

Anthelmintic Activity of *Hypericum japonicum* Thunb.: An *in vitro* and *in silico* Studies

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ABSTRACT

Background: *Hypericum japonicum* Thunb. is a vital medicinal plant in Northeast India, traditionally used by tribal communities to treat helminth infections with leaf extracts. **Objectives:** The present study investigates the *in vitro* and *in silico* studies of anthelmintic activity of *H. japonicum*. **Materials and Methods:** The anthelmintic activity was tested on *Paramphistomum* sp. with a test dose of 5 mg/mL to see the paralysis and death times. Phytochemicals were identified using GC-MS technique. 5 key enzymes-Alkaline Phosphatase (ALP), Acid Phosphatase (ACP), malate-and lactate dehydrogenase, and acetylcholinesterases were assayed using standard protocol. Furthermore, identified compounds were studied for their binding activity with the enzymes. **Results:** Diethyl ether extract of *H. japonicum* showed the most potent anthelmintic activity against *Paramphistomum* sp. in the present study. GC-MS analysis identified 12 compounds in the diethyl ether extract. Of the five enzymes studied, ALP showed highest reduction (42.59%) and the least was found in ACP (16.21%) compared to control. Molecular docking observed strongest binding affinity between compound-2 and AchE (-6.73 kcal/mol) followed by ALP, ACP, and MDH enzymes. **Conclusion:** The findings suggest that *Hypericum japonicum* could be a potential source of anthelmintic agents, warranting further studies to elucidate its exact mechanism of action.

Keywords: Anthelmintic, GC-MS, *Hypericum japonicum*, Molecular docking, Molecular dynamics.

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INTRODUCTION

Plants have been used in traditional medicine since ancient times. Like many other diseases, plants and plant-derived products have been used to control helminth infestation in many parts of the world.^[1] Many studies have established the anthelmintic properties of several plants.^[2-4] Helminthiasis are diseases caused by helminth parasites affecting millions worldwide, especially those living in developing countries.^[5,6] It causes delayed mental and physical development in children, and complications during pregnancy, directly affecting the educational and economic conditions of a country.^[7] Gastrointestinal nematodes are a major limiting factor for the success of livestock production worldwide.^[8,9] Poor social infrastructure, unhygienic livelihood, and climate changes are the critical factors for the high prevalence of helminthiasis.^[10] The use of commercial drugs such as benzimidazole, levamisole, mebendazole, albendazole, praziquantel, etc., are the most common control strategy of helminthiasis.^[11,12] However, there are reports of anthelmintic drug resistance from different parts of the world decreasing the productivity of livestock and also

threatening the success of treatment in humans.^[13,14] However, through continuous drug administration, the helminth parasites resist those particular drugs.^[15] As an alternative to the growing incidence of anthelmintic resistance, there has been considerable interest in searching for effective and safe dewormers in the form of medicinal plants that have their roots in the traditional ethnomedicine system. Many authors documented several medicinal plants against helminthiasis in various parts of the world.^[16-18]

North-east India is rich in flora and fauna covering approximately 43% of the total plant species of India.^[19] Tribal communities of this region have been practicing ethnomedicine for several diseases.^[20-22] Recent studies have found that several medicinal plants are consumed by tribal communities of Assam to treat helminth infections.^[4,16,23] *Hypericum japonicum* Thunb. (Family Hypericaceae) is one such medicinal plant having rich ethnomedicinal properties, including anthelmintic activity.^[24] *H. japonicum* is an annual herb growing 5-35 cm in height and having small diffused branching. The plant is mainly distributed throughout South East Asia, including India (<https://indiabiodiversity.org/>). With a rich source of phytochemicals, the plant has been reported to contain hepatoprotective, antitumor, antibacterial, antiviral, and antioxidant activities.^[25,26] Rural Assam, especially the Bodo community, consume the raw juice



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of plant (on an empty stomach) to expel helminth infestation. A preliminary study has shown that the crude extract of *H. japonicum* possesses better anthelmintic activity compared to the reference drug, albendazole.^[27] The present study investigated the anthelmintic activity of different crude extracts of *H. japonicum* and also explored the alterations in enzymatic activities in response to plant extract treatments.

MATERIALS AND METHODS

Collection and identification plant

The sample plant was collected from the Tinali area of Kokrajhar district of Assam and was identified with the help of botanical taxonomist. The plant was confirmed as *Hypericum japonicum* Thunb. (BUBH2000129).

Preparation of plant crude extract and solvent fractions

Fresh leaves of *H. japonicum* were collected from Kokrajhar area and processed for crude extract preparation following the method of Seidel.^[28] Leaves were washed with distilled water and dried entirely in an oven at $45\pm 2^\circ\text{C}$. Dry leaves were powdered using a grinder machine and macerated with 80% methanol (1:5, w/v) for 72 hr. Next, with Whatman filter paper no. 1, the solutions were filtered, and the filtrate obtained was dried in a rotary evaporator. The solid material was collected as a Methanolic Crude Extract of *H. japonicum* (MCEHJ). MCEHJ was further processed for successive solvent extraction using different solvents such as n-hexane, diethyl ether, and ethyl acetate following the liquid-liquid partitioning method. The solvents were dried in a rotary evaporator, and the solid materials obtained were kept at -20°C till further study.

In vitro Anthelmintic study

The *in vitro* anthelmintic study for different plant solvent extracts followed the methods outlined by Egualé and Giday^[29] and Belemilga *et al.*^[30] Paramphistomum sp. was collected from cow's rumen in 1xPBS (pH 7.4). The parasites were acclimatized at 37°C for 30 min before treatment. Albendazole and plant extracts were dissolved in 100 μL DMSO, then made up to 25 mL with PBS. After washing in PBS, 10-15 adult parasites were incubated at 37°C with a test dose of 5 mg/mL of plant extracts. Albendazole (5 mg/mL) was used as the reference drug. Control parasites were

incubated at 37°C in PBS only. Each treatment set included three replicates, with records taken for the time taken for paralysis and death of the parasites.^[31]

Biochemical study

Preparation of tissue homogenate

A 5% tissue homogenate (w/v) was prepared in an ice-cold buffer solution using a tissue homogenizer. Following homogenization, the mixture was centrifuged at 15000 rpm for 10 min at 4°C . The resulting supernatant were used as enzyme source and was stored at -20°C until further use.

Enzyme assay

Acid Phosphatase (ACP)

The ACP activity was assessed by measuring p-nitrophenol formation following the method outlined by Plummer^[32] with little modifications as described by Swargiary *et al.*^[33] The ACP activity was determined using a standard graph of p-nitrophenol ($y=0.0143x+0.203$, $R^2=0.981$).

Alkaline Phosphatase (ALP)

The estimation of ALP activity was done by estimating the p-nitrophenol formation which was followed by Plummer^[32] with little modifications as described by Swargiary *et al.*^[33] The color formed was observed at 410 nm with the help of a spectrophotometer.

Malate Dehydrogenase (MDH)

The estimation of MDH activity was done following Bergmeyer *et al.*^[34] 850 μL sodium phosphate buffer (100 mM, pH 7.4), 50 μL of 10 mM oxaloacetic acid, 50 μL of 10 mM NADH, and 50 μL tissue supernatant was added to make an assay mixture of 1 mL. The assay mixture was warmed up without NADH at $37\pm 1^\circ\text{C}$ for 1 min. Then the reaction was started by adding NADH. The absorbance was monitored for 5 min at 340 nm. For the preparation of the blank, NaOH was added before adding tissue supernatant and all other steps were the same as the main protocol.

Lactate Dehydrogenase (LDH)

The estimation of LDH activity was done following Bergmeyer *et al.*^[35] 910 μL of phosphate buffer (0.1 M, pH 7.4), 20 μL of 50 mM

Table 1: Anthelmintic activity of different solvent fractions of *Hypericum japonicum*.

Plants	Solvent Fractions	Paralysis time (h: min)	Death Time (h: min)
<i>Hypericum japonicum</i>	Hexane	3:49 \pm 0:11	3:55 \pm 0:12
	Diethyl ether	3:22 \pm 0:17	3:49 \pm 0:21
	Ethyl acetate	13:20 \pm 0:37	14:30 \pm 0:24
	Methanol	9:26 \pm 0:38	9:54 \pm 0:48
Reference chemical	Albendazole	3:50 \pm 0:15	4:21 \pm 0:19

Control parasite lived up to 73:21+0:33 min. Values are expressed as mean \pm SD, $n=3$.

pyruvic acid, 20 μ L of 50 mM NADH and 50 μ L tissue supernatant was added to make an assay mixture of 1 mL. The assay mixture was warmed up without NADH at $37\pm 1^\circ\text{C}$ for 1 min. Then the reaction was started by adding NADH. The absorbance was monitored for 5 min at 340 nm. For the blank solution, NADH was replaced with phosphate buffer.

Acetylcholinesterase (AChE)

The estimation of AChE enzyme activity was done by Ellman *et al.*^[36] 30 μ L of tissue supernatant was added to 430 μ L phosphate buffer (0.1 M, pH 8.0) in a 1 mL assay mixture. The mixture was then incubated for 5 min. After this, 20 μ L of 10 mM Ellman's reagent was added, and then 20 μ L of 75 mM acetylcholine iodide was also added. The addition of AchI increased absorbance. The absorbance was then observed at 405 nm with the help of a spectrophotometer. Changes in the absorbance were read at 30-sec intervals at $37\pm 1^\circ\text{C}$ for 5 min.

GC-MS analysis

The most potent diethyl ether extract of *H. japonicum* underwent GC-MS analysis using a Perkin Elmer system comprising a Clarus 680 GC and Clarus 600 GC MS with TurboMass Ver. 5.4.2 software by following Swargiary *et al.*^[33]

Identification of Peaks

The database software of the National Institute Standard and Technology-2008 (NIST-2008) was used for the interpretation of the peaks. The peaks that appeared in the GC-Chromatogram were made by library search of the mass spectrum of the corresponding peaks. The compounds were identified by name, empirical formula, and molecular weight.

Protein modeling and active site prediction

NCBI database (<https://www.ncbi.nlm.nih.gov/>) was used for retrieving amino acid sequences. The amino acid sequences were ACP (GenBank: THD26168.1), ALP (GenBank: THD21487.1), MDH (GenBank: TPP64788.1), LDH (GenBank: TPP62930.1), and AchE (GenBank: KAA0195891.1). The sequences were

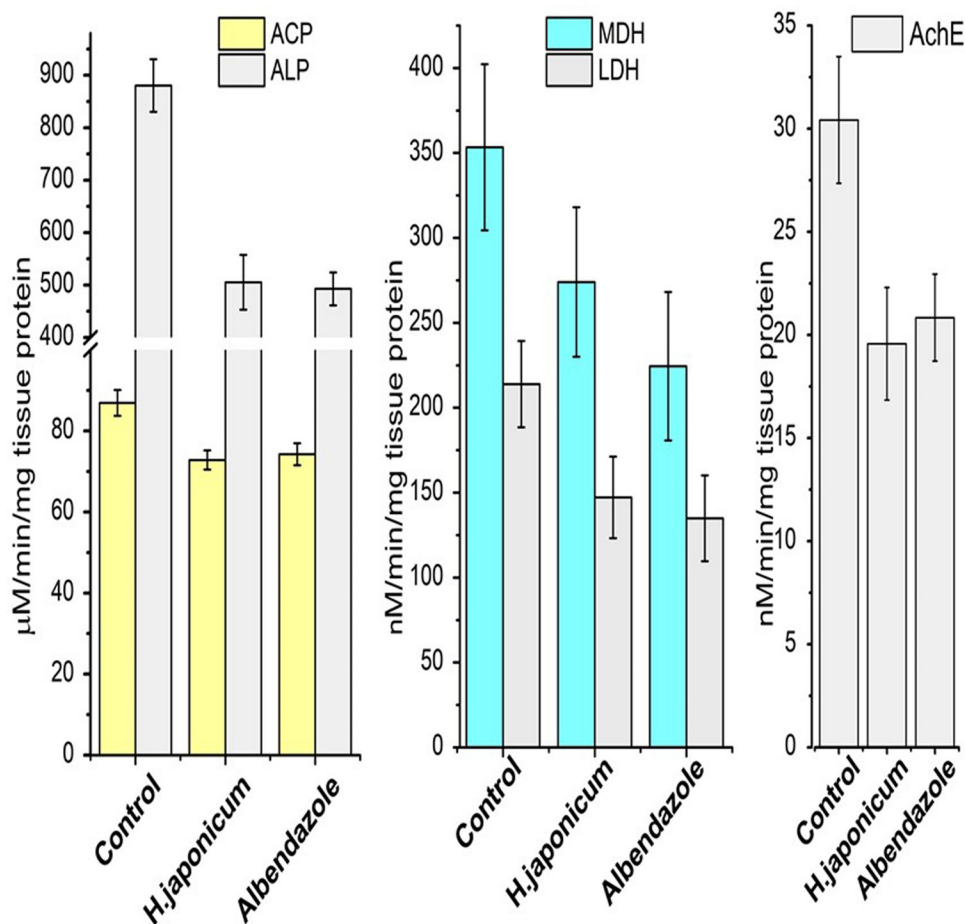


Figure 1: Acid Phosphatase (ACP), Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), Malate Dehydrogenase (MDH) and Acetylcholinesterase (AChE) enzyme, activity of control and plant extract treated parasite. All the enzyme activities showed significant difference between control and plant extract-treated parasites at $p=0.05$ level, except AchE enzyme.

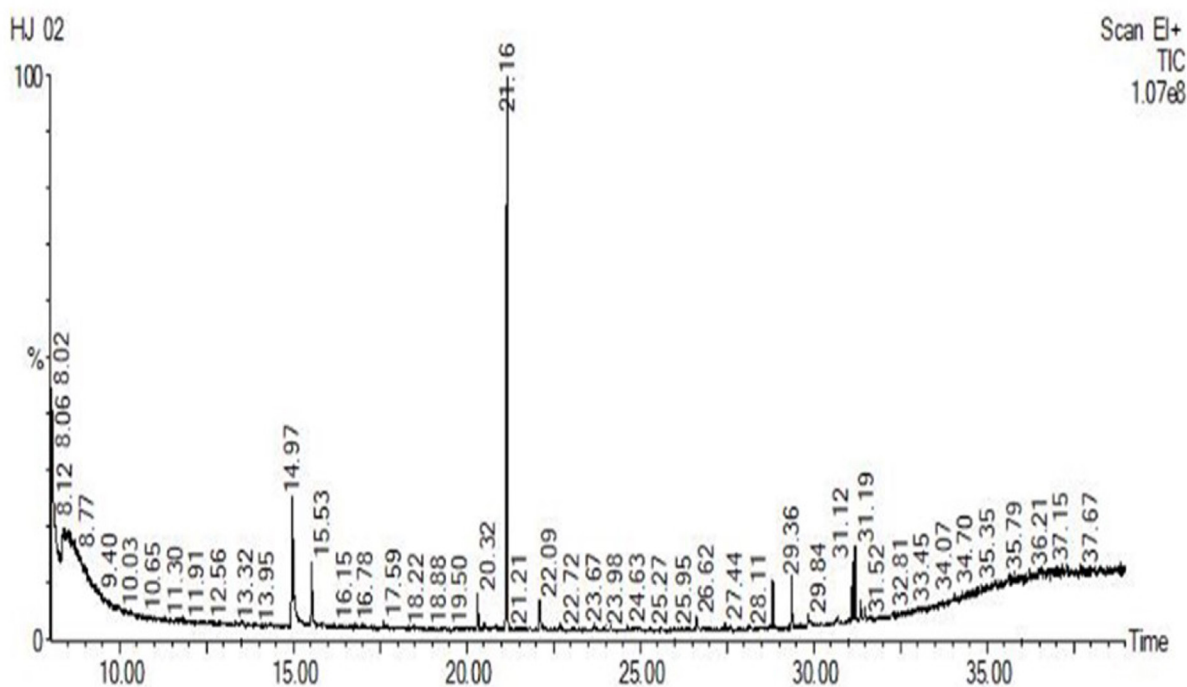


Figure 2: Complete GC-MS spectra profile of diethyl extract of *Hypericum japonicum*.

Table 2: GC-MS identified compounds from the diethyl ether extracts of *Hypericum japonicum*.

Sl. No.	Name of the compounds	RT	PC id	Area (%)	MW (g/mol)	MF
1.	Benzene, (ethenyloxy)- [C1]	15.531	69840	1.212	120.15	C ₈ H ₈ O
2.	1,2-Benzenedicarboxylic Acid, Bis(2-Methylpropyl) Ester [C2]	29.362	6782	0.703	274.34	C ₁₆ H ₂₂ O ₄
3.	Methyl 12,13-Octadecadienoate [C3]	31.118	91694366	0.692	294.5	C ₁₉ H ₃₄ O ₂
4.	Z,Z,Z-8,9-Epoxyeicosa-5,11,14-Trienoic Acid, Methyl Ester [C4]	31.193	71684441	0.982	334.5	C ₂₁ H ₃₄ O ₃
5.	Octadecanoic acid, 11-methyl-, methyl ester [C5]	28.817	554143	0.707	312.5	C ₂₀ H ₄₀ O ₂
6.	Catechol [C6]	14.971	289	3.607	289	C ₆ H ₆ O ₂
7.	Bicyclo[3.1.0]Hexan-2-ONE, 1,5-Bis(1,1-Dimethylethyl)-3,3-Dimethyl- [C7]	20.318	29561	0.492	236.39	C ₆ H ₂₈ O
8.	Butylated Hydroxytoluene [C8]	21.159	31404	7.422	220.35	C ₁₅ H ₂₄ O
9.	1R,2c,3t,4t-Tetramethyl-Cyclohexane [C9]	22.104	94277	0.645	140.27	C ₁₀ H ₂₀
10.	1-Hexyl-2-Nitrocyclohexane [C10]	26.631	544017	0.336	213.32	C ₁₂ H ₂₃ NO ₂
11.	Z,Z-6,28-Heptatriactontadien-2-One [C11]	31.353	5365964	0.417	530.9	C ₃₇ H ₇₀ O
12.	Heptacosanoic Acid, 25-Methyl-, Methyl Ester [C12]	28.817	554101	0.707	438.8	C ₂₉ H ₅₈ O ₂

Table 3: Binding energies (kcal/mol) of *Hypericum japonicum* phytocompounds with different enzymes.

Compounds	AchE	ACP	ALP	LDH	MDH
C1	-5.46	-4.66	-4.50	-4.66	-4.10
C2	-6.73	-6.53	-6.00	-5.33	-5.53
C3	-6.06	-4.80	-5.23	-4.43	-4.46
C4	-5.86	-5.83	-5.63	-4.83	-5.30
C5	-5.03	-5.63	-4.93	-4.40	-4.20
C6	-5.50	-4.80	-4.70	-4.60	-4.13
C7	-6.26	-5.63	-5.60	-5.86	-5.03
C8	-6.33	-6.30	-5.96	-5.53	-5.00
C9	-5.76	-5.03	-4.86	-4.50	-4.80
C10	-6.63	-5.73	-5.43	-5.20	-5.20
C11	-5.90	-4.86	-5.06	-4.56	-3.73
C12	-5.16	-4.90	-4.73	-4.23	-4.30
Albendazole	-6.60	-2.70	-6.46	-5.56	-5.46

submitted to the Swiss-Model server (<https://swissmodel.expasy.org/>) for protein modelling. Enzyme active sites were generated by submitting the model proteins to CASTp 3.0 server.^[37]

Docking study

PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) was used to download the 3D structures of phytocompounds. The 3D protein structures were downloaded from the PDB database (<https://www.rcsb.org/>). Molecular docking was carried out in AutoDock vina.^[38] The grid parameters were set as x, y, z size-coordinate and grid box center-coordinate i.e., 25.511, 10.566, 16.228 and 74, 74, 62 for AchE, 50.440, 65.413, 24.461, and 54, 106, 78 for ACP, 33.323, -9.164 and 44, 44, 50 for ALP, 17.925, -14.018, 27.237 and 62, 48, 54 for LDH and 35.028, 144.289, 42.579 and 52, 44, 46 for MDH. Biovia Discovery Studio was used for the output visualisation. Three replicates were carried out for docking for all the phytocompounds and proteins.

Molecular Dynamics Simulation Study

For Molecular Dynamic (MD) simulations study GROMACS was used for All-atom MD simulations of ligand-protein complexes.^[39,40]

Molecular Mechanics/Poisson Boltzmann Surface Area (MMPBSA) analysis

The free energies of all protein-ligand complexes were analysed by the MMPBSA package.^[39,40] The major energy components such as binding energy (kJ/mol), electrostatic energy, van der Waals energy, polar solvation energy, non-polar solvation energy, and total energy were determined which contributed together to understanding the binding affinity of ligand-protein complexes. The MM/PBSA method-based binding free energy of the protein-ligand systems was calculated using the following equation:

$$\Delta G_{MMPBSA} = \{G_{\text{complex}} - G_{\text{protein}} - G_{\text{ligand}}\}$$

where G_{complex} represents the total free energy of the docking complex, and G_{protein} and G_{ligand} depict the total free energies of the isolated protein and ligand in the solvent, respectively. Solvent Accessible Surface Area (SASA) value was calculated from the non-polar component as follows:

$$G_{\text{non-polar}} = \gamma * \text{SASA}$$

$$\text{Where, } \gamma = 0.0301 \text{ kJ/mol/\AA}^2$$

Statistical analysis

Microsoft Excel was used for all the statistical calculations. OriginPro-8.5 software was used for the test of significance and correlation study. All the experiments were carried out in triplicates ($n=3$), and the results were represented as mean \pm Standard Deviation (SD).

RESULTS

Anthelmintic activity

Table 1 shows the anthelmintic activity of the solvent fractions with a fixed test dose of 5 mg/mL. Compared to the reference drug, diethyl ether extract of *H. japonicum* showed better anthelmintic activity. Among the four extracts, diethyl ether showed the strongest activity with time 3:49 \pm 0:21 hr:min. Hexane extract also showed almost similar activity compared to diethyl ether extract. Ethyl acetate extract showed the weakest activity taking about 14:30 \pm 0:24 hr:min.

Biochemical Enzyme Assay

Two important tegumental enzymes, namely, acid-and alkaline phosphatase, two glycolytic enzymes malate-and

Table 4: Total binding free energies of protein complexes with albendazole. All values are presented in kJ/mol.

Parameters	Ache-C2	Ache-albendazole
VDWAALS	-2537.69±34.4	-3080.12±16.1
EEL	-24609.22±75.84	-24619.6±50.09
EPB	-6730.28±59.61	-6676.16±17.29
ENPOLAR	95.26±0.64	94.51±0.3
Total	-9908.26±38.24	-9925.59±71.66

VDWAALS-Van der Waals Energy, ELL-electrostatic energy, EPB-Polar solvation energy, ENPOLAR -Non-polar Solvation Energy, All values are represented as \pm standard deviation.

Table 5: Free energy changes (Delta values) of protein complexes with albendazole. All values are in kJ/mol.

Parameters	Ache-C2	Ache-albendazole
Δ VDWAALS	-37.41±1.74	-29.48±0.2
Δ EEL	-5.23±1.77	-30.75±0.83
Δ EPB	-24.88±0.67	41.67±0.59
Δ ENPOLAR	-3.94±0.07	-2.9±0.01
Total	-21.7±0.26	-21.46±1.04

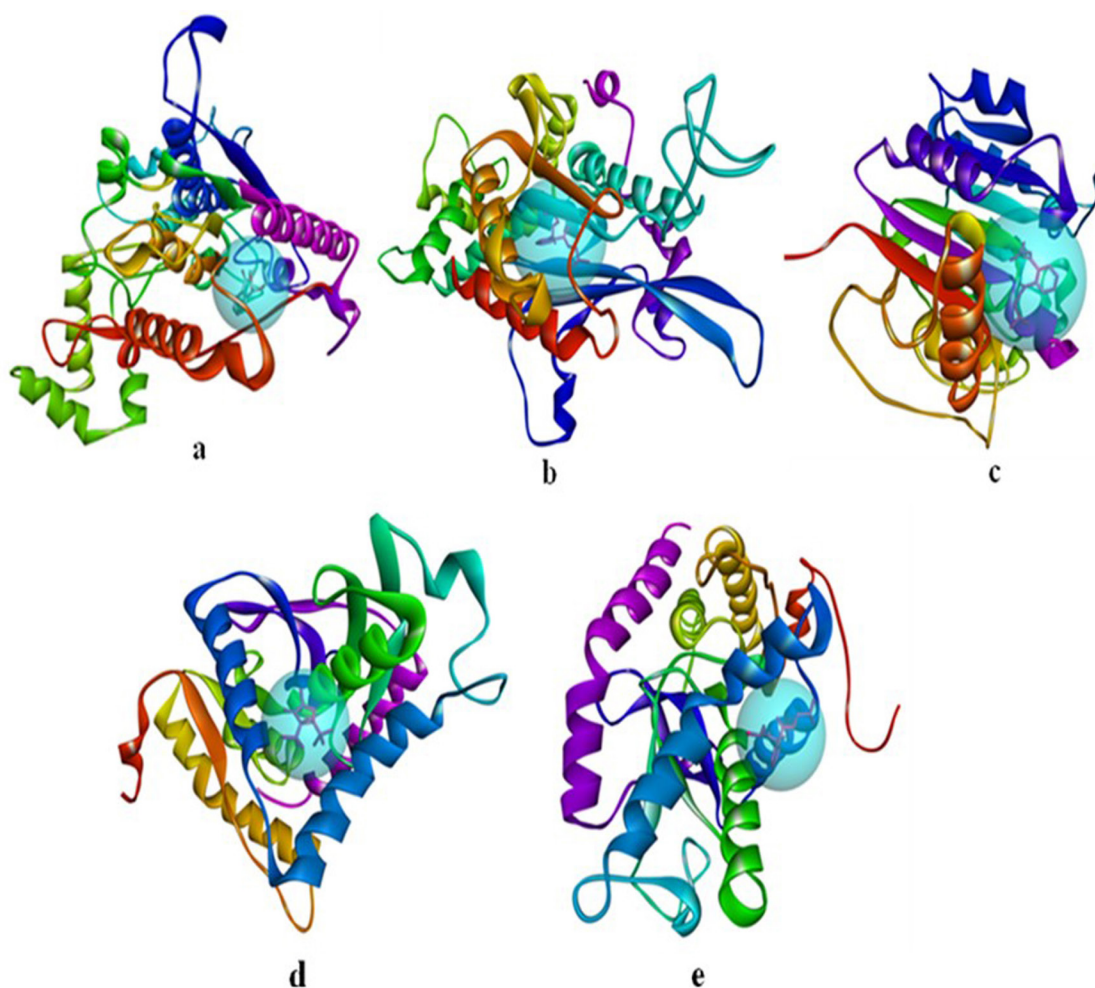


Figure 3: Molecular docking and 3D binding affinities of enzymes and phytochemicals. The circle colour indicates the superimpose binding site between the receptor and the ligand and with the reference drug. (a) AchE with albendazole and C2, (b) ACP with albendazole and C2, (c) ALP with albendazole and C2, (d) LDH with albendazole and C7, and (e) MDH with albendazole and C2.

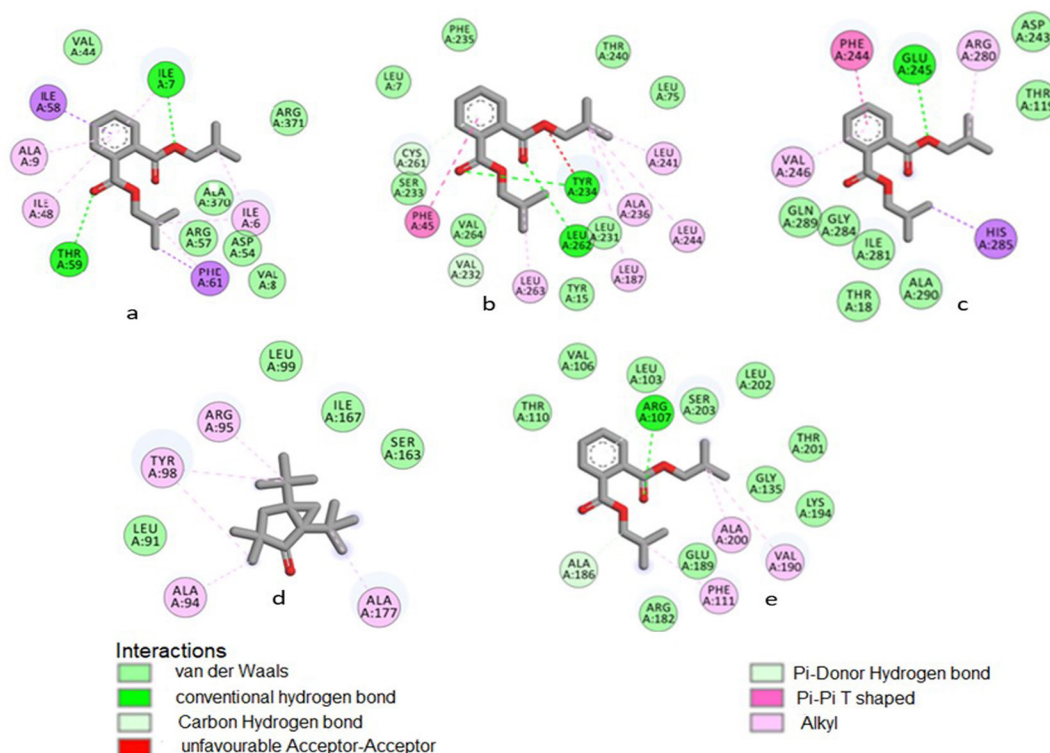


Figure 4: 2D binding affinities of enzymes and phytocompounds. (a) AchE and C2, (b) ACP and C2, (c) ALP and C2, (d) LDH and C7, and (e) MDH and C2.

lactate dehydrogenase, and one neurotransmitter enzyme, acetylcholinesterase have been investigated in the present study (Figure 1). Paralyzed parasites after plant extract treatment showed a decrease in ACP, while control, untreated parasites showed 86.94 ± 3.17 $\mu\text{M}/\text{min}/\text{mg}$ protein, 880.33 ± 50.19 $\mu\text{M}/\text{min}/\text{mg}$ protein, and 30.43 ± 8.07 $\text{nM}/\text{min}/\text{mg}$ protein for ACP, ALP, and AchE enzymes, respectively. Like tegumental enzymes, glycolytic enzymes, MDH, and LDH also showed reduced activity in plant extract and albendazole-treated parasites. The control parasite showed 353.39 ± 38.87 and 213.41 ± 25.41 , $\text{nM}/\text{min}/\text{mg}$ tissue protein for MDH and LDH, respectively. In ALP, LDH, and MDH, the diethyl extract of the plant showed good activity compared to albendazole. Whereas in ACP and AchE, the plant extract showed less activity compared to albendazole. The enzyme activities show significant differences at $p=0.05$ with the control, except AchE. The highest percentage of inhibition of plant extract was found in ALP (42.59%), and the least was found in ACP (16.21%) compared to control.

GC-MS analysis of phytocompounds

The GC-MS study of the diethyl ether fraction of *H. japonicum* observed 12 probable phytocompounds from the plant. The images of the phytocompounds are provided as Supplementary File 1. The names of the compounds with the GC-MS profile are shown in Table 3. The spectra analyzed of the compounds are shown in Figure 2. The highest percentage was shown in peak structure with Retention Time (RT) 21.16. The identified

phytocompounds with the RT, molecular weight, etc., are presented in Table 2.

Protein modeling and Molecular docking

Protein modeling was carried out using the Swiss-Model server. The templates used were 2hpa.1.A, 3mk1.1.A, 4l4s.1.A, 2dfd.1.A, and 5fpq.2.A ACP, ALP, LDH, MDH, and AchE enzymes, respectively. All 12 identified compounds were docked with the model proteins. The binding affinities of compounds with all five enzymes are shown in Table 3. The compound 1,2-Benzenedicarboxylic Acid, Bis (2-Methylpropyl) Ester (C2) showed the best binding affinity with AchE (-6.73 kcal/mol), ACP (-6.53 kcal/mol), ALP (-6 kcal/mol), and MDH (-5.53 kcal/mol). Similarly, Bicyclo[3.1.0]Hexan-2-ONE, 1,5-Bis (1,1-Dimethylethyl)-3,3-Dimethyl- (C7) showed the best binding with LDH (-5.83 kcal/mol). Overall, phytocompounds showed the best binding affinity with AchE followed by ACP, ALP, LDH, and MDH. Figure 3 showed the 3D binding interactions of proteins and compounds with the reference drug (albendazole). Figure 4 showed the 2D binding interactions of proteins and compounds. Albendazole showed the best binding affinity with AchE. A total of 18 residues of ACP interacted with the C2, including 2H-bonds with Ala-234 and Leu A:262 residue. Eight residues formed a van der Waals interaction. MDH involved 15 residues, including a single H-bond with Arg A:245 residue and ten residues of van der Waals interaction. AchE with 13 residues where 2H bond and 6 Van der Waals interaction. ALP has 12 residues with 1 H bond

and 7 van der Waals, and LDH interacted with C7, including 8 residues with four van der Waals and no H bond. Albendazole showed the best binding affinity with AchE.

Molecular Dynamics Simulation Study

The 3D structural conformation of AchE protein with surrounding solvent molecules during the period of MD simulation was shown in Figure. AchE-C2 complex and AchE-Alb complex simulation showed nearly similar backbone RMSD with similar solvent influences in the entire 100 ns simulation, while the apo-protein without ligand showed deviation as shown in Figure 5a. The simulation has shown several structural changes in the protein after ligand binding. However, the protein showed stability throughout the simulation period. When backbone conformation was observed with ligand, RMSDs of two complexes were seen (Figure 5b).

In AchE-C2, the structure showed higher RMSD from 45th ns to 100th ns compared to AchE-Alb. In other words, the AchE-C2 showed stronger binding affinity compared to AchE-Alb. From the RMSF study, it revealed that AchE-C2 showed higher fluctuation in the amino acid residues, while AchE-Alb showed slightly lower fluctuation. Figure 5d shows the radius of gyration of proteins. RG is studied to measure the elastic stability of a protein. RG analysis revealed that all the proteins showed instability in the elasticity of the proteins.

Figure 6a presents an analysis of hydrogen bonds involving AchE-C2, AchE-Alb, and the apo-protein in conjunction with solvent. Both AchE-C2 and AchE-Alb exhibited a range of 700 to 850 hydrogen bonds with the surrounding solvent, whereas the apo-protein displayed a higher count, ranging from over 800 to nearly 900 hydrogen bonds. This indicates that AchE-C2 and AchE-Alb had fewer hydrogen bonds compared to the

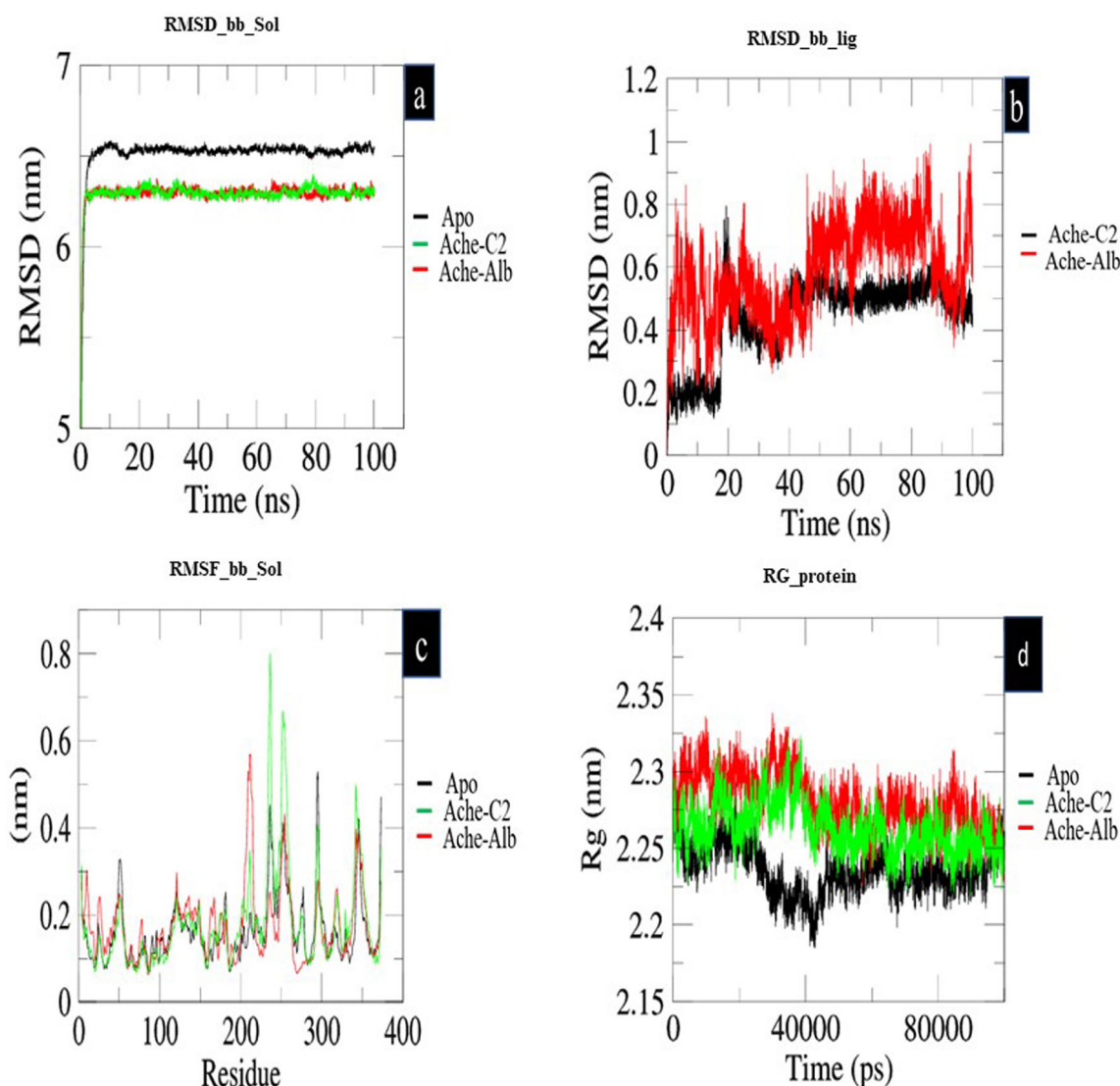


Figure 5: Molecular dynamics simulation (a) RMSD of apo protein (Ache), Ache-C2 and Ache-Alb with solvent, (b) RMSD with ligand (c) RMS Fluctuation of amino acid residues, and (d) Radius of gyration.

apo-protein. The assessment of H-bonds formed between ligands and proteins, depicted in Figure 6c, revealed that AchE-C2 had a greater number of H-bonds than AchE-Alb, with up to 4 and 3 H-bonds detected, respectively, during the simulation period. These bonds persisted in both AchE-Alb and AchE-C2 until the 100th ns. Notably, compound C2 exhibited stronger hydrogen bonding with the ligand than Alb. Additionally, Figure 6b illustrates the protein's total energy throughout the 100 ns simulation time, with the AchE-C2 and AchE-Alb complex displaying nearly identical energy levels, while the apo-protein exhibited lower energy levels compared to the complexes.

MMPBSA Analysis

The total binding energies and binding free energy changes of the complexes are displayed in Tables 4 and 5, respectively. The

thermodynamics property of the complexes has been shown. The contribution of the proteins (receptors) showing binding energies compared to the contribution of the ligands. The binding energy is mainly contributed by Van der Waals Energy, electrostatic energy, polar solvation energy and non-polar Solvation Energy. The total binding free energy of the Alb was found almost slightly higher with value -9925.59 ± 71.66 kcal/mol than compound C2 with value -9908.26 ± 38.24 kcal/mol. All the interaction has shown almost similar binding except for van der Waals force, which has shown higher in Alb with -3080.12 ± 16.1 kcal/mol whereas -2537.69 ± 34.4 kcal/mol in C2.

The free energy changes that is the delta value has shown almost similar result in both the complexes, except for ΔEEL value with -5.23 ± 1.77 kcal/mol in Ache-C2 complex and -30.75 ± 0.83 kcal/mol in Ache-Alb complex and also in ΔEPB value where Ache-C2

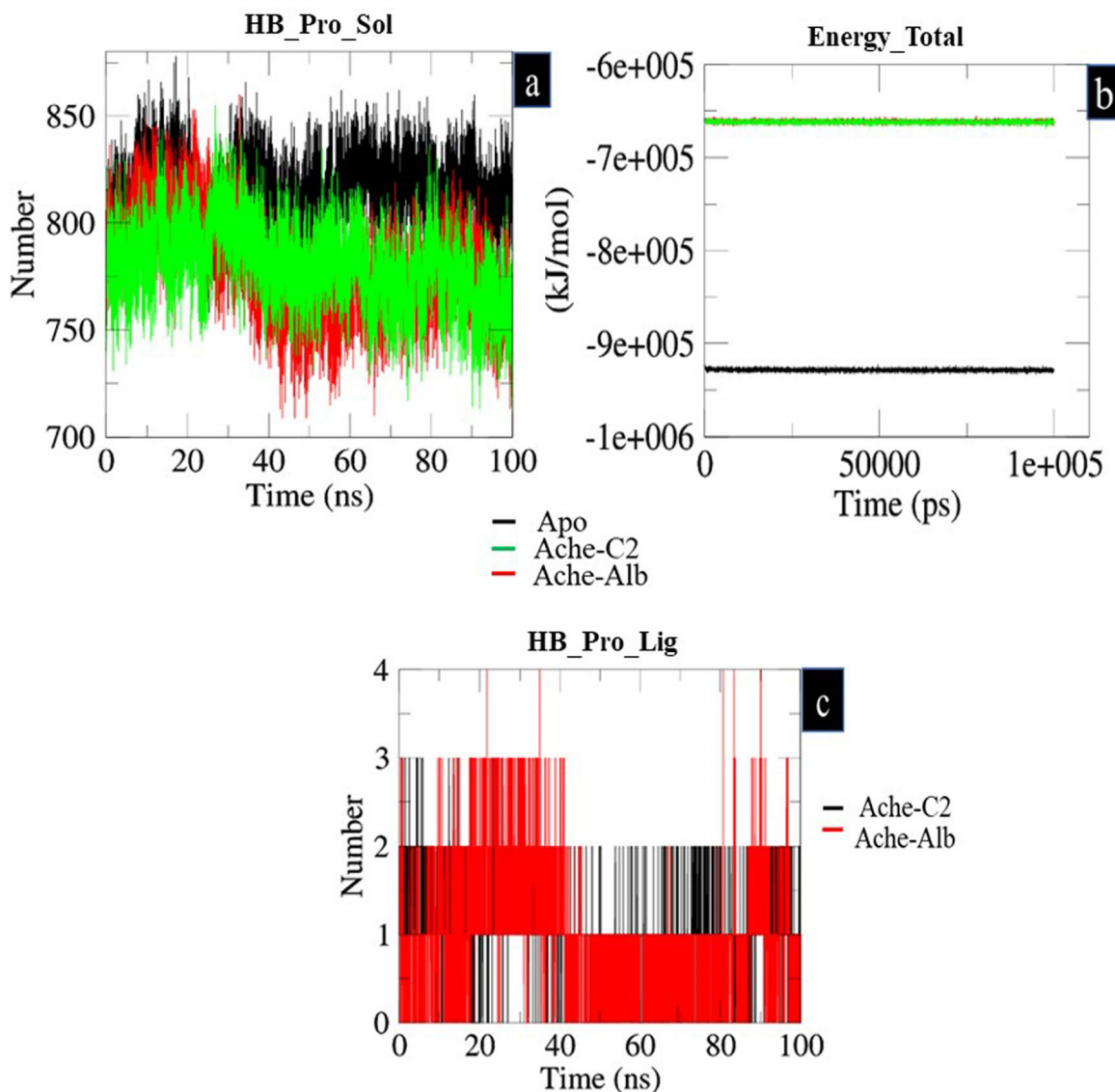


Figure 6: Molecular dynamics simulation (a) total number of H-Bonds (HB) between protein and solvent during the simulation period, (b) total number of H-Bonds (HB) between protein and ligand during the simulation period, and (c) Total energy (-kJ/mol) of proteins during the simulation period.

value was found as -24.88 ± 0.67 kcal/mol and -41.67 ± 0.59 kcal/mol for Ache-Alb. The values of total free energy for both the complexes was found almost similar as shown in the Table 5.

DISCUSSION

Parasites can cause substantial morbidity and mortality in livestock animals resulting considerable productivity losses, mainly to farmers.^[41] Due to helminth infection millions of people are affected worldwide, especially in developing countries. The biochemical analysis and the *in vitro* bioassay exhibited the presence of considerable anthelmintic properties of *H. japonicum*. In the current study, four solvent extracts of *H. japonicum* were prepared, and anthelmintic activity was analyzed. Diethyl ether extract showed the strongest activity, hexane extract also showed almost similar activity against the helminth parasite. The diethyl ether extract of *H. japonicum* extract also showed better anthelmintic activity than albendazole. A study done on the ovicidal and larvicidal activity of *Melia azedarach* extracts found that ethanol extract showed better anthelmintic activity than hexane.^[42] In another study, it was investigated that the anthelmintic property of different solvent extracts of *C. asiatica*, *G. superba*, *P. daemia*, and *P. emblica* extracts showed better activity in methanolic extracts than other solvent extracts.^[43] Similar to our study, a study reported dose-dependent anthelmintic activity of ethanolic extracts of *Platyclusus orientalis* leaves.^[44] Our earlier study on *P. strigosa* on different solvent extracts showed a slightly different result than our present study, where ethyl acetate has shown the strongest activity with a death time of $7:52 \pm 0:24$ hr.^[45]

Metabolic pathways have become a focal point for developing novel drugs targeting checkpoint enzymes, offering promise in combating various infectious diseases.^[46] Within helminth parasites, tegumental enzymes play a pivotal role by mediating crucial reactions such as phosphorylation and dephosphorylation, integral to cell signaling and gene regulation. Acid and alkaline phosphatase, among these enzymes, are vital for functions such as protection, absorption, and secretion within the parasite's outer covering, known as the tegument. Our study unveiled a reduction in tegumental enzyme activity in parasites treated with both plant extract and albendazole. Similar trends were observed in investigations on *Potentilla fulgens* and *Amomum maximum* Roxb, highlighting a noteworthy decline in enzyme activity.^[47,48] Moreover, glycolytic enzymes like malate dehydrogenase and lactate dehydrogenase, crucial for aerobic and anaerobic respiration, displayed decreased activity in parasites treated with *H. japonicum* extract. Similar outcomes were reported in studies on *Lysimachia ramosa* Wall. Ex Duby and *Punica granatum* ethanol extract, where lowered enzyme activity influenced glycogen metabolism.^[49,50] Acetylcholinesterase, a crucial neurotransmitter enzyme, plays a pivotal role in helminths' neuromuscular activity. Hindering its activity can lead to paralysis and subsequent death of the parasites. Essential

oils of *Origanum* have also exhibited anthelmintic effects by inhibiting acetylcholinesterase activity against *Anisakis simplex* L3 larvae.^[51] Twelve potential phytochemicals were identified in the diethyl ether extract of *H. japonicum*, some of which have been reported in other plants for their antimicrobial and antioxidative properties.^[52-54] Molecular dynamics simulations of AchE protein with compound C2 and albendazole suggested similar binding affinity between C2 and the reference drug. Despite structural changes post-ligand binding, protein stability was maintained throughout the simulation. Analysis of radius of gyration indicated protein instability. Apo protein exhibited more hydrogen bonding than C2 and albendazole, suggesting slightly lower binding affinity in the complexes. However, total energy analysis showed comparable energy levels between C2, albendazole, and apo protein.^[55-57] MMPBS analysis revealed negative delta energy, indicating spontaneous and stable binding, essential for predicting biomolecular complex affinities.

CONCLUSION

The present study enlightened *H. japonicum* to be a natural source of antioxidants and a potential anthelmintic agent. The ethyl acetate extract of the plant showed a good source of antioxidant properties. The diethyl ether extract of the plant enlightened to be a strong source of anthelmintic property. Treated parasites showed a significant decrease in enzyme activity. Compound-C2 showed better binding affinity with enzymes compared to other compounds. The highest percentage inhibition of plant extract was found in ALP and the least was found in ACP. Biochemical enzyme assays and *in silico* docking studies suggested significant enzyme inhibitory properties of the plant extracts along with the phytochemicals. MD simulation showed protein stability throughout the simulation period. However, further investigation, including phytochemical isolation and characterization, needs to be done to find out the accurate mode of action.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

MKR carried out the anthelmintic study, biochemical analysis, docking study and writing of manuscript. AS involved in designing the study, statistical calculations, molecular dynamics and writing of the manuscript.

ABBREVIATIONS

H. japonicum: *Hypericum japonicum*; **MCEHJ:** Methanolic crude extract of *H. japonicum*; **PBS:** Phosphate buffer saline; **ACP:** Acid phosphatase; **ALP:** Alkaline phosphatase; **MDH:** Malate dehydrogenase; **LDH:** Lactate dehydrogenase; **AchE:** Acetylcholinesterase; **SD:** Standard deviation; **MD:** Molecular Dynamics; **Alb:** Albendazole; **RMSD:** Root Mean Square Deviation; **RMSF:** Root Mean Square Fluctuation; **RG:** Radius of gyration; **MMPBSA:** Molecular Mechanics Poisson-Boltzmann Surface Area.

SUMMARY

Plants and plant-derived products have been used to control helminth infestation in many parts of the world. *Hypericum japonicum* Thunb. is a vital medicinal plant in Northeast India, traditionally used by tribal communities to treat helminth infections with leaf extracts. The findings suggest that *Hypericum japonicum* could be a potential source of anthelmintic agents, which also showed high reduction in alkaline phosphatase, warranting further studies to elucidate its exact mechanism of action.

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