CHAPTER – 5
DISCUSSION

5. DISCUSSION

5.1. Phytochemical Analysis of Plant Compounds and Their Therapeutic Potential

Plants have long been a valuable source of medicine, with their use in traditional medicine dating back to prehistoric times. Throughout history, plant-based substances have been utilized as phytomedicines due to their diverse therapeutic properties. The compounds derived from various parts of plants, such as leaves, flowers, bark, seeds, fruits, and roots, offer a rich source of bioactive substances (Yadav and Agarwal, 2011). These plant-derived phytochemicals, often referred to as secondary metabolites, exhibit significant biological activity, including enzyme modulation, antioxidant properties, and provision of biochemical substrates, all of which contribute to numerous health benefits (Dillard and German, 2000). Plants are known to produce a wide variety of secondary metabolites that possess impressive biological activities, including cytotoxic, anti-parasitic, and anti-microbial properties (Alagbe et al., 2020; Biswas et al., 2020; Darzuli et al., 2021). Phytochemical analysis of plants has revealed that the most important bioactive compounds found in them include alkaloids, terpenoids, tannins, saponins, flavonoids, steroids, and phenolic compounds (Mustafa et al., 2017; Zhang et al., 2022).

In the present study, a qualitative phytochemical screening of plant extracts was conducted using four different solvents: hexane, diethyl ether, ethyl acetate, and methanol. The analysis revealed the presence of 13 phytochemical compounds out of the 15 tested, with compounds detected in one or multiple solvent extracts. The study found that terpenoids, alkaloids, coumarins, steroids, flavonoids, proteins, and carbohydrates were present in all the solvents, while anthocyanins and glycosides were completely absent. These findings highlight the importance of solvent choice in phytochemical extraction, as different solvents are able to extract different compounds from the plant material. For example, hexane is often more effective at extracting non-polar compounds such as terpenoids and lipids, while methanol tends to extract a broader range of polar and semi-polar compounds such as flavonoids and alkaloids (Pereira et al., 2021). This variability in extraction profiles aligns with previous studies, including research on *Taraxacum officinale*, which revealed a diverse range of phytocompounds, such as tannins, alkaloids, flavonoids, terpenoids, saponins, glycosides, and phenols, with varying extraction profiles

across different solvents (Mir et al., 2006). Another study on *Calotropis procera* showed that 11 out of 12 compounds were present in at least one of the tested solvents, highlighting the effectiveness of solvent choice in extracting bioactive compounds (Abegunde and Ayodele-Oduola, 2015). The complete absence of anthocyanins and glycosides suggests that these compounds are either not present in the plant material under study or are present in very low concentrations that are difficult to extract using the solvents tested. This finding is consistent with other studies, such as those by Pérez et al. (2018), who noted the variation in the presence of specific compounds depending on the plant species and extraction method used. Additionally, the presence of coumarins and proteins across all solvents in our study highlights the plant's potential as a source of bioactive compounds with a wide range of biological activities, including anti-inflammatory, anti-cancer, and antioxidant effects (Liu et al., 2021). Overall, these results underscore the significant role of solvent-based extraction in obtaining diverse and potent phytochemicals, which can be utilized in the development of new therapeutic agents. The quantitative analysis of phytochemicals in *Hypericum japonicum* highlighted the plant's substantial nutritional potential, showing it as a good source of macronutrients, specifically proteins and carbohydrates. These macronutrients are essential for human health and nutrition, supporting various physiological functions such as immune responses, energy production, and cellular repair (Kumar et al., 2017; Singh et al., 2018). The plant's ethyl acetate extract demonstrated the highest protein content (187.47 ± 1.96 µg/mg), while hexane had the highest carbohydrate content (205.93 ± 5.07 µg/mg). These findings suggest that *Hypericum japonicum* is not only a source of bioactive compounds but also a valuable nutrient source that can be considered for therapeutic purposes such as functional foods, protein-based therapeutics, and even vaccines (Chugh et al., 2020). The presence of proteins and carbohydrates also supports the idea that the plant can play a role in developing nutraceuticals, particularly as a source of glycoproteins and polysaccharides known for their therapeutic potential (Srivastava et al., 2019).

Flavonoids and phenolic compounds, key secondary metabolites, are widely recognized for their health benefits, particularly their antioxidant properties. These compounds play a vital role in mitigating oxidative stress by scavenging reactive oxygen species (ROS) and preventing the cellular damage that can lead to various diseases such as cancer, cardiovascular disorders, and neurodegenerative diseases (Pourmorad et al., 2006; Tungmunnithum et al., 2018). In this study, the total phenolic content in the ethyl acetate extract ranged from 68.82 ± 4.91 µg/mg to 242.96 ± 6.25 µg/mg, while the total flavonoid

content ranged from 46.11 ± 2.27 µg/mg to 140.02 ± 3.75 µg/mg. These high levels of phenolic and flavonoid content align with *Hypericum japonicum*'s potential as a natural antioxidant, corroborating findings from studies on other *Hypericum* species that showed significant antioxidant potential (Kızıl et al., 2008; Radulović et al., 2017). The antioxidant properties of flavonoids and phenolic compounds in *Hypericum japonicum* support their potential role in preventing or alleviating chronic diseases, such as diabetes, cardiovascular diseases, and neurodegenerative disorders, where oxidative stress is a contributing factor (Maheshu et al., 2014).

The observed antioxidant potential was further confirmed through assays measuring the plant's ability to reduce ferric ions and its total antioxidant capacity. Similar to other plants like *Clerodendrum infortunatum* and *Citrus grandis*, the ethyl acetate extract exhibited the highest antioxidant activity, suggesting that solvent selection plays a critical role in extracting antioxidant properties (Swargiary et al., 2021a). The lower FRAP and TAC activity in the hexane extract is consistent with other studies that have shown solvent-dependent variations in antioxidant potency (Figueiredo et al., 2013; Priya Darsini et al., 2021). This highlights the importance of extracting antioxidants with appropriate solvents to maximize their bioactive potential. The free radical scavenging assays, including DPPH, ABTS, and TBARS, provided additional insight into the plant's antioxidant capacity. *H. japonicum* exhibited potent antioxidant activity in all assays, with the ethyl acetate extract showing the most robust activity (IC_{50}, 23.04 ± 4.49 µg/ml), while the hexane extract displayed the weakest activity (IC_{50}, 203.19 ± 1.50 µg/ml). This finding supports the idea that high phenolic content is linked to greater antioxidant activity, as seen in studies on other plant species such as *Dillenia suffruticosa* (Yakop et al., 2020). The correlation between phenolic content and antioxidant activity in our study is also consistent with findings from various researchers who have demonstrated that medicinal plants with higher levels of polyphenols tend to exhibit stronger antioxidant properties (Liu et al., 2018; Omeke et al., 2019).

Incorporating the pharmacological significance of *Hypericum japonicum*, the strong antioxidant activity observed in this study supports its potential as a therapeutic agent. Antioxidants are critical in the prevention of oxidative stress-related pathologies such as cancer, neurodegeneration, and aging (Pourmorad et al., 2006). The antioxidant activity observed in *Hypericum japonicum* may help to protect cells from oxidative damage, thus reducing the risk of diseases associated with free radicals. The high phenolic and flavonoid content in the plant suggests that it may also possess other beneficial pharmacological

effects, such as anti-inflammatory, anti-cancer, and neuroprotective activities, as reported in studies on other Hypericum species (Mandrone et al., 2015; Napoli et al., 2018). For instance, *Hypericum perforatum* has been studied for its antidepressant and anti-inflammatory properties, which are linked to its antioxidant components (Sadeghi et al., 2018). Moreover, the plant's potential in alleviating oxidative stress may contribute to its neuroprotective effects, particularly in conditions like Alzheimer's disease, where oxidative damage to neurons plays a significant role in disease progression (Akhondzadeh et al., 2010). The pharmacological significance of *H. japonicum* is also highlighted by its diverse bioactive compounds, which can be further explored for their therapeutic potential. Studies have shown that plants from the *Hypericum* genus possess a wide range of pharmacological activities, including antimicrobial, anti-inflammatory, and anti-parasitic effects (Radulović et al., 2017; Li et al., 2023). The presence of glycosides, alkaloids, and flavonoids in *Hypericum japonicum* aligns with its potential to serve as a rich source of compounds for the development of drugs or functional foods aimed at preventing or treating diseases linked to oxidative stress and inflammation.

Hypericum japonicum exhibits significant nutritional and antioxidant potential, with the ethyl acetate extract demonstrating the highest activity across several assays. The plant's bioactive compounds, particularly phenolics and flavonoids, contribute to its powerful antioxidant effects, reinforcing its pharmaceutical and therapeutic value. The observed antioxidant activity, along with the plant’s high protein and carbohydrate content, suggests its potential for use in functional foods, nutraceuticals, and therapeutic agents targeting oxidative stress-related conditions. Furthermore, the pharmacological significance of *H. japonicum* extends beyond its antioxidant properties, with the plant potentially offering a variety of therapeutic benefits, including anti-inflammatory, anti-cancer, and neuroprotective effects. These findings align with the growing body of evidence supporting the medicinal potential of *Hypericum* species, further validating the plant's use in traditional medicine and its potential for future pharmaceutical applications (Mandrone et al., 2015; Napoli et al., 2018).

While the therapeutic properties of medicinal plants are largely attributed to their bioactive compounds, including essential oils and secondary metabolites, the presence of heavy metals in these plants poses significant health concerns. Heavy metals such as Pb, Cd, Zn, and Ni can contaminate plants, often through environmental exposure, and pose health risks when consumed beyond permissible limits. As noted by the World Health Organization (WHO, 2005), the accumulation of heavy metals in medicinal plants can

affect food quality and mineral nutrition. In particular, toxic metals like Pb can lead to both acute and chronic poisoning, affecting vital organs including the kidneys, liver, vascular system, and immune system. Long-term exposure to heavy metals can induce DNA damage, genetic mutations, and increase the risk of cancer. The International Agency for Research on Cancer has classified several metals, including As, Cd, Cr, Pb, and Ni, as potentially carcinogenic to humans (Sarma et al., 2011). This study aimed to evaluate the heavy metal content in *Hypericum japonicum*, focusing on both essential elements (Zn and Cu) and toxic metals (Cr, Cd, Pb). The results indicated that the heavy metal content in *Hypericum japonicum* was extremely low, suggesting that the plant is relatively safe for use in medicinal preparations. This finding is in agreement with similar studies that have consistently shown that many medicinal plants contain low levels of heavy metals, making them suitable for therapeutic purposes (Khan et al., 2016; Swargiary et al., 2017; Haque et al., 2019; Daimari et al., 2020).

In contrast, studies conducted in areas with industrial, mining, or agricultural activities have shown higher concentrations of heavy metals in plants. For example, research in the Eastern Mediterranean Region of Turkey reported elevated heavy metal concentrations in medicinal plants near industrial and mining areas (Karahan et al., 2020). Such findings emphasize the importance of monitoring and controlling heavy metal levels in medicinal plants to ensure their safety for human consumption. The low levels of heavy metals in *Hypericum japonicum* reinforce its safety for therapeutic use and highlight the importance of sustainable agricultural practices in maintaining the quality and safety of medicinal plants. Overall, the low concentration of toxic metals in *Hypericum japonicum* aligns with its potential for use in medicine, particularly in developing antioxidant-rich formulations. The minimal heavy metal content observed in this study further supports the plant's suitability for use as a source of bioactive compounds, while also reinforcing the need for regular monitoring of contaminants in medicinal plants to ensure public health safety.

5.2. Anthelmintic potential of *Hypericum japonicum*

Parasitic infections, particularly those caused by helminths, continue to represent a major global health challenge, affecting both humans and animals. These infections are responsible for significant morbidity and mortality, particularly in developing countries, where they also contribute to substantial economic losses in livestock industries

(Kumarasingha et al., 2016; Khan et al., 2019). Despite the availability of synthetic anthelmintic drugs, resistance and the ability of helminths to adapt and evade treatment pose a growing concern. Consequently, there has been a growing interest in exploring alternative therapies, particularly from medicinal plants, which are considered to offer a safer, more sustainable option (WHO, 2017). Medicinal plants have been used for centuries in traditional medicine systems, providing effective remedies with fewer side effects, lower toxicity, and minimal risk of addiction (Roy et al., 2009; Swargiary et al., 2016). Many plants with anthelmintic properties have been used in folk medicine and are being scientifically investigated for their potential as natural deworming agents. Traditional knowledge, often passed down through generations, has guided much of this exploration. In this context, *H. japonicum* has emerged as a promising candidate for studying its anthelmintic properties. Several previous studies have highlighted the plant's potential based on its bioactive compounds (Hazarika et al., 2022).

In our study, we investigated the anthelmintic potential of *H. japonicum* through the preparation of four solvent extracts—methanol (Met), diethyl ether (DE), hexane (hex), and ethyl acetate (EA)—and evaluated their dose-dependent effects on parasitic worms. Among the different solvent extracts, the diethyl ether extract showed the most potent anthelmintic activity, followed closely by the hexane extract, which exhibited a similar level of activity compared to the reference drug, albendazole. This suggests that *H. japonicum* may have significant anthelmintic potential, particularly from its diethyl ether and hexane extracts. Interestingly, the ethyl acetate extract, which demonstrated strong antioxidant activity, showed the weakest anthelmintic effect. This finding suggests a potential correlation between the antioxidant and anthelmintic activities of *H. japonicum*. However, further studies are required to confirm whether there is a direct relationship between these two properties. Antioxidants are known for their ability to neutralize free radicals, which could play a role in mitigating oxidative stress in the host organism, but may not necessarily target helminth infections as effectively as other bioactive compounds present in the plant (Hazarika et al., 2022).

Our findings are consistent with several other studies that have evaluated the anthelmintic properties of plant extracts. For example, Maciel et al. (2006) studied the ovicidal and larvicidal properties of *Melia azedarach* extracts and found that the ethanol extract exhibited superior anthelmintic activity compared to the hexane extract. Similarly, Bagavan et al. (2009) reported that methanolic extracts from *C. asiatica*, *G. superba*, *P. daemia*, and *P. emblica* exhibited significantly higher anthelmintic activity compared to

other solvent extracts. These findings support the idea that the solvent used for extraction plays a crucial role in determining the bioactivity of the plant extract, possibly due to the varying solubility of the bioactive compounds in different solvents.

In contrast to our study, previous research on *P. strigosa* found that the ethyl acetate extract exhibited the most potent anthelmintic activity, with a mortality time of 7:52 ± 0:24 h:min (Swargiary et al., 2021a). This discrepancy could be due to differences in the chemical composition of the plant extracts, as well as the type of helminths used in the bioassays. *P. strigosa* may contain more bioactive compounds that are particularly effective against the parasites tested in that study, highlighting the complexity and variability of plant-based anthelmintic activity. The results of this study suggest that *H. japonicum* possesses significant anthelmintic potential, particularly from the diethyl ether and hexane extracts. These findings warrant further exploration of the plant's bioactive compounds and their mechanism of action against helminthic infections. The plant's bioactivity could be attributed to the presence of various secondary metabolites such as flavonoids, alkaloids, and terpenoids, which are known to have anthelmintic properties (Roy and Tandon, 1996; Hazarika et al., 2022). Future studies should aim to isolate and identify these bioactive compounds, and determine their specific modes of action against parasitic worms. Moreover, studies investigating the synergistic effects of different extracts or combinations with synthetic anthelmintic drugs like albendazole could provide insights into developing more effective and sustainable treatments for helminth infections. The low toxicity and reduced adverse effects associated with plant-based remedies make them particularly appealing as adjunct therapies for controlling parasitic infections in both humans and livestock (Khan et al., 2019).

5.3. Identifying bioactive compounds in *Hypericum japonicum*

Chromatography is an essential technique used for the separation, identification, and quantification of compounds within a mixture, enabling both qualitative and quantitative analysis. It involves two phases: a stationary phase (solid or liquid adsorbed on a solid support) and a mobile phase (liquid or gas) (Smith, 2013; Coskun, 2016). Among the various forms of chromatography, Thin Layer Chromatography (TLC) is widely utilized to separate and identify bioactive compounds from medicinal plants. In the present study, TLC was employed to separate and identify bioactive compounds in the diethyl ether (DE) extract of *Hypericum japonicum*, a plant previously noted for its pharmacological

properties, including potential anthelmintic activity (Hazarika et al., 2022). This separation technique was essential for isolating the key bioactive compounds, providing insight into the plant's therapeutic potential. For the TLC separation of compounds from the DE extract of *H. japonicum*, a solvent system consisting of a 1:1 (v/v) mixture of petroleum ether and ethyl acetate was found to achieve the best separation. This solvent mixture was selected after testing multiple combinations and ratios. TLC relies on the interaction between the solute, mobile phase, and stationary phase, where differential migration of compounds is determined by their binding affinity (Bele and Khale, 2011). Similar studies have successfully utilized TLC for separating and analyzing bioactive compounds in medicinal plants. For example, Ahamed et al. (2017) used a hexane: ethyl acetate: acetic acid (4:4:2) solvent system for TLC profiling of medicinal plants, and Cieśla and Waksmundzka-Hajnos (2009) discussed the use of TLC in quality control of herbal medicines. In this study, TLC served as a preparatory step for isolating fractions, followed by further compound isolation using liquid column chromatography (LC).

After TLC separation, the fraction exhibiting the most potent anthelmintic activity—Fraction A—was selected for further analysis using Liquid Chromatography-Mass Spectrometry (LC-MS) and High-Performance Liquid Chromatography (HPLC). The LC-MS analysis revealed a peak at 300.9 m/z, suggesting the possible presence of quercetin, a flavonoid known for its wide range of biological activities, including anti-inflammatory, antioxidant, and anti-parasitic properties. The detection of quercetin in *H. japonicum* is consistent with previous studies that have identified this compound in various medicinal plants. Zhu et al. (2015) detected quercetin in *Nelumbo nucifera* leaves using LC-MS/MS, while Wianowska et al. (2017) and Matei et al. (2015) reported the presence of quercetin and other bioactive compounds in different plants using LC-MS. These findings validate the potential of *H. japonicum* as a source of quercetin.

To confirm the presence of quercetin, HPLC was performed with the TLC Fraction A extract. A standard quercetin run was performed on the HPLC system, and the presence of quercetin was confirmed by comparing the retention times of the peak in the sample with the standard. HPLC, a powerful analytical technique, is commonly used for the isolation, identification, and purification of bioactive compounds from plant extracts (Stefova et al., 2009). In similar studies, HPLC has been employed for quercetin isolation and analysis in various plant species, such as *Ginkgo biloba* (Wang et al., 2014) and *Scutellaria baicalensis* (Ang et al., 2014). The successful identification and quantification of quercetin in *H. japonicum* further confirms its potential therapeutic value. The

combination of TLC, LC-MS, and HPLC provided a robust analytical approach for identifying and isolating quercetin from the diethyl ether extract of *H. japonicum*. These techniques are highly sensitive and specific, which is crucial when analyzing complex mixtures and identifying trace compounds (Mukherjee, 2019). The presence of quercetin in *H. japonicum* not only supports its therapeutic potential as an anthelmintic agent but also highlights the value of using advanced chromatographic techniques for natural product discovery. This study adds to the growing body of evidence that medicinal plants, like *H. japonicum*, can serve as important sources of bioactive compounds with significant pharmacological activities. The findings of this study are consistent with prior research indicating the widespread occurrence of quercetin in medicinal plants. The identification of quercetin from *H. japonicum* strengthens the case for exploring this plant as a source of novel therapeutic agents, particularly for treating parasitic infections. Further research is warranted to explore the exact mechanisms underlying the anthelmintic activity of quercetin and other bioactive compounds in *H. japonicum*, as well as their potential application in drug development. Polyphenols are major dietary phenolics comprising the polyphenols (hydrolysable and condensed tannins), phenolic acids (hydroxybenzoic and hydroxycinnamic acids) and flavonoids. Flavonoids are the most extensively studied group of polyphenols (Awuchi et al., 2021). Quercetin is among the widely occurring polyphenol, found abundantly in nature. It is commonly present in different plant products. Quercetin has anti-oxidative, anti-inflammatory, anti-proliferative, anti-carcinogenic, anti-diabetic, and anti-viral properties (Deepika and Mauriya, 2022). Presence of bioactive compound Quercetin has been reported by many other studies using the chromatography techniques like GCMS, LCMS and HPLC, etc. (Wang et al., 2009; Liu et al., 2014; Peron et al., 2019). Studies have shown that quercetin can have considerable effect on the parasitic worm. It also affects the nervous system of the parasite and alterations in various enzymes (Giovanelli et al., 2018; Goel et al., 2023; Al-Shaebi et al., 2023). In relation with this we can also assume that the bioactive compound, quercetin caused paralysis and death of the parasite affecting the nervous system and also other factors like rupturing of the teguments of the parasite.

5.4. Anthelmintic activity of *Hypericum japonicum* and its bioactive compound quercetin: targeting key enzymes in helminth parasites

Targeting checkpoint enzymes in metabolic pathways has emerged as a promising strategy for developing novel therapeutics, offering new avenues for combating a range of infectious diseases (Tyagi et al., 2019). The external surface of helminth parasites are called tegument which serve various biological function especially nutrient absoption, locomotion, excretion and regulations of electrochemical and osmoregulation. The teguments are membrane-bounded syncytia that contain enzymes found in other transporting epithelia (Thompson and Geary, 1985). Acid phosphatase and Alkaline phosphatase are two tegumental enzymes that possess very close associations with the tegument, sub-tegument, somatic musculature, gut and cuticle of helminth parasites, including in adhesive structures like acetabulum and oral suckers (Swargiary and Roy, 2015; Nagi et al., 2024). Elevated levels of ALP and ACP are observed in the intestinal and sub-cuticular regions of the worm, where they play a crucial role in protein transport. Disruption or inhibition of these enzymes could significantly impact the physiological processes of helminth parasites (Roy et al., 2010; Swargiary et al., 2013). Many studies have revealed that the alterations (reduction) in the ALP and ACP disturbs the helminths in maintaining their pH and also optimal temperature required for their survival (Roy et al., 2010; Elzoheiry et al., 2019). Similarly, we have also found that there is reduction in the activity of ACP and ALP when treated with quercetin, which could have disturbed the functioning of ACP and ALP for their various biological processes and thus resulted in the paralysis and death of the parasites. Similar to our study, a study done on *Potentilla fulgens* revealed that the enzyme activity of ACP, ALP, and ATPase was found to be reduced significantly when treated with the crude extract of the plant (Roy et al., 2010). A similar result was also shown when investigation was done on *Amomum maximum* Roxb. (Chetia et al., 2014).

In the present study, two glycolytic enzymes were also analyzed namely- Malate dehydrogenase and Lactate dehydrogenase with the parasites treated with quercetin. MDH and LDH are glycolytic enzymes which helps the helminths for energy production, regulation of glycolysis and maintenance of redox reaction and are responsible for both aerobic and anaerobic respiration in helminth (Dey and Roy, 2020; Liu et al., 2023). MDH plays a very important role in the life activities of parasites for their survival and metabolic processes. Specifically, MDH catalyzes the conversion of malate to oxaloacetate,

producing NADH and $FADH_2$, which are essential for energy production and glycolysis in helminths (Wang, 2024). Whereas, LDH is responsible for conversion of lactate to pyruvate, generating NADH and ATP which is essential for energy production and glycolysis in helminth (Roy and Giri, 2016). Many studies have revealed that reduction in the activity of LDH and MDH has affected the parasites growth and thus resulted in paralysis to death (Roy and Giri, 2016; Aggarwal et al., 2017; Davuluri et al., 2020). In our study also we have found that both MDH and LDH activities were reduced in parasites when treated with *H. japonicum* extract and also with quercetin when compared with the untreated parasites, which might have disturbed the glycolysis and energy metabolism of the parasites. Disturbance in LDH and MDH also effects the respiration of the parasites which may have directly impacted in paralysis and death of the parasites. Similarly, a study was done on the effect of *n*-butanol fraction of *Lysimachia ramosa* on glycogen content and some energy-related enzymes in the Cestode, *Raillietina echinobothrida*, they found that all the enzyme activity gets decreased, with MDH (66–75%) and LDH (48–60%) (Dey and Roy, 2020). Another study on the effect of *Punica granatum* ethanol extract on the carbohydrate metabolism of *Cotylophoron cotylophorum* suggested the inhibition of LDH and MDH activity (Veerakumari et al., 2014).

AchE is a neurotransmitter enzyme which helps in neurotransmission, movement, feeding and reproduction in helminth (Trang and Khandhar, 2023). AchE plays an important role in regulating the interaction between acetylcholine and the parasite nicotinic acetylcholine receptors by hydrolysing Ach to choline and acetate, allowing ions to pass down electro-chemical gradients into or out of cells (Thompson et al., 2010; You et al., 2017). Inhibition of neuromuscular activity may lead to loss of muscle function and essential activities, including host attachment, feeding and mating, thereby interfering with parasite maturation and, finally, parasite killing in the host (You et al., 2017; Joshi et al., 2018). Studies have found that reduction in the activity of AchE compared to control parasite causes paralysis and death of the parasites (Maity et al., 2022). Similarly in our study also we have found that AchE has reduced its activity when compared with the control parasites hence hindrance of this enzyme activity directly can interfere with the neuromuscular activity which can ultimately result in paralysis of the parasites and thus cause death of the helminth parasites. Furthermore, in a separate study of the essential oils of *Origanum* also showed anthelmintic effects against *Anisakis simplex* L3 larvae as well as the inhibitory activity of the acetylcholinesterase enzyme (López et al. 2018).

5.5. Histological and ultrastructural analysis of *Paramphistomum* species treated with *Hypericum japonicum* extract and quercetin

Histological and ultrastructural analyses play a pivotal role in advancing our understanding of biological systems, particularly in the context of infectious diseases and pharmacological interventions. These techniques allow researchers to investigate the structural and functional changes in tissues at both the cellular and molecular levels, providing invaluable insights into the mechanisms of disease progression and the effects of therapeutic treatments. In the present study, histological and ultrastructural analyses were conducted to evaluate the structural alterations in *Paramphistomum* species following treatment with the diethyl ether (DE) extract of *Hypericum japonicum*, quercetin, and albendazole. These findings were compared with previous studies to better understand the mechanisms behind anthelmintic activity. Histological analysis of the treated parasites revealed significant damage to the tegument, with ruptures and breakages observed in the DE extract and quercetin-treated groups, while the albendazole-treated group exhibited only minimal damage. This observation is consistent with previous research on other helminth species, where natural compounds caused more severe tegumental damage compared to synthetic anthelmintics. For instance, a study on *Orthocoelium parvipapillatum* demonstrated that control parasites had a smooth and intact tegument, while treated parasites exhibited substantial damage (Anuracpreeda et al., 2016). Similarly, treatment of *Fasciola gigantica* with *Cassia fistula* extract showed pronounced tegumental damage compared to the more limited damage induced by albendazole (Sen et al., 2022). These studies suggest that bioactive compounds from plants, such as *H. japonicum* and quercetin, may have superior efficacy in causing tegumental disruption, potentially due to their multifaceted mechanisms of action, including antioxidant, anti-inflammatory, and direct cytotoxic effects. The observed damage in our study aligns with findings from other studies investigating plant-derived compounds. For example, *Cucurbita pepo* (pumpkin) seed extract caused significant tegumental damage in *Fasciola hepatica*, leading to tegumental detachment and rupture, which is a hallmark of anthelmintic activity (Rajesh et al., 2017). Similarly, *Andrographis paniculata* extract induced severe tegumental damage in *Schistosoma mansoni* parasites, leading to the loss of parasite viability (Shalaby et al., 2016). These studies, in combination with our findings, reinforce the idea that plant-based treatments

may have superior anthelmintic potential by targeting the tegumental integrity of helminths.

To further investigate the impact of the treatments at a molecular level, scanning electron microscopy (SEM) was employed to examine the ultrastructural changes of the parasite surface. SEM analysis revealed that the control parasites exhibited a smooth and unaltered tegument, while the treated parasites showed rough, damaged surfaces with extensive breakages and shrinkage. These findings are consistent with those of other researchers who have observed similar ultrastructural changes upon treatment with anthelmintic agents. For example, treatment of *Raillietina echinobothrida* with *Lysimachia ramosa* extract led to visible changes in the surface of the parasites, including body shrinkage and rupturing of the tegument (Bashtar et al., 2011). Furthermore, treatment of *Anisakis simplex* larvae with essential oils from *Origanum* spp. also caused similar structural damage, including roughening of the surface and ruptures in the tegument (López et al., 2019).

The observed changes in the tegumental structure are likely the result of the disruption of important biological functions mediated by the tegument, including nutrient uptake, immune evasion, and protection from the host's immune system (Thompson and Geary, 1985). In addition to the physical damage, the reduced activity of key enzymes such as alkaline phosphatase (ALP) and acid phosphatase (ACP) in the treated parasites provides further evidence of the impact on parasite metabolism and survival. Our biochemical results show a significant reduction in the activity of ALP, with 42.59% inhibition in the DE extract-treated parasites and 48.86% inhibition in the quercetin-treated parasites, compared to the untreated control parasites. This reduction in enzyme activity is likely a consequence of the tegumental damage, which impairs the parasite's ability to carry out essential biological processes, such as nutrient transport and energy production. Previous studies have also reported similar findings, where a reduction in enzyme activity, such as ALP and ACP, was linked to tegumental damage and subsequent parasite death. For example, a study on *Raillietina echinobothrida* showed significant reductions in enzyme activities, including MDH and LDH, in parasites treated with plant extracts, leading to impaired glycolysis and energy production, ultimately resulting in the death of the parasites (Dey and Roy, 2020). Similarly, treatment of *Cotylophoron cotylophorum* with *Punica granatum* extract resulted in a decrease in enzyme activities related to carbohydrate metabolism, further supporting the role of enzyme inhibition in the anthelmintic effects of

plant compounds (Veerakumari et al., 2014). In conclusion, the histological and ultrastructural changes observed in this study, along with the biochemical findings, provide strong evidence for the potential of *H. japonicum* and quercetin as effective anthelmintic agents. The disruption of the tegument, reduction in key enzymatic activities, and impairment of metabolic processes likely contribute to the paralysis and death of the helminth parasites. These results, when compared with other studies, highlight the promising therapeutic potential of natural compounds in the development of alternative anthelmintic treatments. Future studies should focus on isolating and characterizing the active compounds from *H. japonicum* and further elucidating their mechanisms of action to support their use in clinical applications.

5.6. In-silico Analysis of Bioactive Compounds in *Hypericum japonicum* and Their Interaction with Enzymes

In-silico approaches, such as molecular docking and molecular dynamics simulations, have become indispensable in drug discovery, offering insights into the molecular interactions between bioactive compounds and their potential enzyme targets (Shaker et al., 2021; Rashid et al., 2022). These computational methods facilitate the identification of potential drug targets in various diseases, including parasitic infections, by simulating protein-ligand interactions and exploring various binding sites, orientations, and conformations (Jakhar et al., 2020). For molecular docking, Autodock Vina software is widely used due to its reliability and precision in predicting binding affinities and interactions, making it a valuable tool in drug development (Lu et al., 2010; Salamah et al., 2019; Butt et al., 2020).

The present study investigated the binding affinities of quercetin, a bioactive compound in *Hypericum japonicum*, and albendazole, a known antiparasitic drug, to five target enzymes: acetylcholinesterase (AchE), alkaline phosphatase (ALP), acid phosphatase (ACP), malate dehydrogenase (MDH), and lactate dehydrogenase (LDH). The results revealed that AchE had the strongest binding affinity with quercetin (-7.8 kcal/mol), followed by ALP (-7.6 kcal/mol), confirming their potential as therapeutic targets in parasitic infections. These findings are consistent with previous studies, where AchE was identified as a critical target for bioactive compounds due to its role in neurotransmission in parasites (Maule et al., 2006; Abaza, 2024). In addition, albendazole showed the strongest interaction with AchE, followed by ALP, which is in line with the known mode of action of albendazole in parasitic diseases (Horton, 2000; Yereli et al., 2004). Molecular docking

analysis revealed that Van der Waals forces were the predominant interaction type between the ligands and the receptor enzymes, followed by hydrogen bonding and hydrophobic interactions. These findings are consistent with previous reports that highlight the importance of Van der Waals forces in ligand-receptor binding (Swargiary et al., 2023; Macalalad and Gonzales, 2023). While hydrogen bonding also plays a significant role, it was not as dominant as Van der Waals interactions. These results support the notion that the affinity of bioactive compounds for their target enzymes is largely influenced by the nature and strength of intermolecular forces, with Van der Waals forces contributing to the stability of the binding. Interestingly, quercetin showed weaker binding to ACP, with a binding affinity of -3.5 kcal/mol, which was corroborated by our biochemical assays. ACP exhibited the lowest inhibition (26.85%) in our study, indicating that ACP may not be a major target for quercetin. ACP inhibitors generally exhibit lower potency compared to other enzymes involved in parasitic metabolism. This reduced interaction might explain the limited biological activity observed for ACP inhibition in our assays. The role of AchE and ALP as therapeutic targets in parasitic infections has been well documented. AchE inhibitors are widely studied for their potential to interfere with parasitic neurotransmission (Abaza, 2024), while ALP is critical for maintaining parasite physiological functions, including nutrient absorption and signal transduction (Stettler et al., 2001). Our findings suggest that quercetin and albendazole, by targeting these enzymes, could provide an effective means of combating parasitic infections. The low interaction with ACP further underscores the need for future studies to better understand the mechanisms by which these compounds exert their biological effects.

Moreover, other studies have highlighted the antioxidant and anti-inflammatory properties of quercetin, suggesting that its beneficial effects may not be limited to enzyme inhibition but may also contribute to reducing oxidative stress and inflammation in parasitic infections (da Silva et al., 2021; Memariani et al., 2024). Albendazole, as a benzimidazole compound, has been shown to inhibit microtubule polymerization in parasites, leading to their immobilization and death (Shaharyar and Mazumder, 2017; Anichina et al., 2021). Our molecular docking results, combined with these pharmacological insights, suggest that the combined antioxidant and enzymatic inhibitory effects of quercetin and albendazole may work synergistically to combat parasitic diseases. In conclusion, the in-silico analysis of *Hypericum japonicum* bioactive compounds, including quercetin, reveals promising interactions with key enzymes, particularly AchE and ALP. The strong binding affinities observed for quercetin and albendazole suggest

their potential as effective therapeutic agents for parasitic infections. While ACP showed weaker interaction with quercetin, this finding highlights the specificity of enzyme-ligand interactions and underscores the importance of further investigation into the detailed mechanisms of action. Overall, *Hypericum japonicum* represents a valuable source of bioactive compounds that may offer new avenues for the development of effective treatments for parasitic infections.

5.7. Drug-likeness and ADMET analysis of the Bioactive compound

The drug-likenss property of the bioactive compound (quercetin) has been analysed in the present study. This is studied mainly to evaluate the potential of a molecule to become a successful drug. Undesirable pharmacokinetic (PK) properties or unacceptable toxicity are the main reasons for the failure of drug at the clinical trail (Jia et al., 2020). The drug-likeness property has been evaluated using SwissADME (Diana et al., 2017; Riyadi et al., 2021). A drug should follow the Lipinski's rule of five if it is intended to be orally administration in humans. According to Lipinski's rule of five, if a drug to be orally active, the molecular weight of the drug is less than 500g/mol, LogP≤5, Hydrogen Bond Donor (HBD≤5), Hydrogen Bond Acceptor (HBA≤10) and the topological polar surface (TPSA≤ 140 Å^2) (Lipinksi, 2004). From the present findings, it was observed that the bioactive compound has followed the Lipinski's rule of 5, no violaion has been found by quercetin. As it follows the Ro5, the compound can be considered showing a good drug-likeness property and can be assumed as a good oral drug. Similar studies were found where the anthelmintic bioactive compound isolated from the plant *Vitex pedunc*ularis Wall. Leaves followed the Ro5 (Auniq et al., 2019). Again, studies have been found where the bioactive compounds isolated from the plants parts is considered a good antelmintic drug when it follows the Ro5 and also have shown good ADMET properties when ADMET studied were carried out by different authours (Jamkhande and Barde, 2014; Paul et al., 2018; Islam et al., 2023). Along with the drug-likeness analysis, ADMET pharmacokinetic (PK) properties was also evaluated for the bioactive compound. The properties like absopriton, distribution, metabolism, metabolism, excretion and toxicity was studied. These properties are crucial for the concept of drug-likeness and has been widely used to filter out compounds with undesirable properties, especially poor ADMET (absorption, distribution, metabolism, excretion, and toxicity) profiles (Tian et al., 2015). ADMETlab database has been widely used for evaluating the pharmacokinetic (PK) properties which gives the

probability scores of the compound to be accepted as a drug (Dong et al., 2018; Xiong et al., 2021). From the present study, the bioactive compound quercetin has shown good pharmacokinetic properties in most of the parameters, suggesting their good drug-likeness property.

5.8. Molecular Dynamics Simulations for Evaluating the Binding Affinity of ligands to Acetylcholinesterase (AchE)

Molecular dynamics (MD) simulations have emerged as a cornerstone in computational biology, allowing researchers to simulate the motion of atoms and molecules in a system over time. This technique has been invaluable in studying biomolecules such as proteins and nucleic acids, revealing critical insights into their conformational changes, stability, and interactions with ligands. MD simulations provide atomic-level detail about processes such as ligand binding, protein folding, and conformational transitions (Karplus and Petsko, 1990; Karplus and McCammon, 2002). Specifically, MD simulations are crucial for understanding how ligands, including potential drug candidates, interact with enzymes and other proteins in a dynamic and physiologically relevant environment (Hollingsworth and Dror, 2018). In the present study, MD simulations were employed to investigate the interaction of *quercetin* and *albendazole* with acetylcholinesterase (AchE), a key enzyme involved in neurotransmission in parasitic organisms. AchE has been identified as a critical drug target in the treatment of parasitic diseases such as *schistosomiasis* and *neurocysticercosis* (Taman and Azab, 2014; You et al., 2017). By simulating the binding of these ligands to AchE, we sought to understand their potential as inhibitors, and how their binding affinities compare to the reference drug, albendazole.

5.9. Molecular Dynamics Simulations and Protein-Ligand Interactions

MD simulations involve the prediction of atomic-level motions of all components in a system over time, governed by the laws of classical mechanics. The GROMACS software package was used to perform the MD simulations in this study, a popular tool for biomolecular simulations due to its efficiency and scalability (Lu et al., 2010; Pronk et al., 2013). This software has been widely used in various studies involving protein-ligand binding and molecular dynamics simulations of biomolecular systems (Sharma et al., 2021). The results from the MD simulations suggest that quercetin and albendazole both exhibit strong binding affinity for AchE, with quercetin showing binding characteristics

similar to albendazole, the reference drug. Both compounds induced conformational changes in the AchE protein upon binding, although the overall protein structure remained stable throughout the simulation period. These findings align with the general behavior of many drugs that bind to their targets, causing minor structural alterations that enhance binding stability without destabilizing the protein (Karplus and McCammon, 2002; Hollingsworth and Dror, 2018).

5.10. Hydrogen Bonding and Binding Affinity

Hydrogen bonding is one of the most significant forces driving protein-ligand interactions. In this study, hydrogen bonding was analyzed in the context of both quercetin and albendazole binding to AchE. The simulations revealed that the apo-protein (AchE without any ligand) exhibited more hydrogen bonds than either of the ligands. This suggests that while quercetin and albendazole interact strongly with AchE, the number of hydrogen bonds formed was lower than that in the unbound protein. Previous studies have shown that hydrogen bonding is crucial for stabilizing protein-ligand complexes and that a higher number of hydrogen bonds typically correlate with stronger binding affinity (Mobley and Gilson, 2017). Interestingly, both quercetin and albendazole formed hydrogen bonds with key amino acid residues of AchE, but the nature of these interactions varied. Quercetin, a flavonoid, is known for its ability to form stable complexes with enzymes through multiple hydrogen bonds, which can enhance its inhibitory effects (Dolatabadi, 2011). Similarly, albendazole's binding is largely driven by hydrogen bonds and van der Waals forces, which stabilize its interaction with AchE (da Silva Costa et al., 2020).

5.11. Total Energy and Thermodynamics of Binding

Total energy analysis, which includes the evaluation of van der Waals forces, electrostatic interactions, and solvation energy, revealed that quercetin and albendazole both exhibited similar energy profiles, which were higher than the apo-protein. Despite this, both ligands displayed favorable binding interactions with AchE, indicating that their binding is energetically favorable. These results are consistent with the findings of Mobley and Gilson (2017), who emphasized the importance of calculating binding free energy in predicting the efficacy of biomolecular complexes. The MMPBS (Molecular Mechanics Poisson-Boltzmann Surface Area) analysis was performed to further examine the thermodynamics of protein-ligand binding. This analysis revealed that the binding of

quercetin and albendazole to AchE resulted in negative delta energy values, indicating that the binding process was spontaneous and thermodynamically stable. This further supports the potential of quercetin as an effective AchE inhibitor in parasitic diseases, similar to albendazole (Sharma et al., 2021).

5.12. Structural Stability and Protein Flexibility

In addition to the analysis of binding affinity and hydrogen bonding, the radius of gyration (RG) was calculated to evaluate the compactness of the protein structure. Both quercetin and albendazole were shown to induce slight instability in the protein's elasticity, as reflected by a minor increase in RG. This is consistent with previous MD studies that suggest ligands can induce conformational changes, but the overall stability of the protein is often preserved (Karplus and McCammon, 2002; Frimurer et al., 2003). Despite the minor structural deviations, AchE maintained stability throughout the simulation period, which is crucial for its continued function in parasitic systems. This stability, coupled with favorable binding energy and hydrogen bonding, indicates that quercetin could serve as a promising inhibitor for AchE in helminth parasites. This finding is consistent with other studies that have identified flavonoids like quercetin as potential therapeutic agents against various enzymes involved in parasitic infections (Batiha et al., 2020; Oryan et al., 2023). The in-silico study of quercetin and albendazole binding to AchE highlights the potential of quercetin as an inhibitor of this critical enzyme in parasitic organisms. Both compounds exhibited similar binding affinities, with thermodynamically favorable binding interactions, structural stability, and significant hydrogen bonding. These findings suggest that quercetin, a bioactive compound from *Hypericum japonicum*, could be a valuable candidate for further investigation as a therapeutic agent against parasitic infections. Future studies, including in-vitro and in-vivo assays, are necessary to validate these computational predictions and explore the potential of quercetin in combination with existing drugs like albendazole.