CHAPTER – 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

OBJECTIVE 1: Study of phytochemical content, antioxidant, and anthelmintic activity of *Hypericum japonicum* crude extracts and solvent fractions

3.1. Collection, authentication, and preparation of crude extract and solvent fractions

The plant sample utilized in the study was collected from the Tinali area of Kokrajhar district, Assam (26°30'07.1"N 90°19'02.9"E). For taxonomic identification and authentication, a herbarium sheet of the collected plant was meticulously prepared and subsequently submitted to the Department of Botany at Bodoland University. Following a detailed examination of its morphological and taxonomic characteristics, the plant was conclusively identified as *Hypericum japonicum* Thunb by taxonomist Dr. Sanjib Baruah. This identification was officially recorded under the herbarium accession number BUBH2000129, ensuring proper documentation and validation for future reference.

For the preparation of crude extract, fresh leaves of *Hypericum japonicum* were carefully collected and processed following the method described by Seidel (2005; Swargiary et al., 2016) as shown in Photo plate 2. Initially, the leaves were thoroughly cleaned using distilled water to remove any impurities. The cleaned leaves were then dried in an oven at a controlled temperature of 45 ± 2°C to preserve their phytochemical integrity. Once dried, the leaves were ground into a fine powder using a mixer grinder (SINGER, Maxtra model). The powdered leaves were subsequently macerated in 80% methanol in a ratio of 1:5 (w/v) for duration of 72 hours. This process was repeated three times to ensure maximum extraction of bioactive compounds. After maceration, the solution was filtered using Whatman filter paper no. 1 to remove plant debris and obtain a clear filtrate. The filtered solvent was concentrated by removing the methanol through a rotary vacuum evaporator (Buchi type, Optics Technology) under reduced pressure. The resultant dry or semi-solid extract was collected and stored at -20°C for further experimental analysis. This meticulous preparation process ensured the preservation of bioactive compounds for downstream studies.

3.2. Solvent Fractionation

The liquid-liquid fractionation method was employed for the solvent fractionation of the methanolic crude extract of *Hypericum japonicum* (MCEHJ) using a separating funnel, following the method described by Hamzaoui et al. (2013) (Photoplate 3). The detailed procedure is explained as follows:

Steps

i) First, the methanolic crude extract was dissolved in distilled water to form a suspension, ensuring that the extract was adequately dispersed in the aqueous medium for fractionation.

ii) Solvents with varying polarity indices were selected to target a broad spectrum of phytochemicals based on their solubility. The selected solvents were hexane (Hex, polarity index, 0.1), diethyl ether (DE, polarity index 2.8), and ethyl acetate (EA, polarity index 4.1).

iii) An equal volume of hexane was added to the aqueous solution, and the mixture was vigorously shaken to facilitate the separation of compounds into distinct layers based on their affinity for the hexane phase. After allowing the layers to settle, the hexane layer was carefully collected, and this process was repeated multiple times until the hexane layer became completely colorless, indicating the exhaustion of hexane-soluble compounds.

iv) Following the collection of the hexane fraction, diethyl ether was added to the remaining aqueous solution, and the same process of shaking, settling, and collecting the DE layer was carried out until the DE fraction became colorless, ensuring the extraction of moderately polar compounds.

v) Subsequently, ethyl acetate was added to the solution, and the fractionation process was repeated until the EA layer turned colorless, capturing compounds with higher polarity.

vi) Each collected solvent fraction (Hex, DE, and EA) was then subjected to evaporation under reduced pressure using a rotary vacuum evaporator to remove the solvents and yield dry or semi-solid extracts.

vii) The resulting solvent fractions were stored at -20°C to preserve their bioactive components for further phytochemical and pharmacological studies.

3.3. Qualitative and quantitative phytochemical analysis

I. Qualitative phytochemical analysis

Qualitative analysis of phytocompounds refers to the process of identifying the presence or absence of specific bioactive compounds or classes of compounds in plant materials. This type of analysis helps in the preliminary characterization of plant extracts and their potential medicinal properties. It focuses on determining which phytochemicals are present, rather than quantifying their concentration. For qualitative phytochemical analysis, the methods of Trease and Evans (1989) and Sofowara (1993) were followed.

A. Alkaloids test (*Dragondroff's test*)

For the qualitative detection of alkaloids, 5 mg of plant extracts methanolic (Met), diethyl ether (DE), hexane (Hex), and ethyl acetate (EA) were weighed and dissolved separately in 1 mL of distilled water in distinct test tubes. The solutions were thoroughly mixed to ensure complete dissolution of the extracts. To each test tube, 0.5 mL of Dragendorff's reagent was added, and the mixture was gently agitated. The presence of alkaloids was indicated by the appearance of a reddish-brown coloration in the solution. This color change is a positive result, confirming the presence of alkaloid compounds in the plant extracts, as Dragendorff's reagent reacts specifically with alkaloids to produce this characteristic color.

B. Test for Phenolics

For the qualitative detection of phenols, 5 mg of plant solvent extracts (Met, DE, Hex, and EA) were weighed and dissolved separately in 1 mL of distilled water in distinct test tubes. The solutions were mixed thoroughly to ensure complete dissolution of the extracts. To each test tube, 2 mL of distilled water was added to further dilute the solution. Subsequently, 0.5 mL of ferric chloride ($FeCl_3$) solution was added, and the mixture was properly agitated. The presence of phenolic compounds was confirmed by the appearance of a bluish or greenish coloration in the test tube. This color change indicates the presence of phenols, as ferric chloride reacts with phenolic compounds to produce these characteristic colors.

C. Flavonoid test

For the qualitative detection of flavonoids, 10 mg of plant solvent extracts (Met, DE, Hex, and EA) were accurately weighed and dissolved in 2 mL of distilled water in separate test tubes. The solutions were thoroughly mixed to ensure complete dissolution of the extracts. To each test tube, 1 mL of 2N sodium hydroxide (NaOH) solution was carefully added to each test tube. The mixtures were then thoroughly agitated to ensure uniform mixing. The presence of flavonoid was confirmed by the appearance of a distinct yellow coloration in the test tube. This color change is indicative of the presence of flavonoid derivatives, as they undergo a characteristic reaction with NaOH, leading to the formation of a yellow colored complex.

D. Quinones test

For the qualitative detection of quinones, 5 mg of plant solvent extracts (Met, DE, Hex, and EA) were accurately weighed and dissolved in 1 mL of distilled water in separate test tubes. The solutions were thoroughly mixed to ensure complete dissolution of the extracts. To each test tube, 1 mL of concentrated sulfuric acid (H_2SO_4) was added, and the mixture was agitated gently to ensure proper interaction. The presence of quinones was confirmed by the appearance of a distinct red coloration in the test tube. This color change is indicative of the presence of quinone derivatives, as they undergo a characteristic reaction with sulfuric acid, leading to the formation of a red-colored complex.

E. Terpenoid test

For the qualitative detection of terpenoids, 2.5 mg of plant solvent extracts (Met, DE, Hex, and EA) were accurately weighed and dissolved in 0.5 mL of distilled water in separate test tubes. The solutions were thoroughly mixed to ensure complete dissolution of the extracts. To each test tube, 2 mL of chloroform and 1 mL of conc. sulphuric acid were added, and the mixture was agitated gently to ensure proper interaction. The presence of terpenoid was confirmed by the appearance of a distinct red-brown coloration in the test tube. This color change is indicative of the presence of terpenoid derivatives, as they undergo a characteristic reaction with the choloform and conc. sulfuric acid, leading to the formation of a red-brown colored complex.

F. Tannins test (Ferric Chloride Test)

For the qualitative detection of tannins, 5 mg of plant solvent extracts (Met, DE, Hex, and EA) were weighed and dissolved separately in 1 mL of distilled water in distinct test tubes. The solutions were mixed thoroughly to ensure complete dissolution of the extracts. To each test tube, 2 mL of 5% ferric chloride ($FeCl_3$) solution was added, and the mixture was properly agitated. The presence of tannins was confirmed by the appearance of a dark blue or greenish coloration in the test tube. This color change indicates the presence of tannin compounds, as ferric chloride reacts with tannins to form these characteristic colors.

G. Coumarin test

For the qualitative detection of coumarins, 5 mg of plant solvent extracts (Met, DE, Hex, and EA) were accurately weighed and dissolved in 1 mL of distilled water in separate test tubes designated for each solvent extract. The mixtures were thoroughly mixed to ensure proper dissolution. Subsequently, 2 mL of distilled water was added to each test tube, followed by the addition of 0.5 mL of ferric chloride solution. The contents of each test tube were mixed properly to allow for the reaction to occur. The appearance of a bluish or green coloration in the solution was considered a positive indication of the presence of coumarins in the respective extracts.

H. Anthocyanin test

To detect the presence of anthocyanins, 5 mg of plant solvent extracts (Met, DE, Hex, and EA) were dissolved in 1 mL of distilled water in separate test tubes for each solvent extract. The solutions were mixed thoroughly to ensure complete dissolution of the extracts. Subsequently, 1 mL of 2N sodium hydroxide (NaOH) solution was added to each test tube. The test tubes were then heated in a water bath for 5 minutes. After heating, the appearance of bluish-green coloration in the solution was observed, confirming the presence of anthocyanins in the respective extracts.

I. Glycosides test

To test for the presence of glycosides, 10 mg of plant solvent extracts (Met, DE, Hex, and EA) were dissolved in 2 mL of distilled water in separate test tubes, each designated for a

specific solvent extract. The mixtures were thoroughly agitated to ensure complete dissolution of the extracts. Subsequently, 2 mL of chloroform was added to each test tube, followed by the addition of a few drops of 10% ammonia solution. The contents of the test tubes were mixed thoroughly to allow the reaction to occur. The appearance of a pink coloration at the interface of the solution was considered a positive indication of the presence of glycosides in the respective extracts.

J. Anthraquinones test

For the qualitative detection of anthraquinones, 10 mg of plant solvent (Met, DE, Hex and EA) were accurately weighed and dissolved in 2 ml distilled water in in separate test tubes. The solutions were thoroughly mixed to ensure complete dissolution of the extracts. To each test tube, 1 mL of ammonia solution was added, and the mixture was agitated gently to ensure proper interaction. The presence of anthraquinone was confirmed by the appearance of a distinct pink coloration in the test tube. This color change is indicative of the presence of anthraquinone derivatives, as they undergo a characteristic reaction with ammonia solution, leading to the formation of a pink colored complex.

K. Steroids test (*Salkowski's test*)

The presence of steroids was confirmed by the appearance of a red color at the lower level of the solution, while the presence of triterpenoids was confirmed by the development of a yellow color. To perform the test, 5 mg of plant solvent extracts (Met, DE, Hex, and EA) were dissolved in 1 mL of distilled water in separate test tubes for each solvent extract. The mixtures were thoroughly agitated to ensure complete dissolution. Then, 0.5 mL of concentrated sulfuric acid was added to each test tube, and the contents were mixed properly. The color changes observed after mixing indicated the presence of either steroids or triterpenoids in the respective plant extracts.

L. Carbohydrate test

To test for the presence of carbohydrates, 5 mg of plant solvent extracts (Met, DE, Hex, and EA) were dissolved in 1 mL of distilled water in separate test tubes, each designated for a specific solvent extract. The mixtures were thoroughly agitated to ensure complete dissolution. Then, a few drops of Molisch's reagent were added to each test tube. After

mixing, 0.5 mL of concentrated sulfuric acid (H_2SO_4) was carefully added to each test tube, ensuring the acid was layered at the bottom. The contents were mixed gently, and the appearance of a purple or reddish color at the interface was observed, confirming the presence of carbohydrates in the respective extracts.

M. Saponins test

To detect the presence of saponins, 2.5 mg of plant solvent extracts (Met, DE, Hex, and EA) were dissolved in 0.5 mL of distilled water in separate test tubes, each designated for a specific solvent extract. The mixtures were thoroughly agitated to ensure complete dissolution of the extracts. Next, 0.5 mL of olive oil was added to each test tube, and the contents were shaken vigorously for a few minutes. The presence of saponins was confirmed by the formation of a fairly stable emulsion in the solution.

N. Protein test (Ninhydrin)

To test the presence of ninhydrin, 10 mg of plant solvent (Met, DE, Hex and EA) extracts were dissolved in 2 mL distilled water in separate test tubes for each solvent extract. The mixtures were thoroughly agitated to ensure proper dissolution of the extracts. Then, 0.5 mL of ninhydrin reagent was added to each test tube. The solutions were mixed well and heated for five minutes. After heating the formation of a blue precipitate was observed. The appearance of this blue precipitate confirmed the presence of protein.

O. Phlobatannins test

To test for the presence of phlobatannins, 5 mg of plant solvent extracts (Met, DE, Hex, and EA) were dissolved in 1 mL of distilled water in separate test tubes for each solvent extract. The mixtures were thoroughly agitated to ensure proper dissolution of the extracts. Then, 0.5 mL of 2% hydrochloric acid (HCl) was added to each test tube. The solutions were mixed well, and the formation of a red precipitate was observed. The appearance of this red precipitate confirmed the presence of phlobatannins in the respective extracts.

II. Quantitative Test

A. Protein Assay

The protein content of all the plant extracts was estimated following the Lowry method (Lowry et al., 1951).

Principle

Protein quantification by the Lowry method involves two consecutive reactions resulting in the formation of a colored complex. In the first reaction, that is Biuret reaction: In this reaction, copper ions (Cu2+) undergo reduction to cuprous ions (Cu+) when they interact with peptide bonds in proteins under alkaline conditions. In the second reaction, that is Folin-Ciocalteu reaction: In this reaction, the reduced cuprous ions subsequently react with the Folin-Ciocalteu reagent (a mixture of phosphomolybdic and phosphotungstic acids), generating a blue-green chromophore that can be measured spectrophotometrically to determine the protein concentration in the sample (Ranjini et al., 2017).

Chemicals and reagents

Folin reagent, Bovin serum albulim (BSA), Protein reagent, Sodium hydroxide (NaOH), Sodium Carbonate, Sodium-Potassium tartrate, Copper sulphate.

Preparation of protein reagent

The preparation of protein reagent was done by mixing 100 mL of 4% Sodium Carbonate in 0.1N NaOH, 1 mL of 2% Sodium-Potassium tartrate and 1 mL of 2% Copper sulphate.

Procedure

(i) Preparation of Standard Curve for BSA

BSA was taken as a standard for protein estimation. For stock solution 250 µg/mL of BSA was prepared.

Steps

(a) Five different volumes (10, 20, 50, 100, and 200 µL) of the BSA solution were pipetted into separate test tubes, with three replicates for each volume to ensure accuracy.

(b) Distilled water was added to each test tube to bring the final volume to 1 mL, ensuring proper dilution of the BSA solution.

(c) 1.5 mL of Protein reagent was added to each test tube, followed by thorough mixing to ensure complete interaction between the protein and reagent.
(d) 0.5 mL of 10% Folin reagent was added to each test tube, and the contents were mixed thoroughly to facilitate the reaction for color development.
(e) The assay mixtures were incubated for 30 minutes at 37 ± 1°C to allow the chemical reaction to reach completion.
(f) A blank solution was prepared by adding 1.5 mL of Protein reagent, 0.5 mL of Folin reagent, and 1 mL of distilled water to two separate test tubes. This was used to set the spectrophotometer to zero absorbance.
(g) After incubation, the absorbance of each sample was measured at 660 nm using a UV-VIS double beam spectrophotometer (Systronics 2206).
(h) The volumes of BSA were converted to concentrations (2.5, 5, 12.5, 25, and 50 µg). A standard curve was generated by plotting the concentrations of BSA on the y-axis and the corresponding absorbance values on the x-axis.

Calculation

After the formation of standard curve, a linear regression equation, $y = mx+c$ is formed for the calculation of 1 O.D.

where,

y is taken as 1

x is unknown

m is the slope of the curve

c is the intercept

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL.

Steps

(a) 20 µL of plant solvent extracts were added to different test tubes, 3 replicates for each solvent extract.
(b) 980 µL distilled water was then added to all the test tubes at make the volume to 1 mL.
(c) 1.5 mL of Protein reagent was added to all the test tubes.

(d) 0.5 mL of 10% Folin reagent was added to all the test tubes.
(e) Incubation of the assay mixture was done for 30 min at 37 ± 1°C.
(f) After the incubation, reading was taken at 660 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(g) The total protein content was then measured with the help of standard curve of BSA.
(h) The value was represented as per mg plant extract.

Total Protein content of all the plant extract was calculated as follows:

Total protein content = Sample O.D. × µg protein of 1 O.D. (from standard curve)

B. Carbohydrate (Glucose) Assay

The carbohydrate content of plant extract (solvent fractions) was estimated following the Anthrone method (Sadasivam and Manickam, 2008).

Principle

In hot acidic medium, treatment with concentrated sulfuric acid (H_2SO_4) causes dehydration of carbohydrates, resulting in the formation of furfural. The furfural formed then reacts with anthrone, resulting in the formation of a blue-green chromophore, which is quantified colorimetrically. The resulting blue-green solution exhibits an absorption maximum at a wavelength of 620 nanometers (nm) (Guo et al., 2014).

Chemicals and reagents

Anthrone powder, D- Glucose anhydrous, conc. sulfuric acid (H_2SO_4)

Preparation of anthrone Reagent:

The preparation of anthrone reagent was done by mixing 200 mg Anthrone powder in 100 mL ice-cold 95% H_2SO_4 solution.

Procedure

(i) Preparation of Standard Curve for glucose

D-Glucose was taken as a standard for carbohydrate estimation. For stock solution 1mg/mL of glucose was prepared.

Steps

(a) Five different volumes (10, 20, 50, 100, and 200 µL) of D-glucose solution were pipetted into separate test tubes, with three replicates for each volume to ensure reproducibility.

(b) Distilled water was added to each test tube to bring the final volume to 1 mL, ensuring proper dilution of the D-glucose solution.

(c) 2 mL of Anthrone reagent was added to each test tube, and the contents were mixed thoroughly to facilitate the reaction.

(d) The assay mixtures were incubated by heating at 90 ± 1°C for 20 minutes to allow the color reaction to develop.

(e) After incubation, the test tubes were rapidly cooled by placing them in ice-cold water to stop the reaction.

(f) A blank solution was prepared by adding 2 mL of Anthrone reagent and 1 mL of distilled water to two separate test tubes. This was used to calibrate the spectrophotometer to zero absorbance.

(g) After cooling, the absorbance of each test sample was measured at 620 nm using a UV-VIS double beam spectrophotometer (Systronics 2206).

(h) For the construction of the standard curve, the volumes of D-glucose were converted to concentrations (10, 20, 50, 100, and 200 µg). The standard curve was plotted by graphing the concentrations of D-glucose on the y-axis and the corresponding absorbance values on the x-axis.

(i) The calculation of 1 O.D. was performed following the same procedure outlined in the protein assay method (3. II. A).

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL

Steps

(a) 20 µL of plant solvent extracts were added to separate test tubes, with three replicates for each solvent extract to ensure accuracy.

(b) 980 µL of distilled water was added to each test tube, bringing the total volume to 1 mL for proper dilution of the plant extracts.
(c) 2 mL of Anthrone reagent was added to each test tube and mixed thoroughly to allow the interaction between the Anthrone reagent and the plant extract.
(d) The assay mixtures were incubated by heating at 90 ± 1°C for 20 minutes to facilitate the reaction and color development.
(e) After incubation, the absorbance of each sample was measured at 620 nm using a UV-VIS double beam spectrophotometer (Systronics 2206).
(f) The total carbohydrate content was determined by comparing the absorbance of the samples to a standard curve prepared using D-glucose.
(g) The carbohydrate content in the plant extracts was expressed as µg of glucose equivalent (glucose) per mg of plant extract.

Carbohydrate content of all the plant extract was calculated as follows:
Carbohydrate content = Sample O.D. × µg glucose of 1 O.D. (from standard curve)

C. Total Phenolic Content

The total phenolic content (TPC) of crude and solvent fractions was estimated following Iloki-Assanga et al. (2013).

Principle

The principle for the determination of total phenolic content by following Folin-Ciocalteu (F-C) method is based on the formation of a blue-colored complex when the Folin-Ciocalteu reagent (FCR) is reduced by phenolic compounds. The FCR oxidizes phenolic hydroxyl groups, reducing the heteropoly acid to form a blue-colored molybdenum-tungsten complex. This reduction reaction occurs in an alkaline medium, resulting in the formation of a blue-colored chromophore that can be measured spectrophotometrically (Sánchez-Rangel et al., 2013).

Chemicals and reagents

Folin-Ciocalteu reagent (FCR), gallic aicd, sodium carbonate (Na_2CO_3)

Procedure

(i) Preparation of Standard Curve for gallic acid

Gallic acid was taken as a standard for total phenolic content. For stock solution 200 µg/mL of gallic acid was prepared.

Steps:

(a) Five volumes (10, 20, 30, 40 and 50) µL were taken in different test tubes for three replicates each.
(b) Distilled water was then added to all the test tubes at make the volume to 1 mL.
(c) 2 mL of 10% (V/V) FCR was added to all the test tubes.
(d) 0.5 mL of 10% (W/V) Na_2CO_3 was added to all the test tubes.
(e) Incubation of the assay mixture was done for 10 min at 40 ± 1°C.
(f) A blank solution was prepared adding (2 mL 10% (V/V) FCR + 0.5 mL of 10% (W/V) Na_2CO_3 + 1 mL distilled water) in two test tubes for setting the spectrophotometer into zero.
(g) After the incubation, reading was taken at 765 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(h) For ploting the standard curve, the volumes of gallic acid was converted to concentrations (2, 4, 6, 8 and 10) µg and then it was obtained by plotting the concentrations of gallic acid on y- axis and absorbance on x-axis.
(i) Calculation of 1 O.D. was done following same method as mentioned in protein assay (3.II.A).

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL.

Steps

(a) 20 µL of plant solvent extracts were added to different test tubes, 3 replicates for each solvent extract.
(b) 980 µL distilled water was then added to all the test tubes at make the volume to 1 mL.
(c) 2 mL of 10% (V/V) FCR was added to all the test tubes.
(d) 0.5 mL of 10% (W/V) Na_2CO_3 was added to all the test tubes.
(e) Incubation of the assay mixture was done for 10 min at 40 ± 1°C.
(f) After the incubation, reading was taken at 765 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).

(g) The total phenolic content was then measured with the help of standard curve of gallic acid.
(h) Results were expressed as µg gallic acid equivalent (GAE)/mg of plant extract.

Total phenolic content of all the plant extract was calculated as follows:

Total phenolic content = Sample O.D. × µg phenolic content of 1 O.D. (from standard curve)

D. Total Flavonoid Content

The estimation of total flavonoid content (TFC) was done following the procedure reported by Ordonez et al. (2006).

Principle

The principle for the determination of total flavonoid content by Aluminium chloride is that in an alkaline medium, the reaction between aluminum chloride ($AlCl_3$) and flavonoids results in the formation of a yellow-colored complex, stabilized by chelation between $AlCl_3$ and the C-4 keto and C-3 or C-5 hydroxyl groups at a maximum absorbance at 415-440 nm (Chang et al.,2002).

Chemicals and reagents

Quercetin, Ethanol, Alumunium chloride

Procedure

(i) Preparation of Standard Curve for quercetin

Quercetin was taken as a standard for total flavonoid content. For stock solution 1 mg/mL of quercetin was prepared in 80% ethanol.

Steps

(a) Five volumes (10, 20, 30, 50 and 100) µL of quercetin were taken in different test tubes for three replicates each.
(b) 80% ethanol was then added to all the test tubes at make the volume to 1 mL.
(c) 0.5 mL of 2% $AlCl_3$ (prepared in 80% ethanol) was added.

(d) 80% ethanol was added to make the solution mixture 3mL.
(e) Incubation of the assay mixture was done for 30 min at 37 ± 1°C.
(f) A blank solution 3 mL 80% ethanol was taken in two test tubes for setting the spectrophotometer into zero.
(g) After the incubation, reading was taken at 430 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(h) For ploting the standard curve, the volumes of quercetin was converted to concentrations (10, 20, 30, 50 and 100) µg and then it was obtained by plotting the concentrations of quercetin on y- axis and absorbance on x-axis.
(i) Calculation of 1 O.D. was done following same method as mentioned in protein assay (3.II.A).

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL at 80% ethanol.

Steps

(a) 20 µL of plant solvent extracts were added to different test tubes, 3 replicates for each solvent extract.
(b) 980 µL 80% ethanol was then added to all the test tubes at make the volume to 1 mL.
(c) 0.5 mL of 2% $AlCl_3$ (prepared in 80% ethanol) was added.
(d) 80% ethanol was added to make the solution mixture 3mL.
(e) Incubation of the assay mixture was done for 30 min at 37 ± 1°C.
(f) After the incubation, reading was taken at 430 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(g) The total flavonoid content was then measured with the help of standard curve of quercetin.
(h) Results were expressed as µg Quercetin equivalent (QE)/mg of plant extract.

Total flavonoid content of all the plant extract was calculated as follows:

Total flavonoid content = Sample O.D. × µg flavonoid content of 1 O.D. (from standard curve)

3.4. Elemental Analysis

To assess the heavy metal content of the plant, a sample of raw plant powder was submitted to the Sophisticated Analytical Instrument Facility (SAIF) at North-Eastern Hill University (NEHU) for analysis. The trace element analysis was performed using a Graphite Furnace Atomic Absorption Spectrometer (Model: ANALYTIKJENA AG VARIO 6). The following heavy metals were specifically selected for detection in the plant sample: Chromium (Cr), Lead (Pb), Copper (Cu), Zinc (Zn), and Cadmium (Cd). The heavy metal content was determined using the method outlined by Welz and Sperling, ensuring the accurate quantification of trace metal concentrations in the plant material.

Procedure

1 g of plant powder was digested with a mixture of concentrated nitric acid (HNO_3) and hydrochloric acid (HCl) in a 3:1 ratio at 85°C for 3 hours to release the metal contents from the plant material. Following digestion, 1 mL of concentrated perchloric acid ($HClO_4$) was added to the solution to further digest the sample. The solution was then filtered to remove any solid residues and diluted to a final volume of 50 mL with distilled water. The metal content of the plant was analyzed using an Analytik Jena AAS Vario-6 Graphite Furnace Spectrometer, equipped with a PC-controlled 6-piece lamp turret and an argon gas supply. This spectrometer was used for accurate absorption measurements of the metal ions present in the plant sample.

3.5. Antioxidant Study

I. Total Antioxidant Capacity Assay

The phosphomolybdate method was followed for the estimation of total antioxidant capacity (TAC) assay (Huda-Faujan et al., 2009).

Principle

The principle for TAC by phosphomolybdate method is that, the phosphomolybdate assay evaluates a sample's total antioxidant capacity by assessing its capacity to convert molybdenum VI to its reduced form, molybdenum V. As a result of the reduction, a green

phosphate-molybdenum (V) complex is formed. The green colour formed is then measured using a spectrophotometer (Prieto et al., 1999).

Chemicals and reagents

Sodium phosphate (Na_3PO_4), sulphuric acid (H_2SO_4), ammonium molybdate $\{(NH_4)_2MoO_4\}$, ascorbic acid.

Preperation of Reagent solution

600 mM H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate.

Procedure

(i) Preparation of Standard Curve for ascorbic acid

Ascorbic acid was taken as a standard for total flavonoid content. For stock solution 5 mg/mL of ascorbic acid was prepared.

Steps

(a) Five volumes (5, 10, 20, 50 and 100) µL of ascorbic acid were taken in different test tubes for three replicates each.
(b) Distilled water was then added to all the test tubes at make the volume to 1 mL.
(c) 1 mL of reagent solution was added.
(d) 1 mL distilled water was added to make the solution mixture 3 mL.
(e) Incubation of the assay mixture was done for 30 min at 95 ± 1°C.
(f) A blank solution was prepared adding (2 mL Reagent solution + 1 mL distilled water) in two test tubes for setting the spectrophotometer into zero.
(g) After the incubation, reading was taken at 765 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(h) For ploting the standard curve, the volumes of ascorbic acid was converted to concentrations (25, 50, 100, 250 and 500) µg and then it was obtained by plotting the concentrations of ascorbic acid on y- axis and absorbance on x-axis.
(i) Calculation of 1 O.D. was done following same method as mentioned in protein assay (3.II.A).

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL.

Steps

(a) 20 µL of plant solvent extracts were added to different test tubes, 3 replicates for each solvent extract.
(b) 980 µL distilled water was then added to all the test tubes at make the volume to 1 mL.
(c) 1 mL of Reagent solution was added.
(d) 1 mL distilled water was added to make the solution mixture 3 mL.
(e) Incubation of the assay mixture was done for 30 min at 95 ± 1°C.
(f) After the incubation, reading was taken at 765 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(g) The TAC was then measured with the help of standard curve of ascorbic acid.
(h) Results were expressed as ascorbic acid equivalent (AAE)/mg of plant extract

TAC of all the plant extract was calculated as follows:
TAC = Sample O.D. × µg TAC of 1 O.D. (from standard curve)

II. Ferric Reducing Antioxidant Power Assay

For the estimation of the Ferric Reducing Antioxidant Power **(**FRAP) assay, the method followed by Iloki-Assanga et al. (2015) was performed.

Principle

The principle for FRAP assay is that it evaluates the antioxidant capacity of a sample based on its ability to reduce ferric iron (Fe3+) to ferrous iron (Fe2+) in an acidic medium. The ferrous ions (Fe2+) react with a chromogenic reagent to produce a blue-colored compound. The absorbance of this complex at 593 nm is directly proportional to the antioxidant capacity of the sample (Haji et al., 2008).

Chemicals and reagents

Iron (III) chloride ($FeCl_3$), Ferrous sulphate ($FeSO_4$), Sodium acetate (CH_3COONa), Glacial acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), Hydrochloric acid (HCl).

Preparation of working buffer (sodium acetate buffer): 100 mL of acetate buffer was prepared, 46 mL of 200 mM CH_3COONa was taken and mixed with 4 mL 200 mM glacial acetic acid and diluted to a total of 100 mL.

Preperation of Reagent solution (FRAP reagent): 10 mL of acetate buffer (pH 3.6), 1 mL of 10 mM TPTZ in 40 mM HCl, and 1 mL of 20 mM $FeCl_3$**.**

Procedure

(i) Preparation of Standard Curve for $FeSO_4$

$FeSO_4$ was taken as a standard for total flavonoid content. For stock solution 100 µg/mL of $FeSO_4$ was prepared.

Steps

(a) Five volumes (5, 10, 20, 50 and 100) µL of $FeSO_4$ were taken in different test tubes for three replicates each.
(b) Distilled water was then added to all the test tubes at make the volume to 1 mL.
(c) 2 mL of FRAP reagent solution was added.
(d) Incubation of the assay mixture was done for 30 min at 50 ± 1°C.
(e) A blank solution was prepared adding (2 mL FRAP Reagent solution + 1 mL distilled water) in two test tubes for setting the spectrophotometer into zero.
(f) After the incubation, reading was taken at 593 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(g) For ploting the standard curve, the volumes of $FeSO_4$ was converted to concentrations (0.5, 1, 2, 5 and 10) µg and then it was obtained by plotting the concentrations of $FeSO_4$ on y- axis and absorbance on x-axis.
(h) Calculation of 1 O.D. was done following same method as mentioned in protein assay (3.II.A).

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL.

Steps

(a) 20 µL of plant solvent extracts were added to different test tubes, 3 replicates for each solvent extract.
(b) 980 µL distilled water was then added to all the test tubes at make the volume to 1 mL.
(c) 2 mL of FRAP reagent solution was added.

(d) Incubation of the assay mixture was done for 30 min at 50 ± 1°C.

(e) After the incubation, reading was taken at 593 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).

(f) The FRAP was then measured with the help of standard curve of $FeSO_4$.

(g) Results were expressed as µg Fe^{2+} equivalent (FE)/mg of plant extract.

FRAP of all the plant extract was calculated as follows:

FRAP = Sample O.D. × µg FRAP of 1 O.D. (from standard curve)

III. 1,1-Diphenyl-2-Picrylhydrazyl Radical Scavenging Activity

The 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging acvitiy, method described by Mamta et al. (2015) was performed.

Principle

DPPH features a nitrogen atom with an unpaired electron, making it a stable free radical. When any antioxidant that is reacting with DPPH, the antioxidant is donating a hydrogen atom to the nitrogen atom, reducing the radical and changing its color from violet to pale yellow. The change in colour thus can be measured spectrophotometrically (Gulcin and Alwasel, 2023).

Chemicals and reagents

Gallic acid, Methanol, DPPH

Preperation of Reagent solution (DPPH reagent): 0.135 mM of DPPH is dissolved in methanol.

Procedure

(i) Preparation of standard for gallic acid

Gallic acid was taken as a standard for DPPH assay. For stock solution 500 µg/mL of gallic acid was prepared.

Steps

(a) Five volumes (5, 10, 20, 50 and 100) µL of gallic acid were taken in different test tubes for three replicates each.
(b) Methanol was then added to all the test tubes at make the volume to 1 mL.
(c) 2 mL of DPPH reagent solution was added.
(d) Incubation of the assay mixture was done for 30 min at 37 ± 1°C.
(e) A blank solution was prepared with methanol (3 mL) in two test tubes for setting the spectrophotometer into zero.
(f) Positive control (1 mL methanol + 2 mL DPPH reagent) was taken in test tube with three replicates.
(g) After the incubation, development of color change was observed and reading was taken at 517 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(h) For ploting the standard curve, inhibition (%) was calculated and then it was obtained by plotting inhibition (%) of gallic acid on y- axis and absorbance on x-axis with the help of OriginPro 8.5 software.

The calculation of the DPPH activity was done following the formula:

$$Inhibition\ (\%) = [Abs\ O - Abs\ I / Abs\ O] \times 100 \ldots\ldots\ldots\ldots [\mathbf{1}]$$

Here, Abs O is the absorbance of the assay mixture without sample/ standard.
Abs I is the absorbance of the assay mixture with sample/ standard.

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL.

Steps

(a) Five volumes (5, 10, 20, 50 and 100) µL of plant solvent extracts were taken in different test tubes for three replicates each.
(b) Methanol was then added to all the test tubes at make the volume to 1 mL.
(c) 2 mL of DPPH reagent solution was added.
(d) Incubation of the assay mixture was done for 30 min at 37 ± 1°C.
(e) A blank solution was prepared with methanol (3 mL) in two test tubes for setting the spectrophotometer into zero.

(f) Positive control (1 mL methanol + 2 mL DPPH reagent) was taken in test tube with three replicates.

(g) After the incubation, development of color change was observed and reading was taken at 517 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).

(h) For ploting the curve, inhibition (%) was calculated and then it was obtained by plotting inhibition (%) of plant solvent extracts on y- axis and absorbance on x-axis with the help of OriginPro 8.5 software.

IV. Lipid peroxidation inhibition assay

The estimation of Lipid peroxidation inhibition activity was done following thiobarbituric acid reactive species (TBARS) assay (Okhawa et al., 1979).

Principle

The principle of lipid peroxidation assay is based on the the reaction of $FeSO_4$ with egg yolk initiates lipid peroxidation, yielding peroxyl radicals that ultimately lead to the breakdown of lipids into their corresponding aldehydes and other derivative compounds. It generates malondialdehyde (MDA) as a byproduct, which reacts with thiobarbituric acid to form a colored complex that can be quantified spectrophotometrically at 532 nm (De Leon and Borges, 2020).

Chemicals and reagents

Thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), Ferrous sulphate ($FeSO_4$), ascorbic acid, egg yolk homogenate was taken as a lipid-rich media.

Procedure

(i) Preparation of Standard for gallic acid

Gallic acid was taken as a standard for TRABS assay. For stock solution 100 µg/mL of gallic acid was prepared.

Steps

(a) Five volumes (5, 10, 15, 20 and 40) µL of gallic acid and 1 mL distilled water for positive control were taken in different test tubes for three replicates each.

(b) Distilled water was then added to all the test tubes having gallic acid to make the volume to 1 mL.
(c) 0.1 mL of egg homogenate (10% v/v) was added.
(d) 0.05 mL of 75 mM $FeSO_4$ was added to the mixture.
(e) Incubation of the assay mixture was done for 30 min at 37 ± 1°C.
(f) After incubation, 1 mL of 10% TCA and 0.8% TBA (prepared in 1.1% SDS) was added.
(g) The assay mixture was then heated at 95 ± 1°C for 1 h.
(h) For blank solution distilled water (3 mL) was taken in two test tubes for setting the spectrophotometer into zero.
(i) Development of colour change was observed and reading was taken at 532 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(j) For ploting the standard curve, inhibition (%) was calculated and then it was obtained by plotting inhibition (%) of gallic acid on y- axis and absorbance on x-axis with the help of OriginPro. 8.5 software.
(k) The inhibition percentage for the assay was calculated by following the DPPH assay Formula 1.

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL.

(a) Five volumes (5, 10, 20, 50 and 100) µL of plant solvent extracts and 1 mL distilled water for positive control were taken in different test tubes for three replicates each.
(b) Distilled water was then added to all the test tubes having to make the volume to 1 mL.
(c) 0.1 mL of egg homogenate (10% v/v) was added.
(d) 0.05 mL of 75 mM $FeSO_4$ was added to the mixture.
(e) Incubation of the assay mixture was done for 30 min at 37 ± 1°C.
(f) After incubation, 1 mL of 10% TCA and 0.8% TBA (prepared in 1.1% SDS) was added.
(g) The assay mixture was then heated at 95 ± 1°C for 1 hour.
(h) For blank solution distilled water (3 mL) was taken in two test tubes for setting the spectrophotometer into zero.

(i) Development of color change was observed and reading was taken at 532 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).

(j) For ploting the standard curve, inhibition (%) was calculated and then it was obtained by plotting inhibition (%) of plant solvent extracts on y- axis and absorbance on x-axis with the help of OriginPro 8.5 software.

(k) The inhibition percentage for the assay was calculated by following the DPPH assay Formula 1.

V. 2,2'-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonate) Assay

The estimation of the 2,2'-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonate) **(**ABTS) activity was done following the method of Re et al. (1999).

Principle

The principle of ABTS assay is based on the interaction between antioxidants and pre-formed ABTS radical cations (ABTS+), which exhibit a distinctive blue-green color and absorbance peaks at 645, 734, and 815 nm. Upon introduction of an antioxidant, the ABTS+ radical cations are neutralized, causing a visible color shift from blue to green. The degree of color change is quantitatively assessed using spectrophotometry (Kumar et al., 2018).

Chemicals and reagents

Methanol, potassium persulphate ($K_2S_2O_8$), ABTS, gallic acid

Preparation of ABTS working solution

Undiluted ABTS was prepared in which ABTS (7 mM) and potassium persulphate (2.5 mM) were added and the solution was kept for 12–16 h at room temperature in a dark room to generate the free radicals.

Working solution (ABTS): The ABTS solution was diluted using 60% methanol. The working solution was adjusted at an absorbance of 0.70 ± 0.02 at 734 nm using spectrophotometer.

Procedure

(i) Preparation of standard for gallic acid

Gallic acid was taken as a standard for total ABTS assay. For stock solution 100 µg/mL of gallic acid was prepared.

Steps

(a) Five volumes (5, 10, 20, 50 and 100) µL of gallic acid were taken in different test tubes for three replicates each.
(b) Methanol was then added to all the test tubes at make the volume to 1 mL.
(c) 2 mL working ABTS solution was added.
(d) A blank solution was prepared with methanol (3 mL) in two test tubes for setting the spectrophotometer into zero.
(e) Positive control (1 mL methanol + 2 mL working ABTS solution) was taken in test tube with three replicates.
(f) Development of color change was observed and reading was taken at 734 nm in a UV-VIS double beam spectrophotometer (Systronics 2206).
(g) For ploting the standard curve, inhibition (%) was calculated and then it was obtained by plotting inhibition (%) of gallic acid on y- axis and absorbance on x-axis with the help of OriginPro 8.5 software.
(h) The inhibition percentage for the assay was calculated by following the DPPH assay Formula 1.

(ii) For plant extracts

Stock solution for all the plant solvent (Met, Hex, DE and EA) was prepared at 5 mg/mL.

(a) Five different volumes (5, 10, 20, 50, and 100 µL) of plant solvent extracts were pipetted into separate test tubes, with three replicates prepared for each volume to ensure accuracy.
(b) Methanol was added to each test tube to bring the final volume to 1 mL, ensuring proper dilution of the plant extracts.
(c) A volume of 2 mL of the working ABTS solution was then added to each test tube, followed by thorough mixing.
(d) A blank solution was prepared by using 3 mL of methanol in two separate test tubes, which was used to set the spectrophotometer to zero absorbance.

(e) A positive control was prepared by combining 1 mL of methanol with 2 mL of the working ABTS solution in a separate test tube, with three replicates included to ensure consistency.

(f) The development of a color change was observed, and the absorbance of each sample was measured at 734 nm using a UV-VIS double beam spectrophotometer (Systronics 2206).

(g) To construct the standard curve, the percentage inhibition (%) of plant solvent extracts was calculated. The inhibition values were plotted on the y-axis, and the absorbance values were plotted on the x-axis using OriginPro 8.5 software to create the curve.

(h) The inhibition percentage for the assay samples was calculated using Formula 1 from the DPPH assay method.

3.6. Collection of the helminth parasite

Collection of the helminth parasite was conducted using 1X phosphate-buffered saline (PBS), which was prepared by diluting a 10X PBS buffer solution with distilled water in a ratio of 10% 10X PBS to 90% distilled water, and adjusting the pH to 7.4 using a pH meter. The prepared PBS buffer was transported to the collection site to ensure the viability of the parasites. Paramphistomum spp. were collected from the rumen of buffaloes in Kokrajhar and immediately placed in 1X PBS buffer (pH 7.4) as shown in Photo Plate 2 (I) to maintain their viability during transport to the laboratory. Upon arrival at the laboratory, the parasites were properly washed with PBS to remove the dirt particles and were acclimatized for about 30 mins at 37 ± 2°C for further experimental procedures. Taxonomic identification of helminth parasite was done following the taxonomic keys as described by Yamaguti (1954) and Chaoudhary et al. (2015).

In-vitro anthelmintic study

In-vitro anthelmintic study for the different plant solvent extracts was done following Eguale and Giday (2009) and Belemlilga et al. (2016). The parasites were kept at 37 ± 2°C for 30 min before it was given treatment for acclimatization purpose. 100 µL DMSO was used for the dissolving of albendazole and the plant extracts. After dissolving the solution was made to 25 mL with PBS. The parasites were washed in PBS, and after washing 10-15 adult parasites were taken and incubated at 37 ± 2°C with a test dose of 5 mg/mL of plant extracts. The reference drug for the treatment taken was albendazole (5 mg/mL) which is a broad-spectrum anthelmintic drug. The control parasites were also incubated at 37°C only

in PBS. The steps have been shown in Photo plate 4. Three replicates were taken for each set of treatments, and the record was taken for the time taken for attaining paralysis and death of the parasites (Roy and Tandon, 1999).

3.7. Histological study

Chemicals and reagents: Absolute alcohol, Bouin's fluid, xylene, hematoxylin, HCl, ethanol, eosin, Dibutylphthalate Polystyrene Xylene (DPX).

Paraffin-based normal eosin and hematoxylin method of histological study was carried out in control and plant-extract (diethyl ether) and albendazole treated parasites. Paralyzed parasites treated with 5 mg/mL of solvent fractions were collected and washed properly with distilled water. The details of the process are as follows:

Steps

(a) The parasites were fixed in Bouin's fixatives for 24 h.
(b) After fixation, parasites were washed in tape water for another 24 h to remove the fixative color.
(c) Next, the parasites were dehydrated in increasing grades of absolute alcohol (30%, 50%, 70%, 90%, and 100%) for 30 min each.
(d) Dehydrated parasites were infiltrated with a mixture of xylene and wax at 1:1, 1:2, 1:3 ratios for 15 min each, and finally kept in 100% molten wax overnight for complete infiltration.
(e) After infiltration, a paraffin block was prepared.
(f) Sectioning was carried out using a normal microtome machine and cut the section at 8-10 µm thickness.
(g) Tissue sections were stretched in warm water using a water bath.
(h) Tissue sections were collected onto albumin layer slides and kept in the horizontal position overnight for complete attachment of the tissue sections.
(i) Next, the tissue section was deparaffinized in 100% xylene for 15-20 min.
(j) After deparaffinization, tissue sections were rehydrated in a series of alcohol grades (100%, 90%, 70%, 50%, and 30%) for 10 min each.
(k) Tissue sections were dipped into water for 1 – 5 min.
(l) Tissue sections were stained with hematoxylin staining reagent for 15 min.
(m) Tissue sections were washed with acid water (0.5% HCl in 7% ethanol).

(n) After acid wash, dehydration was carried out in graded abs. alcohol in ascending order as follows:

(o) 30% > 50% > Eosin > 70% > 90% > 100% for 10 min in each alcohol grade; Eosin staining 10-15 min

(p) Tissue sections were cleaned in xylene by dipping 4-5 times.

(q) The slide was dried and carefully mounted by DPX.

(r) The tissue sections were observed in a trinocular microscope (Olympus CX43).

3.8. Biochemical study

All the helminth parasites treated with the best solvent (diethyl ether) extract, albendazole treated and control parasites were collected and processed for biochemical enzyme assays.

Tissue Preparation

A 5% tissue homogenate (w/v) was prepared in an ice-cold buffer solution using a Remi motor-driven tissue homogenizer fitted with a Teflon pestle. The homogenized tissue was centrifuged at 15000 rpm for 10 min at 4°C using a refrigerated centrifuge machine (Eppendorf-5424R). Tissue supernatant was collected and used as an enzyme source (Swargiary et al., 2021a).

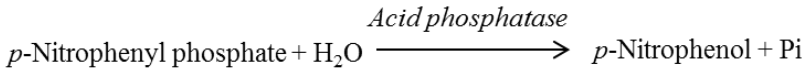
Enzyme assay

I. Acid phosphatase

Acid phosphatise (ACP) activity was measured by estimating the p-nitrophenol formation following Plummer (1988) with minor modifications following Swargiary et al. (2021).

Principle

Acid phosphatase is an enzyme that catalyzes the hydrolysis of phosphate esters at acidic pH levels, releasing inorganic phosphate. The principle of acid phosphatase assay is based on the enzyme's ability to catalyze the hydrolysis of p-nitrophenyl phosphate (pNPP) to p-nitrophenol (pNP) in an acidic environment, releasing inorganic phosphate. The amount of pNP produced is directly proportional to acid phosphatase activity (Bergmeyer, 1984).



Chemicals and Reagents

Sodium acetate buffer ((0.1 M, pH 4.5), Sodium acetate, acetic acid, *p*-nitrophenyl phosphate (*p*-NPP) (2.5 mM), Magnesium chloride ($MgCl_2$) (27 mM), Sodium hydroxide (NaOH) (0.2 N), p-nitrophenol (*p*-NP) and tissue supernatant (5% w/v).

Tissuse Preparation

A 5% (weight/volume) tissue homogenate of control, DE treated and albendazole treated parasites was taken and was prepared in sodium acetate buffer (0.1 M, pH 4.5) using a remi-motor driven homogenizer fitted with Teflon pestle. The homogenate was centrifuged at 15,000 rpm at 4 ± 1°C for 15 mins. The supernatant was taken for the enzyme study.

Procedure

(i) Preparation of Standard Curve for *p*-nitrophenol

p-nitrophenol was taken as a standard for acid phosphatase assay. For stock solution 1 mM *p*-NP was prepared in 10 mL.

Steps:

(a) Five volumes were taken (10, 20, 30, 50 and 100) µL in different test tubes for three replicates each.
(b) Distilled water was then added to all the test tubes at make the volume to 1 mL.
(c) Incubation was done for 10 min at 37 ± 1°C.
(d) 2 mL of 0.2 N NaOH was added to the assay mixture.
(e) For ploting the standard curve, the volumes of *p*-NP was converted to concentrations (10, 20, 30, 50 and 100) µM and then it was obtained by plotting the concentrations of BSA on y- axis and absorbance on x-axis.

Calculation of 1 O.D. (optical density)

After the formation of standard curve, a linear regression equation,

y = mx+c is formed, the value of x will be considered as 1 O.D.

where,

y is taken as 1

x is unkown

m = slope of the curve

c = intercept

(ii) For tissue homogenate

(a) 520 µL of 0.1 M acetate buffer (pH 4.5) was taken for control, DE and albendazole tissue supernatant separately in test tube for three replicates.

(b) 300 µL *p*-NPP and 80 µL $MgCl_2$ was then added to the solution.

(c) Warm up was done for 5 minutes at 37 ± 1°C.

(d) 200 µL of tissue supernatant was added to the mixture.

(e) Incubation was done and allowed to mix the assay at 37 ± 1ºC for 30 min.

(f) Aftter the incubation, the reaction was stopped by adding 1 mL of 0.2 N NaOH to the assay mixture in ice-water.

(g) The color change was read at 410 nm in a spectrophotometer.

(h) A blank assay mixture was prepared in two test tubes following the same steps, except NaOH was added before adding tissue supernatant.

(i) The amount of ACP activity was measured by the amount of p-nitrophenol produced from a standard linear graph of p-nitrophenol ($y = 0.0143x+0.203$ $R^2 = 0.981$).

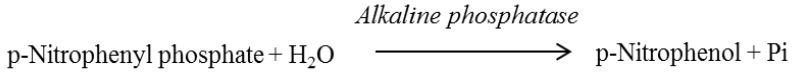
II. Alkaline phosphatase

Alkaline phosphatise (ALP) activity was measured by estimating the p-nitrophenol formation following Plummer (1988) with minor modifications following Swargiary et al. (2021).

Principle

The principle of alkaline phosphatase assay is based on the enzyme's ability to catalyze the hydrolysis of p-nitrophenyl phosphate (pNPP) to p-nitrophenol (pNP) in an alkaline environment, releasing inorganic phosphate. The amount of pNP produced is directly proportional to the alkaline phosphatase activity (Bergmeyer, 1984).

Estimating the p-nitrophenol formation



Chemicals and Reagents

Glycine buffer (0.1 M, pH 10.5), glycine, *p*-nitrophenyl phosphate (*p*-NPP) (2.5 mM), Magnesium chloride ($MgCl_2$) (27 mM), Sodium hydroxide (NaOH) (0.2 N), p-nitrophenol (*p*-NP) and tissue supernatant (5% w/v).

Tissuse preparation

A 5% (weight/volume) tissue homogenate of control, DE treated and albendazole treated parasites was taken and was prepared in glycine buffer (0.1 M, pH 10.5) using a remi-motor driven homogenizer fitted with Teflon pestle. The homogenate was centrifuged at 15,000 rpm at 4 ± 1°C for 15 mins. The supernatant was taken for the enzyme study.

Procedure

(i) Preparation of Standard Curve for *p*-nitrophenol

p-nitrophenol was taken as a standard for alkaline phosphatase assay. For stock solution, 1 mM *p*-NP was prepared in 10 mL. It was followed as mentioned in **Sub-heading 3.7.A**.

(ii) For tissue homogenate

(a) 600 µL of 0.1 M glycine buffer (pH 10.5) was taken for control, DE and albendazole tissue supernatant separately in test tube for three replicates.
(b) 300 µL *p*-NPP and 90 µL $MgCl_2$ was then added to the solution.
(c) Warm up was done for 5 minutes at 37 ± 1°C.
(d) 10 µL of tissue supernatant was added to the mixture.
(e) Incubation was done and allowed to mix the assay at 37 ± 1ºC for 30 min.
(f) Aftter the incubation, the reaction was stopped by adding 1 mL of 0.2 N NaOH to the assay mixture in ice-water.
(g) The color change was read at 410 nm in a spectrophotometer.
(h) A blank assay mixture was prepared in two test tubes following the same steps, except NaOH was added before adding tissue supernatant.

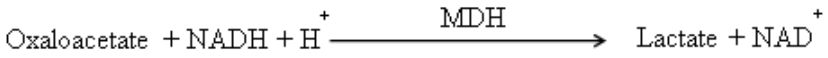
(i) The amount of ALP activity was measured by the amount of p-nitrophenol produced from a standard linear graph of p-nitrophenol ($y = 0.0143x+0.203$ $R^2 = 0.981$).

III. Malate dehydrogenase

Malate dehydrogenase (MDH) activity was measured following Bergmeyer (1974) with littile modification following Swargiary et al. (2021).

Principle

The principle of malate dehydrogenase (MDH) activity is based on the enzyme's ability to catalyzes the conversion of malate to oxaloacetate, reducing NAD+ to NADH. The increase in NADH is directly proportional to MDH activity, measured spectrophotometrically at 340 nm (Bergmeyer, 1984).



Chemicals and Reagents

Tris-HCl buffer (100 mM, pH7.4), Tris-HCl, sucrose (250 mM), Ethylenediaminetetraacetic acid (EDTA, 2 mM), Nicotinamide adenine dinucleotide (NADH, 10 mM), oxaloacetic acid (10 mM).

Tissuse preparation

A 5% (weight/volume) tissue homogenate of control, DE treated and albendazole treated parasites was taken and was prepared in homogenising buffer (consisting of Tris-HCl buffer (100 mM, pH7.4), 250 mM sucrose and EDTA 2 mM). Then the the tissue was homogenated using a remi-motor driven homogenizer fitted with Teflon pestle. The homogenate was centrifuged at 15,000 rpm for 15 mins at 4 ± 1°C. The supernatant was taken for the enzyme study.

Procedure

(a) 850 µL of Tris-HCl buffer (100 mM, pH7.4) was taken for control, DE and albendazole tissue supernatant separately in test tube for three replicates.
(b) 50 µL oxaloacetic acid was then added to the solution.

(c) 50 µL of tissue supernatant was added to the mixture.

(d) Warm up was done for 1 minute at 37 ± 1°C.

(e) 50 µL NADH was then added to the solution.

(f) A blank assay mixture was prepared in two test tubes following the same steps, except NADH was replaced by buffer.

(g) Changes in absorbance were monitored for 5 min at 340 nm with the help of spectrophotometer.

Enzyme calculation

MDH activity = (ΔE x 1000 x X)/ (6.3 x 103 x Y) µM/min/mg protien

Where,

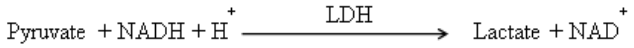
ΔE = absorbance changes per min, 1000 = factor to obtain micro moles, 6.3 x 10^3 = molar extinction co-efficient of chromophore at 340 nm, X = total volume of the assay mixture and Y = volume of enzyme (tissue supernatant) in mL

IV. Lactate dehydrogenase

Lactate dehydrogenase (LDH) activity was measured following Bergmeyer and Bent (1981) with littile modification following Swargiary et al. (2021).

Principle

The principle of lactate dehydrogenase (LDH) activity is based on the enzyme catalyzes the conversion of lactate to pyruvate, reducing NAD+ to NADH. The increase in NADH is directly proportional to LDH activity, measured spectrophotometrically at 340 nm. (Bergmeyer, 1984).



Chemicals and Reagents

Sodium phosphate buffer (100 mM, pH 7.4), sodium phosphate, sucrose (250 mM), Ethylenediaminetetraacetic acid (EDTA, 2 mM), Nicotinamide adenine dinucleotide (NADH, 10 mM), pyruvic acid (50 mM).

Tissuse preparation

A 5% (w/v) tissue homogenate was prepared for the control group, DE-treated group, and albendazole-treated parasites using a homogenizing buffer containing 100 mM sodium phosphate buffer (pH 7.4), 250 mM sucrose, and 2 mM EDTA to ensure optimal conditions for enzyme stability. The tissue samples were homogenized using a Remi motor-driven homogenizer fitted with a Teflon pestle to achieve uniform and efficient disruption of the tissue. The resulting homogenate was then centrifuged at 15,000 rpm for 15 minutes at a temperature of 4 ± 1°C to separate the cellular debris. The supernatant obtained after centrifugation was carefully collected and stored for subsequent enzyme analysis.

Procedure

(a) 910 µL of sodium phosphate buffer (100 mM, pH 7.4) was taken for control, DE and albendazole tissue supernatant separately in test tube for three replicates.
(b) 20 µL pyruvic acid was then added to the solution.
(c) 50 µL of tissue supernatant was added to the mixture.
(d) Warm up was done for 1 minute at 37 ± 1°C.
(e) 20 µL NADH was then added to the solution.
(f) A blank assay mixture was prepared in two test tubes following the same steps, except NADH was replaced by buffer.
(g) Changes in absorbance were monitored for 5 min at 340 nm with the help of spectrophotometer.

Enzyme calculation

$$LDH\ activity = (\Delta E \times 1000 \times X)/ (6.3 \times 103 \times Y)\ \mu M/min/mg\ protein$$

Where,

ΔE = absorbance changes per min, 1000 = factor to obtain micro moles, 6.3 x 103 = molar extinction co-efficient of chromophore at 340 nm, X = total volume of the assay mixture and Y = volume of enzyme (tissue supernatant) in mL

V. Acetylcholinesterase

Acetylcholinesterase (AchE) enzyme activity was estimated following Ellman et al. (1961) with littile modification following Swargiary et al. (2021).

Principle

The principle of Acetylcholinesterase activity measurement is based on the enzyme's ability to catalyze the hydrolysis of acetylcholine to choline and acetate. The AChE activity is typically measured using the Ellman method, which involves the reaction of thiocholine (produced from acetylthiocholine) with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), resulting in a yellow-colored product that is measured spectrophotometrically (Ellman et al., 1961).

Chemicals and Reagents

Sodium phosphate buffer (100 mM, pH 8.0), sodium phosphate, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, 20 mM), acetylthiocholine iodide (AchI), Triton-X (1% w/v). Chemicals were prepared in phosphate buffer.

Tissuse homogenate preparation

A 5% (w/v) tissue homogenate was prepared for the control group, DE-treated group, and albendazole-treated parasites using a homogenizing buffer composed of phosphate buffer and 1% (w/v) Triton-X to ensure proper solubilization of membrane proteins. The tissue samples were homogenized using a Remi motor-driven homogenizer fitted with a Teflon pestle for efficient tissue disruption. The homogenate was subsequently centrifuged at 15,000 rpm for 15 minutes at 4 ± 1°C to separate cellular debris. The resulting supernatant was carefully collected and used for enzymatic studies.

Procedure

(a) 430 µL of sodium phosphate buffer (100 mM, pH 8.0) was taken for control, DE and albendazole tissue supernatant separately in test tube for three replicates.
(b) 30 µL of tissue supernatant was added to the mixture.
(c) Warm up was done for 5 minutes at 37 ± 1°C.
(d) 20 µL DTNB was then added to the solution.
(e) 20 µL of AchI was again added to the assay mixture.
(f) A blank assay mixture was prepared in two test tubes following the same steps, except tissue supernatant was replaced by buffer.

(g) Changes in absorbance were monitored for 5 min at in a double beam spectrophotometer at 405 nm.

Enzyme calculation

AchE activity = (ΔE x 1000 x X)/ (1.36 x 104 x Y) μM/min/mL

Where,

ΔE = absorbance changes per min, 1000 = factor to obtain micro moles, 1.36 x 104 = molar extinction co-efficient of chromophore at 405 nm, X = total volume of the assay mixture and Y = volume of enzyme (tissue supernatant) in mL.

OBJECTIVE 2: Isolation, purification, and characterization of bioactive compound(s) from *Hypericum japonicum* and its anthelmintic study

3.9. Thin Layer Chromatography

The most active solvent fraction (Diethyl ether) of *H. japonicum* having anthelmintic activity was chosen for further study. Thin Layer Chromatography (TLC) was carried out on small slides to select the suitable solvent system as shown in Figure 3. The slurry was made using silica gel. Different solvents were tried (methanol, ethyl acteate, petroleum ether, hexane, chloroform) with various ratios for getting the best solvent system. After getting the suitable solvent system TLC was carried out using larger slides as shown in Figure 4. Based on the banding pattern, four banding fragments (sections) were made on the TLC plate and named A, B, C, and D. Different sections were collected in a different bottle and kept at -20˚ for further use.

Figure 3. Separation of phytocompounds in smaller Thin Layer Chromatography

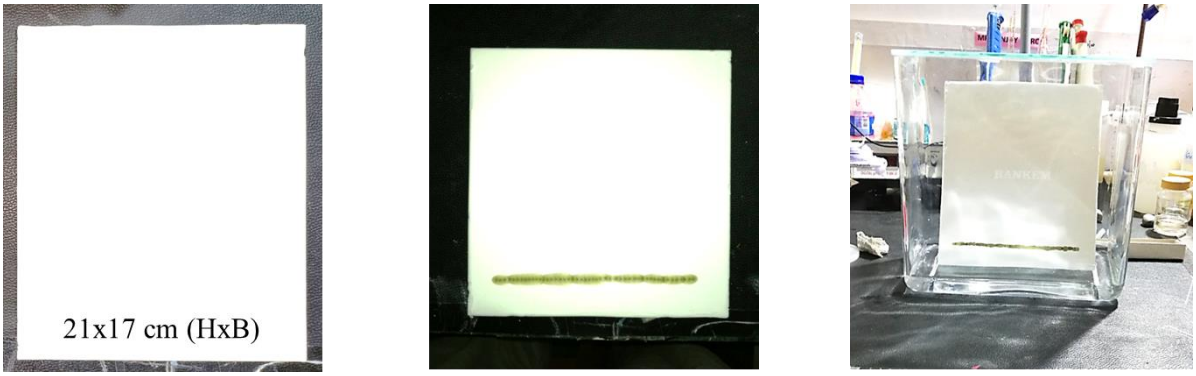


Figure 4. Separation of phytocompounds in larger Thin Layer Chromatography

I. Preparation of extract from the TLC

The collected powder was soaked into 80% methanol and kept for 72 h. The solutions was filtered using Whatman filter paper no. 1. The filtrate obtained was dried in a rotary vaccum evaporator (Optics Technology, An ISO 9100-2008 Certified). The solid material was then collected as a crude extract and kept at deep freezer (Blue Star) at -20ºC for further study.

II. In-vitro anthelmintic study using fractions obtained from TLC

The different fraction obtained from TLC was studied for in-vitro anthelmintic activity. Two doses of solvent fractions (5 mg/mL and 10 mg/mL) were prepared in 1x PBS (phosphate buffered saline, pH- 7.4) for all the fractions including crude methanolic extracts. About 10-15 adult helminth parasites were exposed to each test solution and incubated at 37°C ± 1°C. Paralysis and mortality of the parasites were checked regularly at every 10 to 15 min time intervals. The onset of paralysis was ascertained when the

parasites become immotile and motionless with a small press on their body but become active and motile when put into ice-cold water for some time. The death of the parasites was confirmed when the parasites become completely motionless even in ice-cold water and hot water (50-60°C). The time of paralysis and death was recorded. All the experiments were carried out for a minimum of three replicates. Control, untreated parasites were kept only in PBS solution till death.

3.10. Column Chromatography

I. Liquid Chromatography- Mass spectrometry Analysis

Chemical profiling of the most active fraction was analyzed in detail through target-based Liquid Chromatography- Mass spectrometry (LC-MS) analysis. LC-MS analysis was conducted in National Institute of Pharmaceutical Education and Research, Guwahati (NIPER-G). The analysis was performed with Triple quadrupole LC-MS/MS (Xevo TQ-XS, Waters). The experiment conducted was ESI/APCI-MS analysis method. The method which was followed was MS SCAN NEG.m (Negetive Mode in Mass Spectroscopy). The chromatographic analysis was carried out at a gas temperature of 300 ºC, with a gas flow of 5 l/min with a capillary of 3500 V. Draw speed of the auxiliary with 100 µL/min and at a eject speed of 400 µL/min. Injection wih needle wash was carried out with a injection volume of 10 µL (1 mg/ml) in the developed method. The solvents used were water and acetonitrile (1:1). The results were analyzed using X-Calibur software.

II. High-Performance Liquid Chromatography

High-performance liquid Chromatography (HPLC) analytical analysis for TLC section A for the detection of active compound targeted from LCMS analasysis was carried out in the Technology Incubation Centre (TIC), Bodoland University. It was carried out in Waters 1525 Waters Binary HPLC pump with 2998 PDA detector, shown in the Figure 5 (a). The software used for data analysis was Empower software. The column used for the process was Waters spherisob 5.0 µm ODS2. The dimension of the column used was 4.6 mm × 250 mm. The sample was prepared at 1 mg/mL at methanol and was filtered using Whatman filter paper No 1 before injecting to the HPLC. The chromatographic column used for the process was C18 column. For the mobile phase two solvents were taken, they were

methanol and water (70:30, v/v). 20 µL was then injected to the HPLC and the flow rate was 1 mL/min. The wavelength was detected at 280 nm at 28 °C.

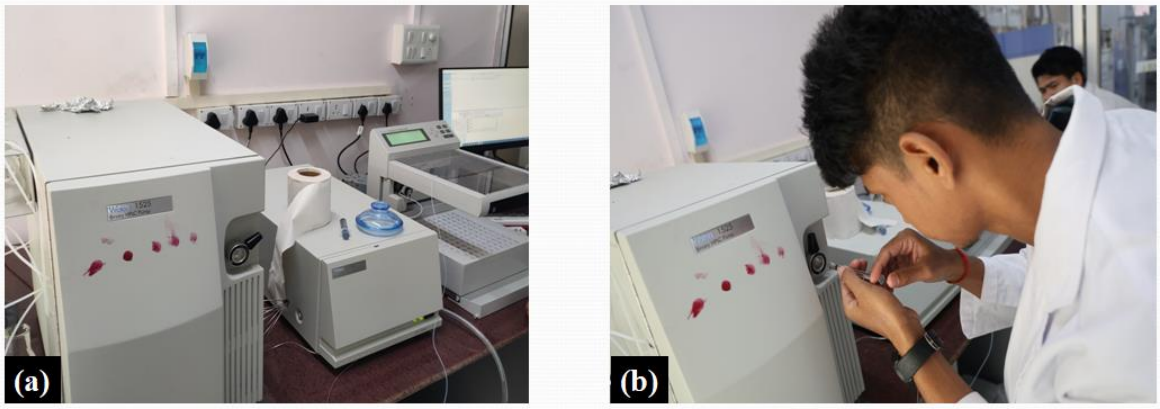


Figure 5. HPLC analysis of TLC (A) extract of *Hypericum japonicum*. (a) The whole set up of HPLC machine, and (b) Injecting of sample

3.11. In-vitro anthelmintic study with active compound

The active compound obtained from the chromatography study was studied for in-vitro anthelmintic activity. Two doses of solvent fractions (5 mg/mL and 10 mg/mL) were prepared in 1x PBS (phosphate buffered saline, pH- 7.4) for all the fractions including crude methanolic extracts. About 10-15 adult helminth parasites were exposed to each test solution and incubated at 37°C ± 1°C. Paralysis and mortality of the parasites were checked regularly at every 10 to 15 min time intervals. The onset of paralysis was ascertained when the parasites become immotile and motionless with a small press on their body but become active and motile when put into ice-cold water for some time. The death of the parasites was confirmed when the parasites become completely motionless even in ice-cold water and hot water (50-60°C). The time of paralysis and death was recorded. All the experiments were carried out for a minimum of three replicates. Control, untreated parasites were kept only in PBS (phosphate buffered saline, pH- 7.4) solution till death.

OBJECTIVE 3: Study of histological, ultrastructural, and biochemical alterations in helminth parasites treated with bioactive compound(s)

3.12. Histological study

Paraffin-based normal eosin and hematoxylin method of histological study was carried out in control parasite and both active compound (Quercetin) and albedazole treated parasites. Paralyzed parasites treated with 5mg/mL and were collected and washed properly with distilled water. For the histology study, the process was followed as as described in **Sub-heading (3.6).**

3.13. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was carried out in the Technology Incubation Centre (TIC), Bodoland University by following (Lanthanpuii and Lalchchhandana, 2020) with little modifications.

Steps

(a) The sample (control, quercetin treated and albendazole treated) parasites were soaked at 4% glutaralaldehyde for fours hours.
(b) Dehydration of the samples were done with ethanol at 30%, 50%, 70%, 80%, 90% and 100% for 20 minutes each.
(c) Then the sample was kept overnight.
(d) Next day, lyophilizer was used for the drying of the samples.
(e) Gold coating of the sample was done using ion-sputtering chamber (Quorum), the chamber is shown in the Figure 6 (b).
(f) The images of the sample after gold coating is shown in Figure 6 (c).
(g) Viewing was done using SEM (EVO- 10 ZEISS model), the image of the model is shown in Figure 6(a).

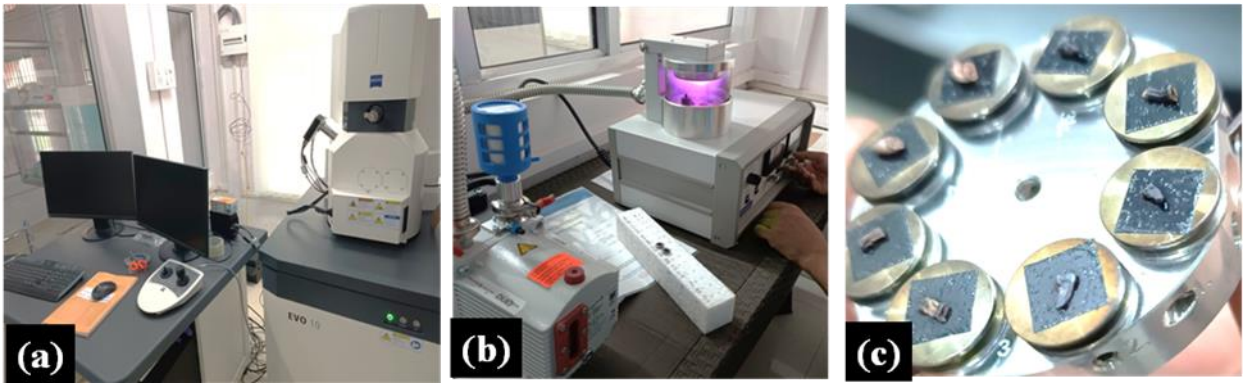


Figure 6. Sample processing and viewing of SEM. (a) The whole set up of SEM, (b) gold coating of the parasite, and (c) Sample after gold coating

3.14. Biochemical study

All the helminth parasites treated with the active compound (Quercetin), albendazole treated and control parasites were collected and processed for biochemical enzyme assays.

Tissue preparation

A 5% tissue homogenate (w/v) was prepared in an ice-cold buffer solution using a Remi motor-driven tissue homogenizer fitted with a Teflon pestle. The homogenized tissue was centrifuged at 15000 rpm for 10 min at 4°C using a refrigerated centrifuge machine (Eppendorf-5424R). Tissue supernatant was collected and used as an enzyme source (Swargiary et al., 2021).

Enzyme assay

I. Acid phosphatase

For Acid phosptase (ACP) assay, the same steps were followed as mentioned earlier in the materials and methods section as described in **Sub-heading 3.8 (I).** Only DE extracted treated parasite tissue homogenate will be replaced by quercetin treated parasite tissue homogenate.

II. Alkaline phosphatase (ALP)

For ALP same steps were followed as mentioned earlier in the materials and methods as described in **Sub-heading 3.7 (II).** Only DE extracted treated parasite tissue homogenate will be replaced by quercetin treated parasite tissue homogenate.

III. Malate dehydrogenase (MDH)

For MDH same steps were followed as mentioned earlier in the materials and methods as described in **Sub-heading 3.8 (III).** Only DE extracted treated parasite tissue homogenate will be replaced by quercetin treated parasite tissue homogenate.

IV. Lactate dehydrogenase (LDH)

For MDH same steps were followed as mentioned earlier in the materials and methods as described in **Sub-heading 3.8 (IV).** Only DE extracted treated parasite tissue homogenate will be replaced by quercetin treated parasite tissue homogenate.

V. Acetylcholinesterase (AchE)

For AchE same steps were followed as mentioned earlier in the materials and methods as described in **Sub-heading 3.8 (V).** Only DE extracted treated parasite tissue homogenate will be replaced by quercetin treated parasite tissue homogenate.

OBJECTIVE 4: Evaluation of molecular interaction and docking of bioactive compound(s) with enzymes using bioinformatics tools

3.15. Protein modeling and active site prediction

Amino acid sequences for the proteins ACP (GenBank: THD26168.1), ALP (GenBank: THD21487.1), MDH (GenBank: TPP64788.1), LDH (GenBank: TPP62930.1), and AchE (GenBank: KAA0195891.1) were retrieved from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). These sequences were obtained in FASTA format, which is widely used for bioinformatics analyses. The FASTA sequences were downloaded and submitted to the Swiss-Model server (https://swissmodel.expasy.org/), a homology modeling platform, to generate the 3D structural models of the proteins. The server analyzed the sequences, selected appropriate templates based on similarity, and generated high-quality 3D models. The templates for the best models were identified and downloaded for further validation. Additionally, protein images were downloaded from the Swiss-Model server for visualization purposes.

For molecular docking studies, SDF (Structure Data File) files of the proteins were downloaded to ensure compatibility with docking tools. The 3D structures of the best protein models were further analyzed to identify their active sites, which are crucial for docking simulations. This was achieved by submitting the 3D models to the CASTp 3.0 server (Tian et al., 2018), a tool designed to identify and characterize protein pockets and active sites. The active site information provided by CASTp allowed precise molecular docking simulations, enabling detailed interaction studies between the proteins and their ligands. These analyses provided a structural and functional basis for understanding protein behavior and ligand binding.

3.16. Docking study

The PubChem database was used to download the 3D structures of the active compounds and the reference drug (https://pubchem.ncbi.nlm.nih.gov/). Additionally, the Protein Data Bank (PDB) database (https://www.rcsb.org/) was utilized to obtain the 3D structures of the proteins. Both the proteins and compounds were then converted into the .pdbqt file format, which is compatible with molecular docking simulations, using OpenBabel software (O'Boyle et al., 2011). AutoDock Vina (Trott and Olson, 2010) was employed to perform molecular docking studies.

For each protein, the grid parameters were carefully set to define the docking area. The grid box coordinates and sizes for each protein were specified as follows: ACP (x: 50.440, y: 65.413, z: 24.461, center: 54, 106, 78), ALP (x: 35.626, y: 33.323, z: -9.164, center: 44, 44, 50), LDH (x: 17.925, y: -14.018, z: 27.237, center: 62, 48, 54), MDH (x: 35.028, y: 144.289, z: 42.579, center: 52, 44, 46), and AchE (x: 25.511, y: 10.566, z: 16.228, center: 74, 74, 62). The docking exhaustiveness parameter was set to 8 to ensure thorough exploration of possible binding sites. The docking results were visualized using Discovery Studio software, which facilitated the analysis of protein-ligand interactions. Docking experiments were performed in triplicate for all proteins and phytocompounds to ensure reproducibility and reliability of the results. This comprehensive approach provided insights into the potential binding affinity and interactions of the compounds with the selected target proteins.

3.17. Analysis of drug-likeness and ADMET profile

The bioactive compound (Quercetin) and the reference drug (Albendazole) was verified using SwissADME (Daina et al., 2017) and Pubchem database. Firstly, the compounds were searched in the Pubchem database and the list of SMILES was copied and pasted in the SwissADME database. The drug likeness property from SwissADME was evaluated based on the Lipinki's rule (Lipinski, 2004). The drug-likeness properties such as molecular weight, H-bond donor, H-bond acceptor, and lipophilicity (LogP value) were used to screen the drug-likeness property. Again, in-silico ADMET properties were also predicted using ADMETlab 2.0 (Dong et al., 2018). For ADMET profile the SMILES of both the compounds were first copied from the pubchem database. Then the SMILES were pasted at the ADMETlab database and the prediction of the compounds were done. The various pharmacokinetic properties such as absorption, distribution, metabolism, excretion, and toxicity (ADMET) of phytocompounds were predicted.

3.18. Molecular Dynamics Simulation Study

For molecular dynamic (MD) simulations study GROMACS was used for All-atom MD simulations of ligand-protein complexes (Abraham et al., 2015). CHARMM27-all atoms with TIP3P water model were used for the forcefield (Lindahl and van der Spoel, 2020). To carry out the solvation, sodium and chloride ions was used. UCSF Chimera was used for the spilt of ligand-protein complex into ligand and protein and converted into mol2 file (Pettersen et al. 2004). For the generation of other ligand parameters, the ligand mol2 file was submitted into the SwissParam server (Zoete et al., 2011). By using DockPrep instruction in Chimera, the protein structures were further optimized by adding Gasteiger charges (Pettersen et al., 2004). The systems were equilibrated by 100 ps position restraint simulations of 1,000 $kJ\,mol^{-1}\,nm^{-2}$ in the NVT and NPT ensembles before the final run. The images of the system has been shown in Figure 7. MD simulation was carried out for 100 nanoseconds (ns). Post-MD analyses were performed to see the root mean square deviation (RMSD), root mean square fluctuations (RMSF), the radius of gyration (Rg), hydrogen bond (HB), and the total energy fluctuations in the protein during the simulation trajectory for all the complexes.

3.19. Molecular Mechanics/Poisson Boltzmann Surface Area (MMPBSA) analysis

The free energies of all protein-albendazole complexes were analysed by the MM/PBSA package (Kumari et al., 2014; Genheden and Ryde, 2015). 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 understand 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:

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

Where, Gcomplex represents the total free energy of the docking complex, and Gprotein and Gligand 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 (Gallicchio and Levy, 2004) as follows:

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

Where, $\gamma = 0.0301$ kJ/mol/A2

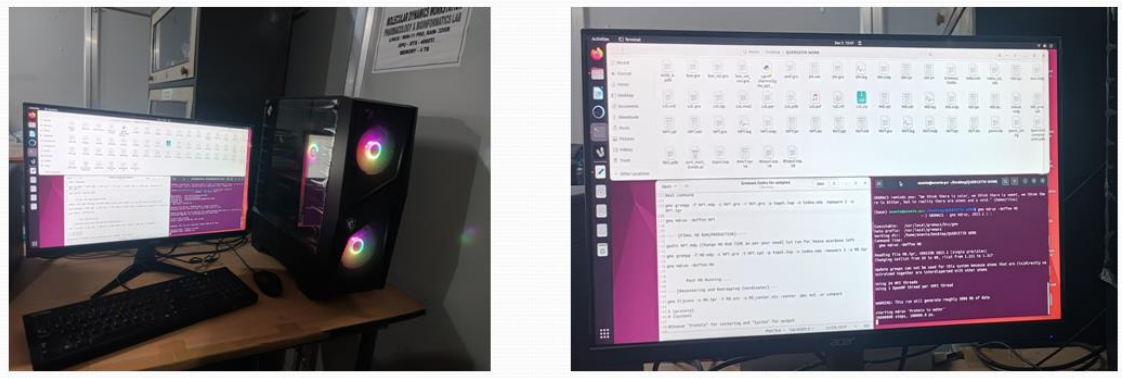


Figure 7. The set up for Molecular Dynamics simulation study

3.20. Statistical analysis

All statistical calculations were performed using Microsoft Excel. For the analysis of significance and correlation studies, OriginPro 8.5 software was utilized. Each experiment was conducted in triplicate (n = 3), and the results were expressed as the mean ± standard deviation (SD) to ensure accuracy and reliability in the interpretation of the data.