3. MATERIALS AND METHODS

3.1. Description of the study site

Kokrajhar is one of the 33rd districts of the state of Assam. It is the district headquarters of the Bodoland Territorial Region of Assam. The district covers an area of 3296 km^2 with the geographical location ranging from 89°46'East to 90°38'East and 26°19'North to 26°54'North. Geographically, it is located in the extreme North of the river Brahmaputra and is a gateway of entire northeast India. There has been a change of district boundaries since 1983, with major change in 2003 due to the creation of Bodoland Territorial Autonomous Council. At present, the district is surrounded by both International and interstate border, Bhutan in the north, Dhubri district in the south, Chirang on the east and West Bengal, with Sankosh river at the boundary on the west. According to a recent population census (2011), the district has a population of 887,142. The district has Hindus (59.64%), Muslims (28.44%), Christians 11.40%, Sikhs (0.01%), Buddhists (0.19%), Jains (0.04%), and other religions (0.28%). The sex ratio of the district is 1000:959 and the average literacy rate is 55.16% (male 60.77% and female 49.3%). The district has two national parks, namely the Raimona National Park and part of the Manas National Park, a Wildlife Sanctuary (Chakrasila Wildlife Sanctuary) and a Forest reserve (Ultapani Forest Reserve). Along with that, three lakes, namely Dheer beel, Diplai beel and Sareswar beel are situated in the vicinity of Kokrajhar district, which provide habitats for different life forms. Several tribal communities are inhabitants of the Kokrajhar district. Of the total population, different people speak different languages namely Bodo (28.39%), Assamese (23.78%), Bengali (17.78%), Santali (11.9%), Rajbongshi (7.62%), Rabha (2.58%), Hindi (1.76%), Nepali (1.65%), and Kurukh and Garo (1.42%) (Census, 2011).

The district is divided into 11 Community Development Blocks (CDBs) and has 1068 villages, of which 15 are uninhabited villages. Kokrajhar district had a forest village of 160 of which Kachugaon stands the highest with 106 numbers (Nath and Mwchahary, 2012). CDBs are the administrative blocks in rural areas for planning and development and the smooth supervisory of the local administrative unit. It provides funding for housing, economic development, and other community development

activities. The names of the CDBs are: i) Kachugaon, ii) Gossaigaon, iii) Hatidhura, iv) Dotma, v) Kokrajhar, vi) Golakganj, vii) Rupsi, viii) Debitola, ix) Mahamaya, x) Bilasipara, and xi) Chappar-Salkocha.

3.2. Antidiabetic ethnomedicinal plants

In the present study, survey work was carried out in the Kokrajhar district to collect ethnomedicinal plants popularly practiced by tribal communities of the district. The survey was done from April to October 2018. The survey was carried out in a block-wise manner. Within every CDB, approximately 20 adjacent villages were taken as a single cluster, and one informant was interviewed and data regarding antidiabetic medicinal plants were collected. The informants include professional ethnomedicinal healers (kaviraja) and older people with ethnomedicinal knowledge. The information was collected via a face-to-face interview with the help of a ready-made questionnaire (Figure 3.1). The information collected from informants included the informer's bio-data, name of the plant, parts used, traditional formulation processes, and mode of administration. The medicinal plants mentioned by the traditional healers were photographed for future uses.

I) Identification of the medicinal plants

The taxonomic identification of plant was carried out following standard taxonomic procedures. The plants were photographed and sample plants were collected from different locations of the district. Herbarium sheets were prepared following the method of Jain and Rao (1977). The plant parts were rinsed thoroughly and washed with distilled water. After the plant is dried, it was dipped into 2% $HgCl_2$ solution for poisoning and preserving. The dried plant part is then pasted on the herbarium sheet and submitted to the Department of Botany, Bodoland University. The identification numbers were collected, and the voucher specimen was preserved.

II) Data analysis

Quantitative analysis to assess the importance of medicinal plants was carried out following Hussain et al. (2018).

Figure 3.1. Template of ready-made questionnaire used during the survey of ethnomedicinal data

A) Frequency of citation (FC): The number of informants who mentioned a certain species.

B) Relative Frequency of Citation (RFC): It is obtained by dividing FC by total number of informants (N). RFC indicates the citing percentage of each species of medicinal plants. RFC was calculated by using the following formula as described by Tardio and Pardo-de Santayana (2008).

$$RFC = FC/N$$

The value of RFC varies from zero (when nobody cites to a plant as important), to one (when all the informants consider a certain species important).

C) Family Importance Value (FIV): FIV indicates the local importance of the families

of plant species and is calculated by counting the percentage of informants mentioning a specific family (Vitali et al., 2013).

FIV = [FC (family)/N] x100

3.3. Phytochemical analysis

I. Collection of plant parts for phytochemical analysis

Alstonia scholaris (Linn.) R. Brown belonging to the family Apocynaceae is a tall tree. The bark of the tree is collected from the Haltugaon area of Kokrajhar district (26° 47′ 29″ N, 90° 34′ 26″). *Rauvolfia tetraphylla* Linn. is a tall slender shrub, belonging to the family Apocynaceae. The roots of the plant were collected from the Tarunguri area Kokrajhar (26° 48′ 26″ N, 90° 34′ 26″ E). *Hydrocotyle sibthorpioides* Lam. is a creeping perennial herb, belonging to the family Apiaceae. The whole plant (including stems and roots) was collected from the University Campus (26° 47′ 26″ N, 90° 29′ 43″ E). *Oroxylum indicum* (Linn.) Bentham ex. Kurz is a tall tree belonging to the family Bignoniaceae. The leaves of the plant are collected from University Campus (26° 47′ 26″ N, 90° 29′ 43″ E). *Phloganthus thyrsiformis* (Roxb. ex. Hardw) Mabb. is a shrub belonging to the family Acanthaceae. The flower of the plant was collected from Debargaon area of Kokrajhar district (26° 47′ 26″ N, 90° 31′ 52″). *Musa balbisiana* Colla is the largest mono-cotyledenous herb belonging to the family Musaceae. The aerial part or the corm of the plant is collected from Thulungapuri area of Kokrajhar, Assam (26° 47′ 26″ N, 90° 29′ 42″ E). *Clerodendrum infortunatum* Linn. is a perennial shrub from the family Lamiaceae. The leaves of the plant are collected from university campus (26° 47′ 26″ N, 90° 29′ 43″ E). *Lindernia crustacea* (Linn.) F. Muell. is an herb that belongs to the family Lindernaceae. The whole plant is collected from the Tarunguri area of Kokrajhar (26° 48′ 26″ N, 90° 34′ 26″ E). *Andrographis paniculata* (Burm.f.) Nees. is small herb, belonging to the family Acanthaceae. The leaves were collected from the Gyanpuri of Kokrajhar (26° 47′ 26″ N, 90° 29′ 43″ E). *Paspalum fimbriatum* Kunth is a small herb and belong to the family Poaceae. The whole plant is collected from the university campus (26° 47′ 26″ N, 90° 29′ 43″ E). *Ficus racemosa* Linn. belongs to the family Moraceae is a tall tree. The fruit of the plant is collected from the Haltugaon area of Kokrajhar (26° 47′ 29″ N, 90° 34′ 26″ E).

PHOTO PLATE: I

Survey, Documentation and Identification of Medicinal plants with the help of traditional healers

II. Preparation of plant extracts

The selected plant samples were collected, washed thoroughly with distilled water, and dried properly in an oven at 50ºC. Dried plant parts were grinded into powdered form and soaked in 80% methanol. The solution was filtered after 24 h of soaking, and the fresh solvent was added. The process was repeated four times, and the filtrate obtained was evaporated to dryness using a rotary evaporator. Dry and semi-solid extracts (crude extract) was obtained and stored at 4ºC for further use (Figure 3.2).

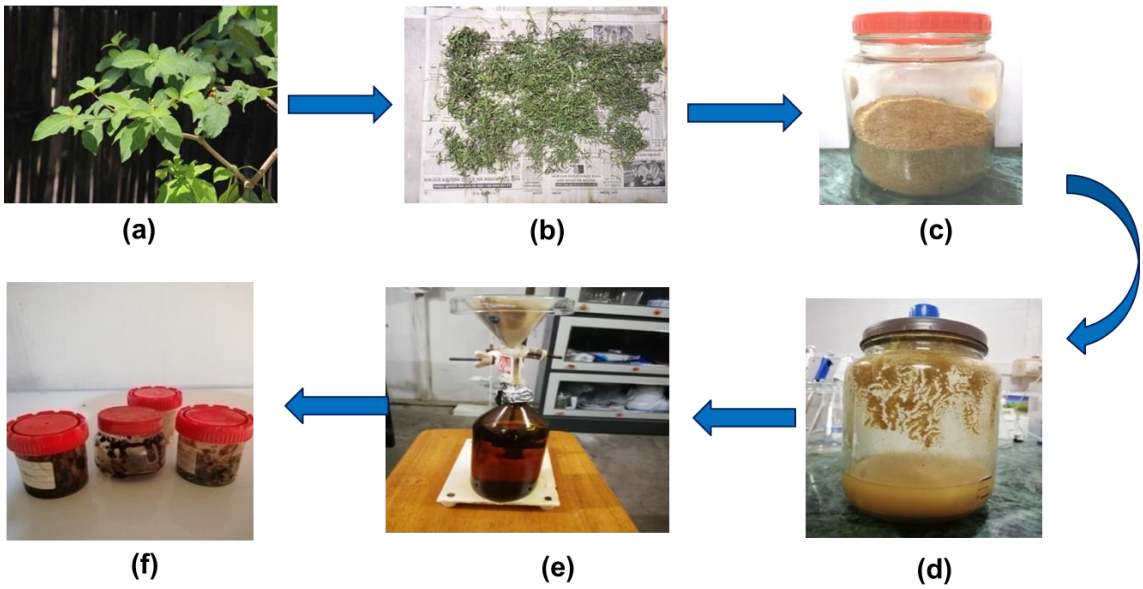

Figure 3.2. Preparation of crude extracts of plants. (a) Plant sample, (b) Air dried plant sample, (c) Powdered form, (d) Powder soaked in methanol, (e) solvent filtration, and (f) Concentrated crude extract obtained

III. Qualitative Phytochemical study

Phytochemical screening of all the plants for the presence of phenol, flavonoids, reducing sugar, saponins, tannins, etc., was carried out following the methods of Trease and Evans (2002) and Sofowara (1993).

Chemicals and reagents

dH_2O, Ninhydrin solution, 37% HCl, NaOH, Fehling's solution, $FeCl_3$, Ethanol, Magnesium chips, Conc. HCl, $FeCl_3$, Mayer's reagent, Potassium iodide, Mercuric chloride, Glacial acetic, H_2SO_4, Plant extracts

A) Test for Protein

5 mg of crude plant extract was dissolved into 1 mL of distilled water. The solution was used as stock solution for the study. In a clean test tube, 1 mL of plant extracts (5 mg/mL) was taken and to it, 2% of ninhydrin solution was added. The mixtures were warmed (60°C) for about 10 min. Appearance of violet color confirmed the presence of protein in the plant extract.

B) Test for Carbohydrate (Reducing Sugars)

500 mg of crude plant extract was taken in clean test tubes. Now, 5 mL of HCl (37%) was added. The solution was boiled for 5 min. The resulting solution was neutralized with 1 mL of 2M NaOH solution. Next, four drops of Fehling's solution were added and then heated on a water bath for 2 min. The appearance of a reddish-brown precipitate indicated the presence of reducing sugars.

C) Test for Phenolic Content

In a clean test tube, 2 mL of distilled water was taken. To it, 3 drops of 10% $FeCl_3$ solution was added. Now, 1 mL (5 mg/mL) of plant extract was added to it. The formation of blue or green color indicated the presence of phenolics.

D) Test for Flavonoid Content

500 mg of each portion of crude extract was dissolved in 5 mL ethanol. The solution was warmed at 40°C and then filtered. Three pieces of magnesium chips were then added to the filtrate, followed by 3 drops of conc. HCl. A pink, orange or red to purple coloration indicated the presence of flavonoids.

E. Test for Saponins

500 mg of plant extract was shaken with 10 mL distilled water in a test tube. The test tube was warmed at 80°C in a water bath for 5 min. The formation of frothing, which persists on warming indicated the presence of saponins.

F) Test for Tannins

About 500 mg each portion was stirred with about 10 mL of distilled water and then filtered. 5 drops of 1% $FeCl_3$ solution were added to 2 mL of the filtrate. Occurrence of a blue-black, green, or blue-green precipitate indicated the presence of tannins.

G) Test for Alkaloids (Mayer's test)

15 mg of each plant extract was separately stirred with 1% HCl (6 mL) on a water bath (40°C) for 5 min and filtered. These filtrates were divided into three equal parts and use for alkaloid test. 1 mL of Mayer's reagent was added to one portion of filtrate. The formation of a cream-colored precipitate indicated the presence of alkaloids. Mayer's Reagent was prepared by mixing 1.36 g of mercuric chloride in 30 mL of distilled water. In another beaker, 5 g of potassium iodide was taken and mixed in 60 mL of distilled water. Both the solution was mixed and the final volume was 100 mL using distilled water.

H) Tests for glycosides

5 mL (0.5 mg/mL) of each of the plant extract was mixed with 2 mL of glacial acetic acid. To the mixture, 1 mL of conc. H_2SO_4 was added. The presence of a brown ring indicated the presence of glycosides.

IV. Quantitative Phytochemical study

A) Protein Assay

Principle: *Under alkaline condition, in the presence of sodium potassium tartarate, nitrogen of the protein reacts with copper sulphate and amino acids tryptophane and tyrosine from the protein reduces the Phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent collectively to form a blue colour. The blue colour so*

developed is read against 660 nm in UV-Vis- double beam Spectrophotometer (Alam, 1992).

Chemicals and reagents

Protein reagent, Folin reagent, BSA, NaOH, Sodium Carbonate, Sodium-Potassium tartrate, Copper sulphate

Preparation of protein reagent: Protein reagent was prepared by mixing 100 mL of A, 1 mL of B, and 1 mL of C.

A. 4% Sodium Carbonate in 0.1N NaOH
B. 2% Sodium-Potassium tartrate
C. 2% Copper sulphate

Procedure

The protein content of all the plant extracts was estimated following the Folin-phenol method (Lowry et al., 1951). Briefly, 1 mL of plant extract (100 µg) was mixed with 1.5 mL protein reagent and 0.5 mL of 10% Folin reagent (v/v). The assay mixture was incubated at 37±1°C for 20 min. The color developed was read at 660 nm in a UV-VIS double beam spectrophotometer against a blank solution. BSA was used as standard. Blank was prepared using all the chemicals while dH_2O replaces the sample or the standard.

Preparation of Bovine Serum Albumin standard curve: (BSA working solution: 250 µg/mL)

Five different concentrations of BSA (2.5, 5, 10, 20, and 50 µg/mL) were taken in five different test tubes. Next, 1.5 mL of Protein reagent was added to all the test tubes. Next, 0.5 mL of 10% Folin reagent was added to all the test tubes. The assay mixture was incubated at 37±1°C for 20 min. A blank solution was prepared by mixing all the reagents, except BSA. After the incubation, the color developed was read at 660 nm in a UV-VIS double beam spectrophotometer (Systronics 2206). Standard curve was obtained by plotting the concentrations of BSA on x- axis and absorbance on y-axis.

Calculation of 1 O.D. (optical density)

Linear regression equation, y = mx+c

where,

y = slope of the curve

x = concentration of the protein or extract

c = intercept

Preparation of sample

Plants extract working solution: 5 mg/mL

In a cleaned test tube 20 µL (i.e., 100 µg extract) of plant working solution was taken and all the chemicals were added as in the standard preparation and the absorbance was measured for the plant extract in triplicates. The total protein content was measured from the standard curve of BSA. The value was represented as per µg plant extract. Total Protein content of all the plant extract was calculated as follows:

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

B. Carbohydrate Assay

Principle**:** *In hot acidic medium starch or di or oligosaccharides is hydrolyzed into simple sugar and dehydrates into furfural or furfural derivatives. The resultant compounds react with Anthrone reagent to form a green color. The color so developed is read at 630 nm (Saravanan and Ravindranath, 1981).*

Chemicals and reagent

Anthrone powder, Glucose, H_2SO_4

Preparation of anthrone Reagent: 200 mg Anthrone powder is dissolved in 100 mL ice-cold 95% H_2SO_4 solution.

Plant extracts working solution: 100 µg/mL

Procedure

The total carbohydrate content in plant extract was estimated following the method as described by Sadasivam and Manickam (2008). Briefly, 1 mL of plant extract was mixed with 2 mL anthrone reagent. Next, the assay mixture was heated in a boiling water bath for 30 min. Then, the mixture was cooled rapidly in ice-cold water. The color developed was measured at 630 nm in a UV-VIS double beam spectrophotometer. Values expressed as µg sugar/mg plant extract. Carbohydrate content of the plant is calculated following the formula of protein content.

Standard curve for carbohydrate: (Working solution of glucose: 100 mg/mL)

Five different concentrations of glucose solution (10 µL, 20 µL, 50 µL, 100 µL, and 200 µL) were taken in test tubes. Required volumes of dH_2O were added in each test tube to make total volume 1 mL. Next, 2 mL of anthrone reagent was added to the test tube and heated in boiling water for 30 min. The assay mixture was cooled rapidly in ice-cold water. The color developed was read in UV-Vis double beam spectrophotometer. Standard graph was drawn by plotting the concentration in the X-axis and absorbance (O.D.) in the Y-axis. Concentration of glucose required to give 1 OD was calculated from linear regression equation, y = mx+c

C. Total Phenolic Content (TPC)

Principle: *In alkaline medium produced by sodium carbonate, Phenols from the plant reacts with Phosphomolydic acid of the Folin-Ciocalteau reagent to form blue colored complex and the color developed in measured at 765 nm (Sadasivam and Manickam, 1996).*

Chemicals and reagents

Folin ciocalteau reagent, sodium carbonate, gallic acid

Plant extracts working solution: 0.1 mg/mL

Procedure

The TPC of plant extract was estimated following Iloki-Assanga et al. (2013). Briefly, 1 mL of plant extract (0.1 mg/mL) was mixed with 3 mL of 10% Folin-Ciocalteau reagent and 0.5 mL of sodium carbonate (10% w/v). The mixture was vortexed for 15 sec and incubated at 40ºC for 30 min. The color was read at 765 nm using UV-VIS double beam spectrophotometer. The amount of TPC was calculated from the calibration curve of gallic acid, and the result was expressed as µg Gallic acid equivalent (GAE) per mg plant extract.

Standard Curve for TPC

Gallic acid stock: Gallic acid was used as standard chemical and the working solution was prepared by dissolving 0.5 mg in 1 mL of dH_2O.

A series of five different concentrations (5 µg, 10 µg, 15 µg, 20 µg, and 25 µg) were used for the preparation of the standard curve. Next, 3 mL of 10% Folin-Ciocalteau reagent and 0.5 mL of sodium carbonate (10% w/v) was added. The mixture was vortexed for 15 sec and incubated at 40ºC for 30 min. The color was read at 765 nm using a spectrophotometer. A standard curve was drawn by following the process described in the protein assay.

D. Total Flavonoid Content (TFC)

Principle: *$AlCl_3$ reacts with the C-4 ketone group and C3- or C-5 hydroxyl group of the flavonoid to stable complex. This reaction formed by the quercetin, rutin or Kaempferol or any other flavones has a maximum absorbance at 415-440 nm (Lindawati and Solikhah, 2018; Tristantini and Amalia, 2019).*

Chemicals and reagents

Ethanol, $AlCl_3$, Quercetin

Plant extract working solution: 0.1 mg/mL (Prepared in 80% ethanol) was prepared.

Procedure

The TFC content was determined following the $AlCl_3$ method as described by Ordonez et al. (2006). The total flavonoid content was measured from the standard curve of quercetin. The value was represented as µg Quercetin equivalent (QE) per mg plant extract.

Preparation of Standard Curve

The working solution of quercetin was prepared by mixing 1 mg of quercetin dissolved in 1 mL of 80% ethanol. Various concentrations of quercetin (10 - 100 µg) was taken. T it, 0.5 mL of 2% $AlCl_3$ (prepared in 80% ethanol) was added. The solution was incubated at 37±1°C for 30 min. The formation of yellow color was measured at 430 nm. A standard curve was obtained by plotting the different concentrations on the X-axis and absorbance of quercetin on the Y-axis.

V. Antioxidant study

A) Total Antioxidant Capacity (TAC) Assay

Principle: The antioxidant molecule reduces the Phosphomolybdate ion or hexavalent molybdate [MO(VI)] to pentavalent form of Phosphomolybdate [MO(V)] resulting in the production of green color complex. The green color so formed is measured at 765 nm (Jan et al., 2013; Olugbami et al., 2015).

Chemicals and reagents

H_2SO_4, sodium phosphate, ammonium molybdate, ascorbic acid

Procedure

The TAC of the plant extracts was assayed by the phosphomolybdate method using ammonium molybdate reagent (Huda-Faujan et al., 2009). Briefly, 1 mL of plant extract (100 µg/mL) was mixed with 1 mL of reagent solution (600 mM H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The mixture was incubated at 95ºC for 30 min, and absorbance was measured at 765 nm against a blank solution. TAC was expressed as µg AAE per mg plant extract.

Standard Curve for Ascorbic Acid

Standard Ascorbic acid was prepared by dissolving 5 mg of ascorbic acid in 1 mL of dH_2O. A series of five increasing concentrations (5 µg, 10 µg, 20 µg, 50 µg, and 100 µg) was taken in a test tube and mixed with 1 mL of the reagent. The mixture is allowed

to incubate for 30 minutes at 95ºC. Blank solution was prepared by adding 1 mL of reagent and 1 mL of dH_2O. A standard curve was obtained by plotting the absorbance on the X-axis and a series of ascorbic acid concentrations (5 - 100 µg) on the Y-axis.

B) Ferric Reducing Antioxidant Power Assay (FRAP)

Principle: The Principle for FRAP assay is based on the reduction of Fe^{3+}*-tripyridyltriazine (TPTZ) complex (colorless complex) to* Fe^{2+} *TPTZ (blue colored complex) formed by the action of electron-donating antioxidants at low pH. Changes in color were measured at wavelength of 593 nm using UV-VIS spectrophotometer (Benzie and Devaki, 2018).*

Chemicals and reagents

Sodium acetate, Glacial acetic acid, TPTZ, $FeCl_3$, $FeSO_4$, TPTZ, HCl

Preparation of FRAP reagent: The FRAP reagent was prepared by mixing 100 mM acetate buffer (pH 3.6), 10 mL TPTZ prepared in 40 mM HCl, and 20 mM $FeCl_3$ in the proportion 10:1:1 at 37°C.

Preparation of acetate buffer: 46 mL of 200 mM Sodium acetate is mixed with 4 mL of 200 mM acetic acid and diluted to a total of 100 mL.

Procedure

FRAP assay was done following the method of Benzie and Strain (1999). Freshly 2 mL of FRAP reagent was pipetted out and mixed with 10 µL of plant extract and 990 µL of distilled water mixed thoroughly. A blue color complex was formed when the Fe^{3+} TPTZ complex was reduced to ferrous (Fe^{2+}) form, and the absorbance at 593 nm was recorded against a reagent blank (2 mL of FRAP reagent and 1 mL of distilled water) after 30 min of incubation at 50°C. All the determinants were performed in triplicates. The FRAP value was obtained by comparing the absorbance change in the test mixture with those obtained from the increasing concentrations of Fe^{3+} and expressed as µg Fe^{2+} equivalent (µg FE)/mg plant extract. Ascorbic acid (5 mg/mL) was used as reference standard.

Standard Curve for FRAP Assay

0.1 mg $FeSO_4$ was mixed in 1 mL of dH_2O to make a stock solution of $FeSO_4$ (0.1 mg/mL). From the stock solution, 5 µL, 10 µL, 20 µL, 40 µL, 80 µL (1 - 8 µg) was taken and followed the procedure as described above. The absorbance of varying concentrations was read against a blank solution at 593 nm. A blank solution was prepared adding all the chemicals except standard/sample. The calibration curve was prepared by plotting the absorbance at 593 nm in the Y- axis, versus different concentrations of $FeSO_4$ (1 - 8 µg) on the X-axis.

C) 2,2- Diphenyl -1- picryl-hydrazyl-hydrate (DPPH) assay

Principle: When a DPPH molecule is mixed with antioxidant molecule, or the substance capable of donating H ion, it reduces into hydrazine complex to DPPH-H. The formation of reduced DPPH-H changes the color from purple to pale yellow. The more the H donor in the chemical or extract the paler the violet color. The reduction of color is measured at 593 nm spectrophotometrically (Gulcin and Alwasel, 2023).

Chemicals and reagents

Methanol, Ascorbic acid, Plant extract, DPPH powder, gallic acid

Plant extracts working solution: 5 mg/mL in methanol

Preparation of DPPH reagent: 0.135 mM of DPPH powder is prepared in methanol

Procedure

DPPH radical scavenging activity of plant extracts was estimated following Mamta et al. (2015). Briefly, 2 mL of DPPH was added to 1 mL of ascorbic acid and plant extracts (concentration range, 25 – 500 µg/mL). After 30 min of incubation at 37±1°C, a decrease in absorbance was read at 517 nm. The scavenging activity of plant extract was calculated using the formula:

$$\text{DPPH scavenging activity} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(1)}$$

Where, Abs control equals to absorbance of DPPH and methanol

Abs sample equals to absorbance of DPPH and ascorbic acid or plant extract.

Standard curve preparation (Gallic acid working solution: 0.5 mg/mL)

In a clean dry test tube, all the chemicals except gallic acid were added. A control absorbance was taken. A series of five gallic acid concentrations (5, 10, 25, 50, and 100 µg) was taken and mixed with all the chemicals. The decreased in absorbance was subtracted from the control absorbance giving the percentage inhibition. A standard curve was obtained by plotting the concentration of ascorbic acid vs percentage inhibition.

D) Lipid Peroxidation Inhibition Assay (Thiobarbituric acid reactive species TBARS) assay

Principle: *$FeSO_4$ reacts induces egg yolk to form lipid peroxide or peroxyl radicals and subsequently decomposing lipid into its aldehyde and derivatives. Malondialdehyde (MDA), a secondary biproduct of lipid peroxidation in heated acidic condition, reacts with two molecules of TBA to form TBA-MDA abduct giving a red pinkish color. Antioxidant molecule reduces the formation of MDA and decreases in color which is best absorbed at 532 nm (Akinpelu et al., 2014; Upadhyay et al., 2014).*

Chemicals and reagents

TBA, TCA, SDS, $FeSO_4$, ascorbic acid

Plant extracts working solution: 5 mg/mL

Procedure

Lipid peroxidation inhibitory activity of plant extract was studied by modified thiobarbituric acid reactive species (TBARS) assay using egg yolk homogenates as lipid-rich media (Ohkawa et al., 1979). Lipid peroxidation was induced in 0.1 mL of egg homogenate (10% v/v) by adding 1 mL plant extract/standard (concentration, 0.05-1.0 mg/mL) and 0.05 mL of 75 mM $FeSO_4$. The mixture was incubated at 37±1°C for 30 min. Then 1 mL each of 10% TCA and 0.8% TBA (prepared in 1.1% SDS) were added, and the solution was vortexed and heated for one hour at 95°C. After cooling,

the mixture was centrifuged at 3000 rpm for 10 min. The color formation was measured at 532 nm using a spectrophotometer. Ascorbic acid was used as a standard. Lipid peroxidation scavenging activity was calculated following the same calculation of the DPPH assay.

Standard curve preparation

5 mg ascorbic acid was dissolved in 1 mL of dH_2O. In a clean dry test tube, all the chemicals except ascorbic acid were added. A control absorbance was taken. A series of ascorbic acid concentrations (25 µg, 50 µg, 75 µg, 100 µg, and 200 µg) was taken and mixed with all the chemicals. The decreased in absorbance was subtracted from the control absorbance giving the percentage inhibition. A standard curve was obtained by plotting the concentration of ascorbic acid vs percentage inhibition.

E) 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) Assay

Principle: A free radical $ABTS^{\bullet+}$ generated from the reaction of ABTS and potassium persulphate reacts with organic compounds present in the plants to reduced form, resulting from the dark green color to colourless.

Chemicals and reagents

ABTS, potassium persulphate, gallic acid, methanol

Preparation of ABTS working solution: 200 mL of ABTS (7 mM) and potassium persulphate (2.5 mM) were mixed and allowed to react for 12 – 16 h in the dark at room temperature to generate the free radicals. The ABTS solution was diluted with 60% methanol, and the absorbance of the working solution was adjusted to 0.700 at 734 nm using a spectrophotometer.

Procedure

The ABTS activity was measured following Re et al. (1999) using gallic acid as a reference chemical. Five different concentrations of plant extract (50, 100, 250, 500 and 1000 µg/mL) were prepared in distilled water. Next, 2 mL of the working ABTS

solution was added, and the absorbance was read at 734 nm. The ABTS radical scavenging activity was calculated following the calculation of the DPPH assay.

Standard curve preparation

Gallic acid was used as a reference chemical (working solution, 100 µg/mL)
In a clean dry test tube, all the chemicals except gallic acid were added. A control absorbance was taken. A series of gallic acid concentrations (1 µg, 2 µg, 5 µg, 10 µg, and 20 µg) were taken and mixed with all the chemicals. The decrease in absorbance was subtracted from the control absorbance giving the percentage inhibition. A standard curve was obtained by plotting the concentration of gallic acid vs. percentage inhibition.

VI. Elemental analysis

Seven elements such as Pb, Cr, Ni, Cd, Cu, Zn, and Mn were analyzed using AAS (Shimadzu AA-7000) following the method of Zheljazkov and Nielson (1996). Briefly, 1 g dry powder of each plant sample was digested with conc. HNO_3, at 90°C for 45 min. The temperature is then increased up to 100°C and boiled for 6 - 7 h by adding 5 mL HNO_3 till complete digestion. The process was continued until the extract was colorless. The solutions were filtered by Whatman filter No.1 and diluted to 100 mL of distilled water. The values were expressed in ppm. Heavy metal analysis was carried out at the Institute of Advanced Science and Technology, Boragaon, Guwahati, Assam.

VII. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The phytochemical components of the methanolic plant extracts were analyzed by the GC-MS system (TQ-8030 Shimadzu Corporation Kyoto, Japan) following the method as described by Kalita et al. (2016). Extraction is the main process by which bioactive compounds may be obtained. Polar solvents like methanol has the capacity to dissolve maximum amount of desired active constituents specifically hydrophilic compounds (Sasidharan et al., 2011). A highest GC was run on an EB-5MS capillary column (30m x 0.25 mm i.d.; 0.25 µm) at 57.4 kPa pressure with an initial temperature of 50°C and maintained at the same temperature for 2.5 min. Next, the oven temperature was raised

to 300°C, at the rate of 15°C/min, and maintained for 8 min. Injection port temperature was ensured at 300°C and Helium flow rate was 1 mL/min. The ionization voltage was 70eV. The plant sample was injected in split mode as 20:1. The Mass spectral scan range was set at 0 - 700 (m/z). Compound identification was carried out by comparing the spectra with the databases (NIST-11) using a probability-based algorithm.

3.4. Enzyme Inhibition Assays

I) α-Amylase inhibition Assay

Amylase is the enzyme secreted by salivary amylase and the pancreas, which hydrolyses the breakdown of carbohydrates. Pancreatic amylase is the major enzyme for the digestion of oligosaccharides in the small intestine after the initial breakdown of carbohydrates in the buccal cavity by the action of salivary amylase.

Principle: Starch is converted into maltose by the α-amylase enzyme. Maltose released from starch is measured by the reduction of 3, 5-dinitro salicylic acid provided the pH and temperature remains constant (pH 6.9 at 37±1°C).

$$\text{Starch} + H_2O \xrightarrow{\alpha-\text{Amylase}} \text{Maltose (reducing agent)}$$

Chemicals and reagents

α -amylase ex.porcine pancreas (code no. 28588), Acarbose (code no. 65457), Sodium potassium tartarate, NaOH, Dinitrosalicylic acid, Starch

Plant extracts working solution: 5 mg/mL

Preparation of DNS reagent: DNS reagent was prepared by making a solution of 1.5 M Sodium potassium tartarate in 2.8 M NaOH and to it, 1 g of DNS was added and stirred continuously to obtain orange-yellow coloured solution.

Procedure

The inhibition of α-amylase enzyme activity of all the plant extracts was done following Kwon et al. (2008) with slight modification. Acarbose was used as a reference standard. Starch was used as substrate. Different concentrations of plant extracts and reference

inhibitors acarbose (5 mg/mL) were mixed with 200 µL of α-amylase enzyme (2U enzyme). The assay mixture was incubated at 25°C for 10 min. Next, 0.5 mL 1% starch solution was added and re-incubated for another 20 min at 37ºC. After the incubation, 0.5 mL DNS reagent was added to stop the reaction, and the assay mixture was boiled for 5 min. The reaction mixture was then diluted after adding 5 mL distilled water, and the absorbance (Abs) was measured at 540 nm in UV-VIS double-beam spectrophotometer. The control samples were prepared without any plant extracts/compounds. The inhibition (%) of α-amylase activity was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{Abs } control - \text{Abs } sample)}{\text{Abs } control} x\ 100 \ldots\ldots\ldots\ldots (2)$$

Where, Abs control means absorbance of assay mixture without extract and acarbose
Abs sample means absorbance of assay mixture with extract or acarbose

II) α-Glucosidase Inhibition Assay

Principle: α-Glucosidase catalyses the conversion of p-nitrophenyl-α-D-glucopyranoside into α-D-glucopyranoside and p-nitrophenol under specified conditions (pH 6.9 at 37°C). The color developed was measured at 405 nm using spectrophotometer.

pnG + α-glucosidase → α-D-glucopyranoside + p-nitrophenol

Chemicals and reagents

α-glucosidase (Maltase) (code no. 75551), Acarbose, glucopyranoside, DMSO, Na_2CO_3, p-nitrophenyl-α-D-glucopyranoside (12735)

Plant extracts working solution: 5 mg/mL

Procedure

α-Glucosidase inhibition assay was carried out following the method of Elya et al. (2012). The plant extract was dissolved in 5% DMSO and α-glucosidase in 100 mM sodium phosphate buffer, pH 6.9. Different concentrations of the plant extracts and

acarbose (5 mg/mL) were mixed with 50 µL α-glucosidase (25 Units) and incubated for 10 min at 37°C. In the mixture, 100 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside was incubated for another 20 min at 37°C. The reaction was stopped by adding 2 mL of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the absorbance of p-nitrophenol released from pNPG at 405 nm using a UV-VIS double beam spectrophotometer. Inhibition (%) of α-glucosidase activity was calculated using Eq. (2).

3.5. Preparation of different solvent extracts

The most potent plant extract showing most potent α-amylase and α-glucosidase inhibitory plant was selected for further study. The crude extract of *Ficus racemosa* showed the most potent inhibitory activity against the two antidiabetic enzymes. The crude extract of *F. racemosa* was further processed for solvent fractionation with three other solvents selected based on the polarity index of the solvents – **hexane**, **diethyl ether** and **ethyl acetate**. The solvents were selected based on the increasing polarity of the solvents and following other standard protocols. Solvent fractionation was carried out following the method of Sarker et al. (2006). Briefly, in a 1 L capacity beaker, five grams of *F. racemosa* crude plant extracts were mixed with 250 mL of distilled water and shaken and mixed properly using a magnetic stirrer properly and allowed to stand overnight to dissolve completely. Next, the solution was poured into a separating funnel. Another 250 mL of hexane was poured into the solution and shaken properly. The solution was allowed to stand overnight to form two clear and distinct layers – the upper hexane and lower aqueous layers. After the formation of transparent layers, hexane layer was collected and kept in a glass bottle. Another 250 mL of fresh hexane was poured into the aqueous layer and shaken properly. The collection of hexane layers and the addition of fresh hexane to the aqueous layer were repeated several times until the hexane layer became colorless. The exact process was repeated with both diethyl ether and ethyl acetate as well. After the collection, all the solvents- hexane, diethyl ether and ethyl acetate solutions were evaporated to dryness using a rotary evaporator. The dried material obtained was recognized as solvent fractions and kept at -20°C till further use.

3.6. Phytochemical and antioxidant study

The different solvent extracts of *F. racemosa* were carried out for quantitative analysis of crude protein, carbohydrates, and total phenolic and flavonoid contents. Antioxidant activity was investigated following phosphomolybdate assay, FRAP, DPPH, ABTS and TBARS assays. All the protocols were followed as described in **Subheading 3.3.** (IV and V)

3.7. Enzyme inhibition assays

I) Inhibition of α-amylase activity

The inhibition of α-amylase enzyme activity of all the plant extracts was done following Kwon et al. (2008) with slight modification. All the protocols were followed as described in the subheading **3.4. (I)**

II) Inhibition of α-glucosidase activity

α-Glucosidase inhibition assay was carried out following the method of Elya et al. (2012). All the protocols were followed as described in the Sub-heading **3.4. (II).**

3.8. Toxicity study

Healthy Wistar male rats weighing 200 - 230 g of 8 - 12 weeks old procured from Chakraborty Enterprise Certified Laboratory Animal Supplier, Kolkata, West Bengal (Regd. No.1443/PO/b/11/CPSEA) were used for the study. The rats were acclimatized for 15 days in alternating 12 h light/dark cycles provided with a standard diet and water *ad-libitum*. Proper ethical clearance for the use of animals was taken from the institute (Ref No. IAEC/ZOOL/2019/1) (Annexure III). All the experiments were done according to the CPSEA guidelines and approved by the expert of the Institutional Ethical committee, Bodoland University.

PHOTO PLATE: II

Scientific validation of the plant fraction in in-vivo model

I) Acute Oral Toxicity (AOT)

AOT refers to the adverse effects occurring within 24 h of oral administration of single or multiple doses of a drug. AOT study was done following Organisation for Economic Co-operation and Development guidelines for testing chemicals 420 (OECD, 2001).

Experimental Procedure

A total of 24 rats were used for the study. Rats were divided into 4 groups (n = 6) and orally fed with 3 different doses of the best fraction *Ficus racemosa* diethyl ether fraction (FRDF) as follows. Before drug treatment, rats were fasted overnight.

Group I : Group treated with 500 mg/kg body weight (bw)
Group II : Group treated with 1000 mg/kg bw
Group III : Group treated with 2000 mg/kg bw
Group IV : Control, received only vehicle distilled water

Animals were frequently observed during the first 30 min and then at 1, 4, 8, 12, and 16 h over the first 24 h. The body weights were recorded and changes in their skin, fur, eyes, mucous membranes, nervous systems, movement, and behavior was noted.

II) Sub-acute toxicity

A chronic oral toxicity study was done to determine the toxicity of the extract by observing changes that become apparent when the extract is administered to the animal continuously over a long period. In the present study sub-acute toxicity was carried out by treating the animal groups with the plant extracts for 28 days.

Experimental Procedure

24 Wistar albino rats (8-12 weeks old, 200 - 230 g bw) were divided into four groups (n = 6).

Group I : Animal group treated with 100 mg/kg bw
Group II : Animal group treated with 200 mg/kg bw
Group III : Animal group treated with 500 mg/kg bw
Group IV : Control group with vehicle distilled water

Treatment was done orally with the help of oral gavage. Animals were observed individually for 28 days and the body weight of rats was measured on 0, 7, 14, 21, and

28th day post-treatment. After 28 days, the rats were subjected to gross necroscopy. The blood was collected in EDTA and non-EDTA-coated vials and used for biochemical and hematological studies.

A) Biochemical analysis

For lipid profiling such as total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and triglycerides were analyzed. For liver function, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin were studied. For kidney function, parameters such as creatinine and albumin were analyzed. All the biochemical test was done using Diatek kit on AVH analyser/05 at the ICMR Branch, Guwahati Medical College and Hospital, Guwahati.

B) Haematological Analysis

Parameters, such as total red blood cells, total white blood cells, hemoglobin, platelet count, lymphocyte, hematocrit, mean corpuscular haemoglobin and mean corpuscular hemoglobin concentration were done using Sysmex XP 300 hematology analyzer.

C) Assessment of Cardiovascular Risk Index

Cardiovascular risk indices such as Coronary artery index, Cardiac index, and atherogenic index were done following Kang et al. (2004) and Adebayo-Gege et al. (2022) cardiovascular risk indexes are calculated as follows:

Castelli Risk Index-I or Cardiac index (CRI) = Total Cholesterol/HDL Cholesterol

Castelli Risk Index Coronary artery index (CR-II) = LDL Cholesterol /HDL Cholesterol

Atherogenic index of plasma (AIP) = log (triglyceride/ HDL)

Atherogenic coefficient (AC) = (TC-HDL)/HDL

3.9. Oral Glucose Tolerance Test (OGTT)

OGTT test was carried out in two ways: i) normal rat group and ii) diabetic rat group. Both groups were treated with 3-doses of FRDF.

3.9.1. Assessment of the hypoglycemic effect of plant extract in normal rats

OGTT test was done following Prakasam et al. (2003). Briefly, rats were divided into five groups with three numbers (n = 3) in each group. Rats were fasted overnight and Fasting Blood Sugar (FBS) was estimated from the tail vein using a glucometer. The groups were subjected to different doses of FRDF extracts.

Group-I : Served as control and received dH_2O

Group-II : Received 100 mg/kg bw

Group-III : Received 200 mg/kg bw

Group-IV : Received 500 mg/kg bw

Group-V : Received standard chemical glibenclamide (600 µg/kg bw)

After 30 min of the dose, blood glucose was taken from the tail vein. After the blood was collected, all the groups were injected with 2 g/kg bw glucose intraperitoneally. Blood was collected from the tail vein at 0, 30, 60, 90, and 120^{th} min, and glucose was estimated.

3.9.2. Assessment of the hypoglycemic effect of plant extract in diabetic rats

Induction of diabetes

TIIDM was induced following the method of Rai et al. (2010). The rats fed with a high-fat diet for 15 days (except normal control rats) were injected with a single dose of STZ (50 mg/kg bw, prepared in citrate buffer, pH 4.5). Five days after the STZ injection, the rats were fasted overnight. The blood glucose level was estimated; rats having glucose levels ≥ 250 - 350 mg/dL were screened as TIIDM and used for further studies. The OGTT test in diabetic rats was done following Prakasam et al. (2003).

Experimental procedure

In the experiment, a total of 30 rats (24 diabetic, 6 normal rats) will be taken. The rats were divided into five groups of six rats each (n = 6) as given below. Rats were divided into four groups of three rats each.

Group I : Served as diabetic control administered with vehicle (dH_2O)
Group II : Diabetic rats administered orally with 100 mg/kg bw
Group III : Diabetic rats administered orally with 200 mg/kg bw
Group IV : Diabetic rats administered orally with 500 mg/kg bw
Group V : Diabetic rats administered orally with glibenclamide 600 µg/kg bw

Rats were fasted overnight and fasting blood glucose was taken from the tail vein of the rat. After 30 min of dosing, all the rats were injected with 2 g/kg bw glucose intraperitoneally. Blood glucose was taken from the tail vein, just before and at the 0, 30, 60, 90, and 120^{th} min.

3.10. Study of antidiabetic effects of the best fraction on rat model

Wister male healthy rats weighing between 200 - 230 gm, procured from Chakraborty Enterprise, Kolkata (CPCSEA certified, registration no. 1443/PO/Br/s/11/CPCSEA) were utilised for the study. The rats were acclimatized for 15 days in an alternating 12 h light/dark cycles providing with standard diet and water *ad-libitum*. Ethical clearance from the institute for the use of animals was taken. All the experiments were performed using the guidelines from the expert of Institutional ethical committee, Bodoland University and the details of the work plan was approved by forest department, Manas Choudhury, expert from Tezpur University and CPSEA guidelines. In the experiment, a total of 30 rats (24 diabetic rats, 6 normal rats) were taken. The rats were divided into five groups of six rats each (n = 6) as given below

Group-I : Normal control rats administered with vehicle dH_2O daily for 28 days
Group-II : Diabetic control rats administered with vehicle dH_2O daily for 28 days
Group-III : Diabetic rats administered orally with 100 mg/kg bw daily for 28 days
Group-IV : Diabetic rats administered orally with 200 mg/kg bw daily for 28 days
Group-V : Diabetic rats administered orally with glibenclamide 600 µg/kg bw daily for 28 days

For the induction of diabetes, STZ (55 mg/kg bw) in ice-cold citrate buffer, pH 4.5 was used. After three days, rats having blood glucose levels in between 400 - 500 mg dL^{-1}) were screened as diabetic rats and used for further study. The crude extract is dissolved in the vehicle containing 100 µL DMSO and dH_2O. Treatment was given between 8 a.m. to 10 a.m. orally using an intragastric feeding tube once daily for 28 days continuously. The blood glucose was examined weekly (i.e., after 7 days) with blood glucose meter on call plus G133-115. Food and Water consumption was observed daily. The changes in body weight were also observed weekly.

I) Histology

After 28 days of daily treatment with crude extract and glibenclamide (33 days after STZ treatment), all the rats were anesthetized and sacrificed by cervical dislocation, and tissue samples - liver and kidney were collected. For histological study, tissue samples - liver and kidney were collected in Bouin's fluid and kept in the fixative for 72 h. After 72 h, the tissue samples were washed overnight in tap water and after usual dehydration, clearing and infiltration processes, the organs were embedded in paraffin wax and sectioned into thin slices (4 µm) through a microtome machine. The tissues were stained with haematoxylin and eosin.

Preparation of Blouin's fluid: To 75 mL of saturated picric acid, 25 mL of 40% formaldehyde is added, to give a total volume of 100 mL. To the mixture, 5 mL of glacial acetic acid is added.

II) Biochemical analysis

The blood glucose and body weight of rats were measured on 0, 7, 14, 21, and 28 days after treatment. At the end of the 28^{th} day, blood samples were collected under light ether anaesthesia. Blood was collected in three vials: EDTA coated, non-EDTA coated and Fluoride vials. The blood samples were then used for the analysis of total cholesterol, high-density lipoprotein, low-density lipoprotein, very low-density lipoprotein, triglycerides.

A) Liver function tests

Liver function tests include serum AST, ALT, ALP, and bilirubin in the liver. Briefly, the blood of the rats was collected in non-EDTA coated tubes and centrifuged at 3000 rpm for the separation of serum. The serum was then used for analyzing AST, ALT, and ALP using Diatek kit on AVH analyzer/05 at ICMR branch, Guwahati Medical College and Hospital.

B) Antioxidant marker enzymes

After 28^{th} days of treatment, all the rats were anesthetized and sacrificed. Organs such as liver, kidney and pancreas were collected, washed, and homogenized in Tris– HCl buffer (pH 7.4) and stored at -20°C for the analysis of antioxidant marker enzymes. Antioxidant marker enzymes such as Catalase, Lipid peroxidation, and Glutathione-S-transferase were analyzed.

i) Catalase

Principle: H_2O_2 in the presence of catalase is converted into water (H_2O) and oxygen molecule (O_2). Catalase activity is measured based on the ability of the tissue (enzyme source) to break down H_2O_2. The decrease in absorbance was read at 240 nm. Catalase activity was calculated following the method of Abei et al. (1984) with slight modification.

Chemicals and reagents

Hydrogen peroxide (H_2O_2), Potassium phosphate buffer, Ethanol, Triton-X

Tissue homogenate: Tissue homogenate was prepared following the method of Cohen et al. (1970) with slight modification. Briefly, 10% of tissue homogenate was prepared in potassium phosphate buffer (pH 7.0, 0.1 M). To it, ethanol was added to a final concentration of 0.17 M. After 30 min, Triton-X was added to a final concentration of 1%. The tissue homogenate was centrifuged at 15000 rpm in a refrigerated centrifuge

machine for 15 min. The tissue supernatant was transferred carefully and used as enzyme source.

Assay mixture

In an assay mixture containing 1 mL, 990 µL of H_2O_2 (13.6 mM) and 10 µL of tissue supernatant were added. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm for 3 min at an interval of 30 sec. Phosphate buffer was used as a blank. Catalase activity was expressed as µmol/min/mg protein.

Catalase activity = Decrease in absorbance/min/43.6µM/min/mg tissue protein

ii) Lipid Peroxidation Inhibition Assay (Malondialdehyde level assay)

Principle: One molecule of MDA reacts with two molecules of thiobarbituric acid to form an MDA-TBA adducts. The colour so formed absorbs strongly at 532 nm. The reaction is measured by observing the conjugation of MDA with thiobarbituric acid (TBA). To rule out a false reaction or interfering compounds, that absorbs in 532 nm, leading to overestimation of MDA values, the absorbance is also measured at 600 nm.

Chemicals and reagents

Thiobarbituric acid, Potassium phosphate buffer, dH_2O, tissue supernatant

Tissue homogenate: A 10% of tissue homogenate was prepared in potassium phosphate buffer (pH 7.0, 0.1 M). The tissue homogenate was centrifuged at 15000 rpm in a refrigerated centrifuge for 15 min. The tissue supernatant was collected and used as an enzyme source.

Assay mixture

MDA level was estimated following the method of Hodges et al. (1999) and Esterbeur and Cheesemen (1990) with slight modification. Briefly, 800 µL of 1% TBA was mixed with 200 µL of tissue supernatant. The mixture was boiled at 100°C for 30 min. Blank was prepared by adding 200 µL of dH_2O instead of tissue supernatant. The assay

mixture is then centrifuged at 5000 rpm. The supernatant is collected and is read at 532 nm and 600 nm spectrophotometrically. The activity of MDA is determined by

$$MDA\ equivalent\ (mmol^{-1}ml^{-1}) = [(A_{532} - A_{600})/155]$$

Where, A_{532} is the maximum absorbance of the TBA-MDA abduct at 532 nm. A_{600} is the correction of nonspecific turbidity. 155 mM^{-1} is the molar extinction co-efficient for MDA.

iii) Glutathione S- transferase (GST)

GST activity was analyzed following the method of Habig et al. (1974).

Principle: GST is a major class of enzymes that is involved in the detoxification of wide range of chemicals. The reaction is measured by observing the conjugation of CDNB with GSH. The increase in absorbance was observed at 340 nm.

Gluthathione-SH + CDNB □ Gluthathione-S-CDNB

Chemicals and reagents

1-chloro, 2-4-dinitrobenzene (CDNB), reduced glutathione (GSH)

Tissue homogenate: A 10% of tissue homogenate was prepared in sodium phosphate buffer (Ph 7.4, 0.1 M). The tissue homogenate was centrifuged at 15000 rpm in a refrigerated centrifuge for 15 min. The tissue supernatant was collected and used as enzyme source.

Assay mixture

In a total volume of 1 mL, 960 µL of phosphate buffer, 20 µL of GSH reagent (0.1 M, prepared in buffer), 10 µL of CDNB (0.1M, dissolved in ethanol and adjusted with buffer) and 10 µL of tissue was added. The assay mixture except tissue supernatant was warmed up at 37°C. The addition of tissue supernatant initiated the reaction. The change in absorbance was read for 3 min, in a time interval of 30 sec, using a UV-VIS double beam spectrophotometer (Systronics 2206). GST activity is calculated by

GST activity = Adjusted Δ340/min/0.0096μM/min/mg tissue protein

Where,

Δ340/min = O.D. change per min

0.0096 μM = molar extinction co-efficient of CDNB

III) Hematological Analysis

Parameters, such as total red blood cells, total white blood cells, hemoglobin, platelet count, lymphocyte, hematocrit, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were done using Sysmex XP 300 hematology analyzer.

3.11. Statistical Analysis

All the experiments conducted were statistically analysed using different statistical tools. Biochemical experiments were carried out in triplicate. The results were expressed in mean ± Standard Deviation (SD). Statistical calculations such as mean and standard deviation were calculated using MS. Excel 2019. IC_{50} calculation, significant test and ANOVA were carried out using Origin Pro-8.5 software (OriginLab Corp., USA). The results are considered to be significant at $P \leq 0.05$ level.