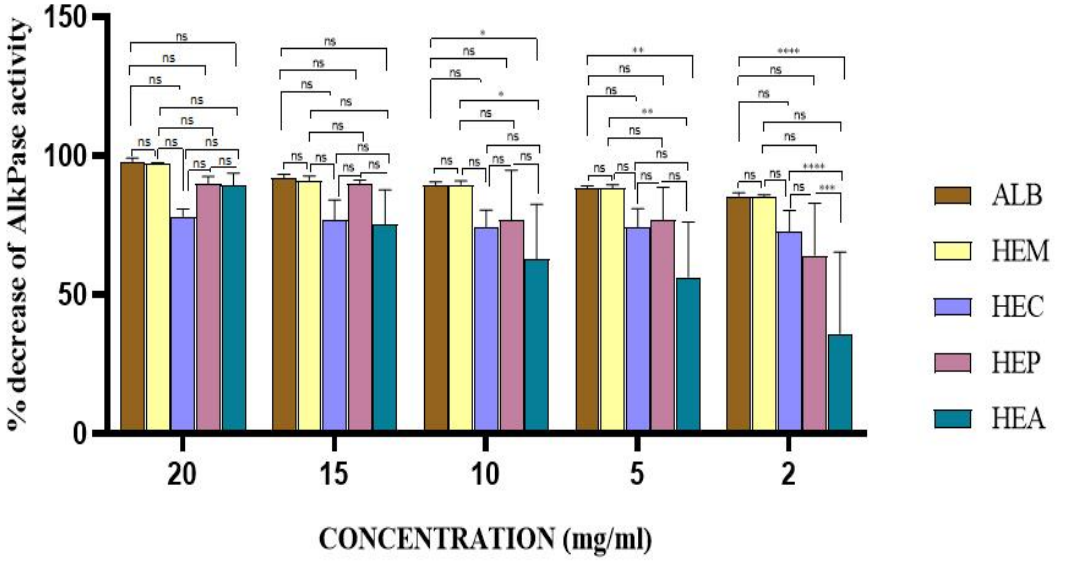

Figure 6.23: A graphical representation of the percentage (%) reduction activity alkaline phosphatase activity of the tegumental enzyme of *R. echinobothrida* treated with different dose of the *H. excelsa* extracts (HEM, HEC, HEP, HEM) in reference with the standard drug i.e. albendazole.

SUMMARY

Identification and extraction of the selected plant

1. The plant selected for this thesis is an important ethnomedicinal plant species traditionally used by Mizo community for the treatment of various ailments. A careful morphological, molecular, online and book based identification was carried out.

2. Morphological based identification: BSI identified the herbaria of the plant as *Helicia excelsa* with identification number: BSI/ERC/Tech/2021-22/389. Online based identification: Additional authentication was performed with specimens maintained at the Natural History Museum, London (herbarium numbers BM013822430 and BM000951124) and the Royal Botanic Gardens, Kew (numbers K000009323 and K001110658).
Book based identification: A renowned local taxonomist, M. Sawmliana also identified it as *H. excelsa* in his book *The book of Mizoram Plants.*
Molecular based identification: The final sequence of *ITS* gene based isolation of *H. excelsa* leaf was deposited to NCBI GenBank under accession no PX245743. By using this generated sequence, since no exact matching sequence was not found, and four nearest genera under the family Proteaceae, *viz*, *Helicia* sp., *Grevillea* sp., *Virotia* sp., and *Protea* sp. retrieved from NCBI GenBank were used for phylogenetic analysis. Based on constructed ML tree, each genus under Proteaceae clustered cohesively forming a monophyletic group. The generated sequence showed closest similarity with *H. nilagirica* (MW044388).

3. Extraction of the plant: The yield of extraction of *H. excelsa* leaves using four solvents of varying polarities is in the ascending order of methanol extract>petroleum ether extract>aqueous extract>chloroform extract.

Phytochemical profiling of *H. excelsa*

4. Preliminary phytochemical screening: The analysis of *H. excelsa* extracts using phytochemical screening characterized a diverse class of secondary metabolites having significant pharmacological activities. The primary phytochemical analysis revealed that methanol and aqueous extracts of the plant are remarkably rich in essential bioactive compounds such as alkaloids, phenolics, flavonoids, glycosides, saponins, and phytosterols, which take part in their bioactivity including antioxidant, anti-inflammatory, antimicrobial, and antiparasitic effects.

5. The presence of sterols and cardiac glycosides offers complementary support for the therapeutic application of this traditional plant and highlights its potential as a source for novel drug discovery.

6. GC-MS analysis: This analysis strengthened the findings mentioned above by identifying a comprehensive structurally wide range of compounds belong to different compound classes across all the extracts with their biological activity potentials. Meanwhile, methanol and petroleum ether usually extracted terpenoids and lipophilic compounds, whereas aqueous favored the extraction of sugars, sugar alcohols and oxygeneted aromatics, which clearly showed that solvent polarity considerably influenced the extract composition.

7. Since the identification and detection of various chemical compounds of *H. excelsa*, it is then believed that this plant offers valuable lead compounds appropriate for target-driven drug screening and formulation design.

Antioxidant activity of *H. excelsa*

8. Total phenolic content (TPC): The TPC quantified based on Folin-Ciocalteu assay highlighted the methanolic and aqueous extracts were richest in phenolics which indicates high polyphenolic content, an important contributor to antioxidant activity.

9. Total flavonoid content (TFC): The TFC of methanol extract showed the highest flavonoid content, closely followed by chloroform and aqueous

extracts of the plant, suggesting substantial presence of flavonoid antioxidants.

10. Total antioxidant capacity (TAC): In TAC, methanol extract exhibited marvellous antioxidant capacity which equivalent nearly four times that of aqueous extract and approximately nine times that of the chloroform and petroleum ether extracts.

11. Ferric reducing antioxidant power (FRAP): In the $Fe^{3+} \rightarrow Fe^{2+}$ reduction ability test using FRAP assay, methanol extract continued to dominate other extracts across all concentrations, followed by aqueous extract, while chloroform and petroleum ether extracts exhibited comparatively lower reducing activity.

12. Diphenyl-1-pycrylhydrazyl (DPPH) scavenging activity: In the antioxidant activity assessed by IC_{50} of DPPH activity using serial extract concentrations, methanol extract maintained to express the highest activity over the other extracts with no statistically significance when compared with standard BHT which clearly shows its potent free radical scavenging activity.

13. To conclude the plant antioxidant activity, the methanol extract displayed exceptional antioxidant properties for all the activity assays which was comparable to known synthetic standards. These findings implies strong potential for the development of *H. excelsa*-derived antioxidant agents for therapeutic or preservative use.

Antimicrobial activity of *H. excelsa*

14. Antibacterial activity: The extracts prepared in four different concentrations such as 500, 250, 125 and 62.5 mg/ml was used to assess the antibacterial activity of *H. excelsa* by following agar well method. This assessment showed that the methanol extract exhibited concentration-dependent activity across all the tested bacterial strains, while the chloroform showed no activity against *M. luteus* and the petroleum ether extract expressed no antibacterial activity at all.

15. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): Regardless of the activity expressed by the above-mentioned assay, MIC and MBC of these extracts exhibited unexpected turn when performed by resazurin-based 96-well plate and agar streak methods, respectively. The methanol extract showed MIC at the concentration ranging from 1.95 - 15.63 mg/ml, while the chloroform extract has in between 0.98 - 7.813 mg/ml, and the aqueous extract ranging from 7.813 - 125 mg/ml. In the meantime, MBC at the concentrations ranging from 3.91 - 62.5 mg/ml, 3.91 - 31.25 mg/ml and 15.63 - 125 mg/ml were observed in the methanol, chloroform and aqueous extracts, respectively. Notably, the lowest MIC was expressed against a gram-positive *B. cereus* in all extracts.

16. Antifungal activity: The antifungal activity determined by poisoned food technique was tested in four different concentrations viz., 1, 0.5, 0.25 and 0.125% against four pathogenic fungal strains. The result showed that dose-dependent activity upon the treatments of the methanol, chloroform and aqueous extracts. While the other fungal strains responded based on serial doses of the chloroform extract, 0.5 and 0.25% concentration exhibited almost similar activity against *F. oxysporum*. Both the methanol and aqueous extracts were found to have the highest efficacy against *P. oryzae,* simultaneously, the petroleum and chloroform extracts showed their highest antifungal activities against *F. keratoplasticum* and *F. oxysporum*, respectively.

17. The outcomes suggested that *H. excelsa* possesses a rich reservoir of bioactive antimicrobial agents, which later may serve as a source of novel antimicrobial drugs with deeper studies on their mechanism of action and further phytochemical exploration.

Anthelmintic activity of *H. excelsa*

18. Survival test of the parasites: This study revealed that the anticestodal activity of different extracts of *H. excelsa* leaves and albendazole engaged in the dose-dependant viability of the cestodes. The parasites upon the highest

dose of treatment (20 mg/ml) of different extracts of the plant and reference standard albendazole, including control showed a survival time of the parasites in the order of albendazole (4.893 ± 0.697 h)>methanol extract (16.323 ± 3.047 h)>petroleum ether extract (24.033 ± 4.090 h)>aqueous extract (24.889 ± 4.630 h)>chloroform extract (26.53 ± 4.72 h)>control (82.200 ± 7.217).

19. Acid phosphatase activity (AcPase): The AcPase of the tegumental enzymes of *R. echinobothrida* showed concentration dependent activity across all the concentrations prepared, except petroleum ether extract, with almost completely inhibited the enzyme activities in some extracts, including albendazole. The efficacy of enzymes inhibitory property of the highest dose (20 mg/ml) of plant extracts and albendazole is in the order of methanol extract>albendazole>chloroform extract>aqueous extract>petroleum ether extract for AcPase.

20. Alkaline phosphatase enzyme activity (AlkPase): Upon treatment of the highest concentration of four extracts of the plants and albendazole, the AlkPase of the tegumental enzyme of R. echinobothrida was found to be in the order of albendazole>methanol extract>petroleum ether extract>aqueous extract>chloroform extract.

21. Scanning electron microscopic (SEM) and histological studies of the treated parasites: The findings of the phosphatase enzyme activity inhibited by the extracts and albendazole supported by the microscopic studies of the worms by SEM and light microscopy are the clear indication of the the anthelmintic efficacy of the plant.

22. In addition to the extracts exhibiting significant anthelmintic activity, the present investigation is the second ever documented anthelmintic activity from the entire family of Proteaceae, after *Roupala montana* from the genus *Roupala* against platyhelminthes (*Schistosoma mansomi*) (Takahashi *et al*., 2004).

23. The overall anthelmintic effects suggest that the plant consists active phytochemicals which potentially possess distinguished chemical and biological properties responsible for the said activity.

24. Based on the overall results, the phytochemical profiling validates *H. excelsa* being used as an ethnomedicinal plant for its potency in various therapeutic purposes. Even though only a limited applications of therapeutical properties and biological activities of the plant were carried out in this study, it somehow is given the outstanding potential of the plant. Therefore, the further exploration, such as mechanism of action of *H. excelsa* extracts is recommended as it may contribute to an important discovery of novel plant-derived drugs to treat various diseases.

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APPENDIX - I

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
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No. PUC/IEC/2019-21/029 06 August 2020

To

K. Lalchhandama
Department of Life Sciences
Pachhunga University College

Subjects: Application for Ethics Approval Certificate.

I refer to your application to the IEC of Pachhunga University College (dated 03/08/2020), and I am pleased to inform you that your proposed project, after scrutiny, is duly approved by the committee.

The details are given below:

Project title	Characterisation of medicinal plants and their effects on helminth parasites, bacteria and fungi
Animal/organism handling involved	1. Chickens from market and their parasites 2. Lab sub-cultures of bacteria 3. Lab sub-cultures of fungi
Approval number	PUC-IEC-2020-Z65
Approval date	06/08/2020

The approval is on the conditions that you shall:

- use and handle the biological samples, i.e., laboratory mice, chicken parasites and microbes according to the ethical guidelines laid down by the Committee for the Purpose of Control of Supervision of Experiments on Animals (CPCSEA), and Animal Dissection Monitoring Committee of Pachhunga University College;
- submit annual progress report of the research;
- and facilitate inspection of the research environment from time to time.

Dr H. Lalthanzara
i/c Chairman

Dr Laramliana
Secretary

Institutional Animal Ethics Committee of Pachhunga University College

BIO-DATA

Name: LALBIAKNGHETI TLAU

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Educational qualifications:

Exams	Year	Board/University	Subject	Division	Percentage
HSLC	2009	Mizoram Board of School Education	General	Second	51.2
HSSLC	2012	Mizoram Board of School Education	Science	Second	56.6
B. Sc	2015	Mizoram University	Botany	First	72.08
M. Sc	2017	Mizoram University	Botany	First	74.32

PAPER PRESENTED IN SYMPOSIUM/ CONFERENCE

INTERNATIONAL CONFERENCE/ SEMINAR

1. Oral presentation on the topic Conservation and Medicinal Studies of **"Helicia excelsa from Mizoram, Northeast India"** at International Conference on biodiversity and Conversation (ICBC) held at St. Anthony's College, Shillong on the 14th & 15th December, 2022.

2. Oral presentation at 'PUC Research conclave 2023 (An international Workshop on Research and Development organized by Research and Development Cell, Pachhunga University College) on the topic **"Phytochemical Analysis and Evaluation of the Medicinal Properties of Helicia sp. (Proteaceae)"**.

3. Oral presentation on the topic "**Antioxidant, antimicrobial and anthelmintic activity of *Helicia excelsa* leaf extracts from Mizoram, india"** at the International Conference on Conservation of Biodiversity in Genomics Era Held at Pachhunga University, Aizawl, Mizoram, during 22nd - 23rd March, 2024.

4. Oral presentation on the topic "**Unveiling the Potential Antioxidant and Antimicrobial Activity of Mizo Traditional Medicinal Plant (*Helicia excelsa*)"** at the International Conference on Advances in biological Sciences (ICABS) at Pachhunga University College, Aizawl, during 6th - 7th February, 2025.

5. Oral presentation on the topic "**Ethno-medicinal Survey of an Important Mizo Traditional Plant - *Helicia excelsa* from Mizoram, India"** on 3rd International Symposium on Plant Taxonomy, Ethnobotany, Botanical Gardens and Biodiversity Conservation during 13th - 15th February, 2025, Kolkata.

NATIONAL CONFERENE/SEMINAR

1. Oral presentation at 'Mizoram Science Congress held at Aijal Club, Aizawl during 24-25 November, 2022 on the topic **"Qualitative Phytochemical Screening, Assessment of Antioxidant Property and Antibacterial Activity of Helicia excelsa Leaf".**

2. Poster presentation on the topic "**Quantification of Phytochemical Property, Antibacterial and Anthelmintic activity of *Helicia excelsa* Leaf"** at the National Conference on Challenges and Approaches in Biological Sciences (CABS) held on March 7 & 8, 2024 at Madras Christian College, Chennai.

TRAINING/WORKSHOP ATTENDED

1. Participated in the UGC STRIDE Workshop on "Molecular Phylogeny and Molecular Docking" held from 19th - 27th January, 2021 at Mizoram University, Aizawl, Mizoram.

2. Attended the "Workshop on Traditional Medicine" under the aegis of the International Conference on Biodiversity and Conservation held on 15th December, 2022 at St. Anthony's College, Shillong, Meghalaya.

3. Participated at the Hands-on "Molecular Phylogenetics Workshop" during 9th – 14th March 2023 sponsored by DBT- Advance State Level Biotech Hub & Mizoram University.

LIST OF PUBLICATIONS

1. Tlau L, Lalawmpuii L. Analysis of the Phytochemical and Antibacterial Properties of the Indigenous Mizo Medicinal Plant, *Helicia excelsa*. *Pharmacognosy Journal.* 2023; 15(5): 823-828. DOI: 10.5530/pj.2023.15.157.
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ABSTRACT

PHYTOCHEMICAL ANALYSIS AND EVALUATION OF THE MEDICINAL PROPERTIES OF *HELICIA* SP. (PROTEACEAE)

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

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ABSTRACT

PHYTOCHEMICAL ANALYSIS AND EVALUATION OF THE MEDICINAL PROPERTIES OF *HELICIA* SP. (PROTEACEAE)

BY

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Submitted

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

Plants have been long used for medicine since prehistoric time. Some of the firsts recorded medicinal plants and their applications were documented in ancient Mesopotamia, Ebers papyrus, Ayurveda in ancient India, as well as in Chinese writings. Traditional medical systems are still widely practiced across the world. Among many other reasons, due to population growth, burdening treatment costs, adverse effects of synthetic drugs, and an emerging resistance to several drugs for infectious diseases lead to resurgence in medicinal plants which threatens some plants to near extinction. To this matter, preventive measures are required for sustainability. India is a rich reservoir of medicinal plants and to which, Mizoram contributes approximately 160 plant species across various families.

The therapeutic properties of plants rely on their phytocompounds known as secondary metabolites. These secondary metabolites of plants are organic compounds produced by plants which play a vital role in signaling, defense, and ecological interactions of the plants. Several classes of these compounds including alkaloids, flavonoids, phenols, tannins, terpenes, saponins, glycosides, and others are responsible for the biological properties of medicinal plants. The trans-formative force for discovering more compounds from plants was started when morphine was isolated from opium poppy for the first time in 1920s, which was followed by the isolation of quinine and caffeine from cinchona bark and coffee, respectively. The isolation of these alkaloids finally led to modern pharmaceutical industry with the help of more accelerated isolation processes using GC-MS, HPLC, LC-MS/MS and various other methods. The compound isolation then advances the understanding of molecular profiling of the compounds which provide a fundamental principle for the development of modern pharmaceutical products that can be working as drugs to treat diseases.

In 1928, Alexander Flemming's accidental discovery of penicillin from *Penicillum notatum* set a ground-work for the modern antibiotic era which further led to the discovery of many other antibacterial and antifungal drugs. However, as though these drugs have been saving countless lives, the overreliance on it causes a global phenomenon called antibiotic resistance worldwide which poses a threat to human health on the verge of eliminating years of hard works and achievements in

medical world. To minimize and avert this challenge, researchers explore possible remedies in medicinal plants acknowledging that plants possess secondary metabolites of various classes which are potent of several medicinal properties. When these compounds act as antioxidants, they help prevent or slowing the process of cell damage by oxidative stress that takes place in the human body. Moreover, several studies had proved that phytochemicals such as phenols, alkaloids, and terpenes possess antimicrobial properties which can be leading molecules to develop novel antimicrobial drugs to overcome resistant microbes. Accordingly, the other phytocompounds have been investigated for numerous other diseases, including communicable and non-communicable diseases.

Among communicable diseases, helminthiasis becomes a critical public health concerns which causes an illness to approximately 450 million individuals each year. Most of the parasitic infections are mainly caused by *Ascaris lumbricoides*, *Giardia lamblia*, and *Trichuris trichiura.* Depends on the contaminated environments, in association with environmental factors including temperature, humidity, rainfall and others, the frequency of the infection can be varied. If the parasitic infection is not treated, it often results to death. Other animals such as chicken are also affected by parasites. *Raillietina echinobothrida* is one of the major parasites which causes intestinal blockage, cellular damage, anemia, malnutrition, secondary infections by virus and bacteria, and in some cases, leading to death of the fowl. These infections also impact adverse economic consequences in poultry farming as they cause reduction in productivity through retarding growth rate, reducing production levels and quality of meat and egg, decrease feed effectiveness, and lower laying performance. Due to the long-term dependency on synthetic anthelmintic drugs like albendazole, ivermectin, and fenbendazole for treating helminthiasis, the resistance has been recognized in human and livestocks and its frequency has become a global phenomenon. Since the limited availability of antiparasitic drugs, there is a considerable demand for new anthelmintic medications from medicinal plants that have strong anthelmintic action.

Many plants have been investigated for their pharmacological properties according to their medicinal applications by particular ethnic or cultural groups.

Since the effectiveness of allopathic medicines have been declined, accompanied by the continuous emergence of resistance, the ethnopharmacological assessment of plants captures many researchers' attention. Despite being diverse group of plants consisting of around 83 genrea with approximately 1,600 species, the family Proteaceae is barely studied. A few plants from *Persoonia*, *Lomatia*, *Hakea*, *Banksia*, *Leucodendron*, and *Macadamia* are the prominent studied genera. Majority of these plants are commonly found in Australia, South America, and South Africa. These plants have various ethnomedicinal importance especially among indigenous Australians for the treatment of various diseases including cold, gastrointestinal ailments, anti-inflammatory, diabetes, respiratory diseases, skin infections, and others. Additionally, some plants from Proteaceae also provide economic value by producing nutritional, horticultural, and structural materials. The Mizo people from Mizoram, India also use plants from *Helicia* genus such as *H. nilagirica*, and *H. excelsa*, locally known as Pasaltakaza, and Sialhma, respectively. They are predominantly ethnomedicinally used to treat stomach problems in most parts of Mizoram. Sialhma is commonly used to treat other health problems such as fever and diarrhea, urinary problems, convulsions, parasitic infection like scabies, and in removing retained placenta. Since the morphological resemblance of the two plants, they are always misidentified. However, the herbaria prepared from Sialhma sent to Botanical Survey of India, Shillong, India cleared this doubt and identified the plant as *H. excelsa.* The molecular identification of the plant also showed that there is variation between the two while sharing the closest similarity with *H. nilagirica.* The final sequences of *H. excelsa* were also deposited to NCBI GenBank under accession no PX245743.

Since *H. nilagirica*, the most studied species of *Helicia*, has made a substantial contribution to pharmaceutical science, it is quite likely that the other species of *Helicia* will also have some pertinent medicinal qualities. Despite numerous records of ethnomedicinal significance, *H. excelsa* was chosen for this study due to the relatively restricted number of investigations and literature evaluations. The leaves of the selected plant were then collected and air dried in a room temperature. Coarsely ground dried leaves were loaded in Soxhlet apparatus

and the plant was extracted in four different solvents of varying polarities such as petroleum ehter, chloroform, methanol, and water. The crude extracts of the plant were subjected to various tests by following the objectives of the study:

Objective 1: Identification and Analysis of the Chemical Constituents of *Helicia* sp.

The analysis of *H. excelsa* extracts using phytochemical screening characterized a diverse class of secondary metabolites known to have significant pharmacological activities. The analysis revealed that methanol and aqueous extracts of the plant are remarkably rich in essential bioactive compounds such as alkaloids, phenolics, flavonoids, glycosides, saponins, and phytosterols. The presence of sterols and cardiac glycosides offers complementary support for the therapeutic application of this traditional plant and highlights its bioactivity. By detecting a broad structural range of compounds from several chemical classes in all extracts with possible biological activity, the GC-MS analysis validated these findings. It was evident that solvent polarity had a significant impact on the extract composition since aqueous preferred the extraction of sugars, sugar alcohols, and oxygeneted aromatics, while methanol and petroleum ether typically extracted terpenoids and lipophilic chemicals.

Objective 2: Determination and Quantification of the Antioxidant Property of *Helicia* sp.

The absorbance value of a serial concentration (10, 20, 40, 60, 80, 100 µg/ml) of gallic acid, quercetin, and ascorbic acid at 765, 510, and 695 nm, were used to calculate the total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant capacity (TAC), respectively of the extracts of *H. excelsa.* The TPC of the plant were found to be 11.768 ± 0.078, 4.516 ± 0.085, 2.392 ± 0.068 and 11.711 ± 0.346 GAE mg/g of methanol, chloroform, petroleum ether and aqueous extracts of the plant, respectively. The TFC for methanol, chloroform, petroleum ether and aqueous extracts of the plant, respectively, were found to be 69.48 ± 5.80, 64.27 ± 1.04, 47.6 ± 2.76 and 60.00 ± 7.64 QE mg/g, respectively. The TAC were calculated

as 100.901 ± 2.823, 11.395 ± 0.445, 8.802 ± 0.123, 26.364 ± 0.360 AAE mg/g of methanol, chloroform, petroleum ether and aqueous extracts of the plant, respectively. The electron-transfer based antioxidant assay was also carried out by using ferric reducing antioxidant power (FRAP) and 2, 2 -diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity. In FRAP assay, the methanol extract surpassed other extracts across all concentrations, followed by aqueous extract, while aqueous and petroleum ether showed less comparatively lower activities. DPPH activity assessed by IC_{50} also exhibited highest activity in methanol extract with 5.677 ± 0.363 which is almost equivalent to the activity of standard antioxidant, butylated hydroxytoluene (BHT).

Objective 3: Antimicrobial Property Assessment of *Helicia* sp. against Pathogenic Bacteria and Fungi.

The assessment of antibacterial activity of the extracts against eight pathogenic bacteria by preparing four different concentrations (500, 250, 125, and 62.g mg/ml) by following agar well method showed that methanol extract exhibited concentration-dependent activity across all the tested bacterial strains, while the chloroform showed no activity against *M. luteus* and the petroleum ether extract expressed no antibacterial activity at all. The minimum inhibitory concentration (MIC) for the methanol extract ranged from 1.95 to 15.63 mg/ml, the aqueous extract from 7.813 to 125 mg/ml, and the chloroform extract from 0.98 to 7.813 mg/ml. Meanwhile, minimum bactericidal concentration (MBC) was found in the methanol, chloroform, and aqueous extracts at concentrations ranging from 3.91 to 62.5 mg/ml, 3.91 to 31.25 mg/ml, and 15.63 to 125 mg/ml, respectively. Interestingly, across all extracts, the lowest MIC was expressed against a gram-positive *B. cereus*. Using four different concentrations such as 1, 0.5, 0.25, and 0.125%, the antifungal activity was determined against four pathogenic fungal strains by the poisoned food technique. The dose-dependent activity was observed in methanol, chloroform, and aqueous extract treatments. 0.5 and 0.25% concentrations of the chloroform extract showed nearly identical action against *F. oxysporum*, but the other fungal strains reacted to serial dosages of the extract. The petroleum and chloroform extracts had their

strongest antifungal effects against *F. keratoplasticum* and *F. oxysporum*, respectively, while the methanol and aqueous extracts were determined to be the most effective against *P. oryzae*.

Objective 4: Determination of the Antiparasitic Property of *Helicia* sp. against Intestinal Helminths.

The survival test of the intestinal helminths of local fowl (*Raillietina echinobothrida*) upon the treatment of the plant extracts and the standard drug, albendazole, showed dose-dependant activity across various treatment doses (20, 15, 10, 5, and 2 mg/ml). By taking the highest dose from each treatment group, survival time of the parasites was in the order of albendazole>methanol extract>petroleum ether extract>aqueous extract>chloroform extract>control. The inhibition activity of the highest dose of the extracts and albendazole on acid and alkaline phosphatases of the tegumental enzymes of the parasites are in the order of methanol extract>albendazole>chloroform extract>aqueous extract>petroleum ether extract, and albendazole>methanol extract>petroleum ether extract>aqueous extract>chloroform extract, respectively. The results of the microscopic examinations of the worms using scanning electron microscopy and light microscopy corroborate the findings of the phosphatase enzyme activity inhibited by the extracts and albendazole, which amply demonstrate the plant's anthelmintic effectiveness.

Further, the present study is the second ever anthelmintic activity investigation from the entire family of Proteaceae, after *Roupala montana* from the genus *Roupala* against platyhelminthes.

In summary, the chemical profiling of *H. excelsa* confirms that it contains numerous crucial phytocompounds belonging to different classes which could be the leading compounds for its medicinal properties. The determination and quantification of the antioxidant properties showed that it has high contents of TPC, TFC, and TAC. In addition, the extracts also exhibited significant activities in FRAP and DPPH assays. Moreover, various compounds present in the plant also validates the notable antimicrobial activity of the plant against pathogenic bacterial and fungal strains.

Lastly, the inhibition properties of the extracts against tegumental enzymes of the parasites support SEM and histological assessment of anthelmintic activity of the plant. Therefore, the findings and results from the present study will possibly be providing future researchers on the evaluation of ethnomedicinal properties of the plant and helping them in better understanding of its therapeutic effects on human health which may partake in the discovery of novel plant-derived drugs to treat various diseases.