Chapter 3
MATERIALS AND METHODS

3.1 Survey:

The survey was conducted in a systematic way, having a structured research questionnaire framework.

3.1.1 Survey area

The study was conducted in the five districts of Bodoland Territorial Region (26° 7'12" N to 26° 47' 50" N Latitude and 89° 47' 40" E to 92° 18' 30" E Longitude) which includes Kokrajhar, Chirang, Baksa, Tamulpur and Udalguri. BTR is located in the north-eastern part of India and had an area of 8,970 km^2.

3.1.2 Offline survey method

The questionnaire was devised to know the demographic information, preparation process, duration of medication, benefits/side effects of medication, precautions and suggestions from the local traditional healers of BTR.

A semi-structured trilingual questionnaire (Boro, Assamese, and English) was used during personal visits to collect data on every significant aspect (Appendix-A8). Respondents were invited to share information in their native or regional language, based on their personal knowledge and experiences. Casual conversations were used to clarify any ambiguities. The collected data was then translated into English in order to develop conclusions. Geotag photographs were captured to ensure the survey's legitimacy.

3.1.3 Respondents

The survey covered respondents of different ages, caste, creed, or religion. An offline survey was conducted using snowball sampling technique by visiting particular regions in all five districts of the BTR where the local traditional healers were present. Local medicinal practitioners were questioned during interviews, and the questionnaires were duly filled in. The questionnaires were signed or provided with a thumb impression by the respondents.

3.2 Collection and authentication of plant samples

The selection of plant materials was based on the disclosed substances used by the local practitioner of BTR, Assam. The following plant species were collected from various localities in the Bodoland Territorial Region: *Centella asiatica* (whole plant), *Hydrocotyle sibthorpioides* (whole plant), *Drymaria cordata* (whole plant), *Oroxylum indicum* (bark), *Alstonia scholaris* (bark), *Senna hirsuta* (leaf), *Senna occidentalis* (leaf), *Solanum indicum* (root), and *Stephania japonica* (tuber). Initially, they were recognised by Plant Taxonomist Dr. Sanjib Baruah from the Department of Botany at Bodoland University. The voucher specimens were sent to the Botanical Survey of India, namely the Central National Herbarium in Howrah, for the purpose of final authentication and identification. The specimens were assigned the reference numbers CNH/Tech.II/2021/42 and CNH/Tech.II/202/62. Following verification, all the botanical specimens were meticulously cleansed and let to dry naturally in a shady location. Subsequently, the materials were individually pulverised using a mechanical grinder into a granular consistency. They were then amalgamated in a container and well blended. The proportions of each constituent are specified in table 3.1.

3.3 Formulation of Polyherbal medicine (PHF) and naming it:

Each of the above ingredients were blended in various ratios to obtain effective formulation. The novel formulation is named as *LivSwasthya* where the word '*Liv*' stands for liver and '*Swasthya*' is a Sanskrit term for health and is translated as being rooted in the self.

3.4 Extract preparation

The selected individual plant parts such as the bark of *Alstonia scholaris*, and *Oroxylum indicum,* the whole plant of *Centella asiatica*, *Drymaria cordata,* and *Hydrocotyle sibthorpioides*, the leave of *Senna hirsute* and *Senna occidentalis*, the root of *Solanum indicum* and the tuber of *Stephania japonica* were dried at room temperature. They were then pulverized with an electric grinding machine, passed through a 600 μm mesh size sieve to obtain the fine powder and mix them together in a ratio. It was subjected to soxhlation for 72 hrs or 3 cycles using aqueous as a solvents. The extracts obtained were concentrated using a rotary evaporator under reduced pressure and stored in air-tight bottles at a temperature of 4°C for future use.

Table 3.1: The name of components of *LivSwasthya* and their GPS coordinate.

Botanical Name	Parts Used	Vernacular names		GPS Coordinates	
		Bodo Name	Assamese Name	Latitude	Longitude
Alstonia scholaris	Bark	Sitaona	Chatiyana, Sotiyana, Chatim	26.470425ºN	90.296998ºE
Centella asiatica	Whole plant	Manimuni geder	Bor manimuni	26.4706114ºN	90.2969722ºE
Drymaria cordata	Whole plant	Japsri	Thunthuni, Laijabori, Lai Jabori	26.534360ºN	90.015519ºE
Hydrocotyle sibthorpioides	Whole plant	Manimuni pisa	Soru manimuni	26.4697987ºN	90.2968682ºE
Oroxylum indicum	Bark	Karokandai	Dingdinga, Toguna, Bhatghila	26.463155ºN	90.297376ºE
Senna hirsuta	Leaf	Sumu bipang	N/A	26.797191ºN	90.540234ºE
Senna occidentalis	Leaf	Gangrim bipang	Hat thenga, Jonjoni goch, Kusum	26.47872ºN	90.3053733ºE
Solanum indicum	Root	Kuntainara	Deuri tita, Tit bhek, Bhot bengena	26.79414ºN	90.5303609ºE
Stephania japonica	Tuber	Dibaolu	Galdua, Tubuki Lota, Tubuka-lot	26.790827ºN	90.5300655ºE

3.5 Determination of yield extract

The yield of evaporated extracts (w/w) was estimated using the standard protocol of Goyal et al. (2010).

3.6 *In vitro* studies

3.6.1 Preliminary phytochemical screening:

The presence or absence of phytoconstituents in *LivSwasthya* powdered was determined using the following standard procedures by Trease and Evans with slight modification by Shaikh et al., (2020)

3.6.2 Detection of alkaloids:

3.6.2.1 Dragendroff's test: A few millilitres of extracts were mixed with 1 millilitre of Dragendroff's reagent. The occurrence of a reddish-brown solid implies the existence of an alkaloid.

3.6.2.2 Hager's test: A few millilitres of extracts and 1-2 drops of Hager's reagent were put next to the test tube. The occurrence of a creamy white precipitate indicates the existence of an alkaloid.

3.6.2.3 Mayer's test: A few millilitres of extracts, and 1-2 drops of Mayer's reagent were applied to the test tube, specifically along the sidewalls. The occurrence of a smooth white or yellow precipitate shows the existence of alkaloids.

3.6.2.4 Vitali-Morin test: To 1 millilitre of extract, Add concentrated nitric acid and alcoholic potassium hydroxide. The occurrence of violet colour shows the existence of alkaloid/atropine.

3.6.3 Detection of carbohydrates

3.6.3.1 Molisch's test: To 1mL or 2mL of the extract, three drops of Molisch's reagent were added. The mixture was well agitated and then 1mL of concentrated H_2SO_4 was gently poured along the walls of the test tube. The test tube was then left undisturbed. The detection of carbohydrates was suggested by the appearance of a violet ring.

3.6.3.2 Test for Starch: Five millilitres of a 5% potassium hydroxide (KOH) solution were added to 1mL of the extract. A cinary colouration serves as an indication of the presence of carbohydrates.

3.6.3.3 Test of Pentose: Add a little quantity of Phloroglucinol to 2mL of concentrated hydrochloric acid, then add an equal quantity of extract. Heat the mixture over a flame. The occurrence of a crimson precipitate indicates the presence of carbohydrates.

3.6.4 Detection of reducing sugars

3.6.4.1 Benedict's test: 1 mL of extract was combined with 1 mL of Benedict's reagent and subjected to boiling water for a duration of 2 minutes. The formation of a green/yellow/red coloured precipitate indicates the presence of sugar.

3.6.4.2 Fehling's test: A volume of 1 millilitre of extract was combined with 1 millilitre each of Fehling's solution A and B, and the mixture was heated to boiling for a brief period of time. The observation of a crimson precipitate serves as an indication of the presence of sugar.

3.6.4.3 Aqueous NaOH test: Combine a few mL of alcoholic extracts with 1mL of water and then introduce a small quantity of aqueous NaOH solution. The presence of sugar is indicated by a yellow precipitate.

3.6.4.4 Conc. H_2SO_4 test: Combine 5 millilitres of extract with 2 millilitres of glacial acetic acid. Then, add a single drop of 5% $FeCl_3$. Next, gently add a few

millilitres of concentrated H_2SO_4 down the walls of the test tube. Allow the mixture to stand.

3.6.5 Detection of cardiac glycosides

3.6.5.1 Keller-Killani test: Add 1.5mL of glacial acetic acid and 1 drop of 5% ferric chloride to 1mL of the extract. Then, carefully pour a few millilitres of concentrated H_2SO_4 down the edge of the test tube. The presence of glycosides is indicated by a solution that has a blue hue.

3.6.6 Detection of proteins and amino acids

3.6.6.1 Biuret test: The 2 mL extract was subjected to treatment with a single drop of a 2% solution of copper sulphate. Subsequently, 1mL of 95% ethanol is added, followed by an excessive amount of potassium hydroxide pellets. The presence of proteins was suggested by the pink hue seen in the ethanolic layer.

3.6.6.2 Millon's test: A few drops of Millon's reagent were added to 1mL of the extract. The observation of a white precipitate serves as evidence for the existence of proteins.

3.6.6.3 Ninhydrin test: Add 2 drops of Ninhydrin solution to a 1 millilitre extract and apply heat for a duration of 5 minutes. The presence of amino acids is indicated by a distinct purple coloration.

3.6.7 Detection of flavonoids

3.6.7.1 Alkaline reagent test: Add 2mL of a 2% NaOH solution and a few drops of diluted HCl to 1mL of the extract. This will result in the formation of a strong yellow hue, which will become colourless at the addition of a few drops of diluted acid. This colour change indicates the presence of flavonoids.

3.6.7.2 Lead acetate test: Add a little amount of 10% lead acetate solution to the 1m extract. Flavonoids are present when there is a yellow glow.

3.6.7.3 Shinoda's test: Take a little strip of magnesium ribbon and apply a few drops of concentrated hydrochloric acid over it. Then, add 1 millilitre of the extract to the mixture. The presence of flavonoids is indicated by the pink to scarlet coloration of the solution.

3.6.7.4 Shibata's reaction: Add 1 millilitre of extract to a solution of 1-2 millilitres of 50% methanol, heated, and containing metal magnesium. Then, introduce 5 drops of concentrated hydrochloric acid. The presence of flavonoids is indicated by a red or orange hue.

3.6.7.5 Ferric chloride test: Add a little amount of 10% ferric chloride solution to 1mL of the extract and observe for a vivid green hue.

3.6.7.6 Pew's test: Add 1 mL of extract, metallic zinc, and a little amount of concentrated H_2SO_4 (1mL) beside it. The presence of flavonoids is indicated by a red coloration.

3.6.7.7 Zinc-hydrochloride reduction test: Add a little amount of zinc dust and a small quantity of concentrated hydrochloric acid to 1 millilitre of the extract, pouring the acid down the edge of the test tube. The hue magenta indicates the presence of flavonoids.

3.6.7.8 Ammonia test: Add 1 millilitre of diluted ammonia solution and 1 millilitre of concentrated sulfuric acid to a 1 millilitre extract, pouring the acid down the side. Flavonoids are present when the colour is yellow.

3.6.8 Detection of phenolic compound

3.6.8.1 Add a few drops of a 10% $FeCl_3$ solution to 1 mL of the extract. The presence of phenolic chemicals was indicated by a hue of dark green.

3.6.9 Detection of tannins

3.6.9.1 Braymer's test: Add 3 millilitres of distilled water to 1 millilitre of extract, followed by the addition of 3 drops of a 10% Ferric chloride solution. The presence of phenolic compounds was indicated by the blue-green hue.

3.6.10 Detection of phlobatannins

3.6.10.1 HCl test: Add 2mL of 1% hydrochloric acid to a 2mL aqueous extract and heat it till boiling. The existence of phlobatannins compounds was confirmed by the formation of a crimson precipitate.

3.6.11 Detection of saponins

3.6.11.1 Foam test: The 2mL extract was diluted with distilled water and then brought up to a final volume of 10 mL. The suspension was agitated in a graduated cylinder for a duration of 15 minutes. The presence of saponins was indicated by a layer of foam measuring two centimetres.

3.6.12 Detection of phytosterol

3.6.12.1 Salkowski's test: Add 5 mL of chloroform to a 1 mL extract and treat the sample with a little amount of concentrated solution. The presence of steroids is indicated by the red colour in the bottom layer of H_2SO_4, whereas the creation

of a yellow-coloured lower layer shows the presence of phytosterol.

3.6.12.2 Libermann-Burchard's test: To 2mL acetic acid, add 2mL chloroform, 1mL extract, and 1-2 drops of strong sulphuric acid were applied gently down the walls of the test tube. The presence of phytosterols is indicated by an array of colour changes.

3.6.13 Detection of triterpenoids

3.6.15.1 Horizon test: Add 1 mL of extract to 2 mL of trichloro acetic acid. The presence of triterpenoids is indicated by a crimson precipitate.

3.6.14 Detection of lignins

3.6.14.1 Labat test: Add gallic acid to the side of a 1mL extract. The presence of lignins is indicated by an olive-green hue.

3.6.15 Detection of quinines

3.6.15.1 Alcoholic KOH test: Combine gallic acid with a 1mL extract. Lignins are identified by the presence of an olive-green coloration.

3.6.16 Detection of anthraquinones

3.5.16.1 Borntrager's test: Add a little amount of filtrate, obtained by passing a liquid through a filter, to a 10 mL solution containing 10% ammonia. Shake the mixture rapidly for 30 seconds. The presence of anthraquinones was suggested by the formation of a solution with a pink, violet, or red hue.

3.6.17 Detection of coumarins

3.6.17.1 NaOH paper test: Take 1 gm of moistened extract in a test tube. Cover the mouth of the test tube with filter paper prepared with 1N NaOH. Heat the test tube for a few minutes. If the paper develops a yellow fluorescence under UV light, it indicates the presence of coumarins.

3.6.17.2 NaOH test: Add 1 mL of plant extract to 1 mL of 10% NaOH and 1 mL of chloroform. The presence of coumarins is indicated by a yellow hue.

3.6.18 Detection of resins

3.6.18.1 Turbidity test: The plant extract, dissolved in acetone, was added to 1mL of distilled water.

3.7 Microscopic study

The powdered samples were affixed to a transparent glass slide using a small amount of water and then covered with a protective coverslip. The slides were seen

using a binocular microscope (Labomed Vision 2000) and the images were captured using a Samsung Galaxy phone.

3.8 Determination of physical characteristics of powder

3.8.1 Bulk density:

Bulk density refers to the measurement of the density of a powder after it has been aerated and then allowed to settle in a gentle manner. The mass-to-volume ratio refers to the relationship between the specified mass and the unused volume. The 15 g powdered sample was put into a 100 mL cylinder. The beginning volume was recorded, and the ratio of the weight of the occupied volume was calculated using the prescribed formula

$$Bulk\ density = \frac{W}{V0}$$

Where, W = powder mass V0 = untapped volume.

3.8.2 Tapped density:

The tapped density was determined by introducing 15 g of powdered material into a measuring cylinder and physically tapping it about 500 times. The loudness was recorded both before and after tapping. The tapped density was determined using the established formula (Madhavi et al., 2019; Anilkumar et al., 2020).

$$Tapped\ density = \frac{W}{Vf}$$

Where, W = mass of the powder Vf = tapped volume.

3.8.3 Carr's index:

This refers to the powder's inclination to be crushed based on its perceived bulk and tapping density. The Carr's index was calculated using the conventional formula, following the established approach (Barbosa-Ferreira et al., 2005). The result is presented as a percentage.

$$Carr's\ index = \frac{Tapped\ density - Bulk\ density}{Tapped\ density} \times 100$$

3.8.4 Hausner's ratio:

The flow qualities of the powder are determined by Hausner's ratio. The tapped density to bulk density ratio of a powder is defined as the ratio between its tapped density and bulk density (Madhavi et al., 2019; Anilkumar et al., 2020).

$$Hausner's\ ratio = \frac{Tapped\ density}{Bulk\ density}$$

3.8.5 Angle of repose:

The angle of repose is the highest possible angle between the pile of powder and the horizontal plane. The powder was funnelled via a fixed tripod stand at a height of 2.9 cm. The funnel had a mouth diameter of 100 mm and a stem diameter of 10 mm. Graph paper was positioned underneath the tripod on the table, and the dimensions of the stack, including its height and radius, were measured (Tripathi et al., 2013; Madhavi et al., 2019). The formula used for the computation is

$$\theta = tan^{1}\frac{h}{r}$$

Where, h = height of the pile, r = radius of the pile.

3.9 Determination of Total Phenolic Content:

The total phenolic content was assessed using the method described by Singleton and Rossi (1965) using the Folin-Ciocalteu (FC) reagent, with a minor adjustment as outlined by Goyal et al. (2010). Concisely, the 0.5 mL extract was mixed with 0.5 mL of FC reagent (previously diluted 1:1 with distilled water) and left at room temperature for 5 minutes. Subsequently, the mixture was combined with 1 mL of 2% Na_2CO_3 solution. After incubating for 10 minutes at room temperature, the absorbance was quantified at a wavelength of 730 nm. The trials were conducted in duplicate. Gallic acid monohydrate was used as a control. The total phenolic content was quantified as grams of gallic acid equivalents (GAE) per 100 g of extract .

3.10 Determination of Total Flavonoid Content:

The quantification of total flavonoid content was conducted using the technique described by Goyal et al., (2010), which use the aluminium chloride ($AlCl_3$) method and a quercetin standard. The plant extract (0.25 mL) was diluted with 1.25 mL of distilled water (DDW) and then combined with 75 µl of a 5% solution of sodium nitrite ($NaNO_2$). After 5 minutes at room temperature (RT), 0.15 mL of $AlCl_3$ (10%) was introduced. After a duration of six minutes at ambient temperature, the reaction mixture was subjected to the addition of 0.5 mL of a 1 mM NaOH solution. Finally, 275 mL of distilled deionized water (DDW) were introduced into the reaction mixture. Following an additional duration of 20 minutes at ambient temperature, the optical density at a wavelength of 510 nm was quantified. The experiments were conducted in groups of three. The quantification of flavonoid content was conducted by using a quercetin standard curve.

3.11 DPPH radical scavenging activity:

The antioxidant activity of the extracts and standard was evaluated by measuring their ability to scavenge the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical, using the method described by Goyal et al., (2010). A DPPH solution with a concentration of 0.006% w/v was prepared using a 95% solvent. The *LivSwasthya* extract was mixed with a solvent consisting of 95% concentration to create a stock solution with a concentration of 1 mg/mL. The freshly made DPPH solution was put in test tubes, and extracts were added. Subsequently, each test tube underwent consecutive dilutions (ranging from 100 to 1000 g) until the total volume reached 2 mL. After an incubation period of 30 minutes in the absence of light, the degree of discoloration was evaluated at a wavelength of 517 nm. The Themo UV1 spectrophotometer, manufactured by Thermo Electron Corporation in England, UK, was used to collect measurements, which were conducted at least three times. Ascorbic acid was used as a standard and was diluted in distilled deionized water (DDW) to create the stock solution with a concentration of 1 mg/mL. For the control sample, an equivalent volume was prepared without any extract, and a blank was created using a solvent with a concentration of 95%. The equation provided was used to compute the proportion of DPPH free radical scavenging.

$$DPPH\ scavenging\ effect\ (\%) = \frac{Ao - A1}{Ao} \times 100$$

Where, Ao represents the absorbance of the control, whereas A1 represents the absorbance when the sample is present.

The observed reduction in absorption caused by the test substances was compared to the positive controls. The IC_{50} value was determined by analysing the dosage inhibition curve.

3.12 Reducing power assay:

The *LivSwathya's* reducing power was assessed using the method described by Oyaizu (1986), with some modifications. The extract was mixed with different quantities (ranging from 250 to 2,500 µg) in 1 mL of DDW, together with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The solution was subjected to incubation at a temperature of 50°C for a duration of 20 minutes. The mixture was thereafter subjected to centrifugation at a speed of 3,000 rpm for a duration of 10 minutes, with the addition of 2.5 mL of Trichloroacetic acid (10%). The solution from the top layer (2.5 mL) was combined with distilled deionized water (2.5 mL) and Ferric chloride (0.5 mL, 0.1%). The absorbance of the resulting mixture was then measured at a wavelength of 700 nm.

Increased absorbance of the reaction mixture showed a greater capacity for reducing substances. Ascorbic acid served as the benchmark standard. A phosphate buffer with a pH of 6.6 was used as a control. The mean absorbance of the final reaction mixture in the two parallel experiments was computed and reported together with the standard deviation.

3.13 Hydrogen peroxide scavenging:

The determination of this activity was conducted using a pre-existing methodology with minor adjustments. A portion of H_2O_2 (2 mM) and various sample concentrations (100 - 1000 µg/mL) were mixed together at a ratio of 1:0.6 v/v and left to incubate at room temperature for 10 minutes. After the incubation period, the measurement of hydrogen peroxide absorbance at a wavelength of 230 nm was compared to a blank solution containing phosphate buffer but no hydrogen peroxide. A distinct blank sample was used for background removal at each concentration. The scavenging activity of hydrogen peroxide by the *LivSwasthya* extract was assessed using the following method.

$$(\%)scavenging\ activity\ of\ H_2O_2 = \frac{Abs\ (control) - Abs\ (standard)}{Abs\ (control)} \times 100$$

Where, Abs (control): Absorbance of the H_2O_2 (2 mM) as control Abs (standard): Absorbance of the extract/standard

3.14 Phytoconstituent profiling of different extracts by GC-MS/MS analysis:

The study used an Agilent Technologies 7890B GC and Triple Quadrupole mass spectrometer 7000D series, equipped with a mix silica column consisting of 5% biphenyl and 95% dimethylpolysiloxane (Elite-5MS). This column was combined with a capillary column measuring 30 m in length, 0.25 mm in diameter, and 1 m in thickness. The test sample was separated into distinct components using helium quench gas and nitrogen collision gas as carrier gas. The helium quench gas was used at a flow rate of 2.25 mL/min, while the nitrogen collision gas was used at a flow rate of 1.5 mL/min. The separation process was conducted at a pressure of 8.745 psi. The temperature of the injector was consistently set at 260°C for every chromatographic run. A 2µ L volume of the 100-fold diluted sample was introduced into the device, with a split ratio of 10:1. The oven temperature varied between 20°C and 260°C. The specifications for the mass detector were as follows: the transfer line temperature was set to 300°C, the ion source temperature was set to 260°C, the ionisation mode used was electron impact at 70 eV, the scan period was 0.2 seconds,

and the scan interval was 0.1 seconds. The sample's mass range spanned from 50 m/z to 1000 m/z. The solvent delay varied between 0 and 2 minutes, whereas the total duration of the GC-MS/MS procedure was 44 minutes. The mass spectrum of each peak in the total ion chromatogram was cross-referenced with the databases of The National Institute of Standards and Technology (NIST) 14 MS Library, which has a comprehensive collection of 276,248 spectral databases for various chemicals. Therefore, the IUPAC names, molecular formulae, molecular weights, and structures of the HMPGL metabolites were established.

3.15 *In vivo* studies

3.14.1 Experimental approval:

The experimental procedures were carried out subsequent to obtaining the necessary approval from the Animal Ethical Committee (1368/ac/10/CPCSEA/BT-SKM/06) and were meticulously executed in accordance with the protocol sanctioned by the Maharani Lakshmi Ammanni College Ethical Committee, Bangalore, pertaining to the maintenance and utilisation of laboratory animals.

3.15.2 Experimental animals homogenization:

The provision of veterinary care is a crucial aspect of ensuring the health and well-being of animals. Male Wistar albino rats, with an average weight ranging from 200 to 250 grams and an age of four months, were procured from the esteemed Indian Institute of Science located in Bangalore, India. The animals were housed in groups of four within polypropylene, polycarbonate, and stainless-steel cages for a duration of 7 days prior to the commencement of the experiment. This housing arrangement was implemented within the animal facility of MLACW, Bengaluru, where the environmental conditions were maintained at a temperature of 23°C ± 2°C, relative humidity of 68% ± 1%, lighting intensity of 350 lx, and a light/dark cycle of 12 hours. During the course of the investigation, the subjects under scrutiny were provided with a conventional dietary regimen and were granted unrestricted availability to water resources, as documented by Middha et al. in 2015 and S. K. Middha et al. in 2019. The enclosures, measuring 421 mm 290 mm 190 mm with a 7 mm interstice between wires, were periodically replaced with fresh bedding to maintain optimal hygiene conditions for the subjects. This practice aligns with the guidelines set forth by the esteemed National Institutes of Health (NIH 2010), ensuring the animals' well-being by keeping them dry and clean. Daily monitoring

was conducted to oversee the well-being of the animals, and no adverse occurrences were documented.

3.15.3 Acute toxicity test:

Swiss albino mice (25-30 g) were put into five groups of 10 each. The animals were fasted overnight, although water was provided before to the experiment. The experimental groups were given an aqueous extract of *LivSwasthya* at various dosage levels [5.0, 6.0, 7.0, 8.0, and 9.0 g/kg BW/mL]. The mice were examined for 24 hours, mortality was recorded. As per OECD guidelines 2000 mg/kg BW/ml was found to be safe and demonstrated no observed adverse effect. The acute toxicity (LD_{50}) was calculated using the formula:

$$LD_{50} = LDy - ^{I-r} \sum \frac{(Dd \times Md)}{n}$$

Where LD_y = highest dose and n = number of animals per group (n = 10), Dd = dose difference, Md = mean dead.

3.15.4 Hepatotoxicity study group:

A group of thirty male rats was subjected to random allocation, resulting in the formation of five distinct groups, each comprising six rats. The delineation of therapeutic interventions for each cohort is explicated in the subsequent sections. An oral administration of 3 mL/kg of carbon tetrachloride (CCl_4) was administered as a single dose on the initial day. The *LivSwasthya* extract was solubilized in Millipore water, and subsequently, two distinct concentrations (100 mg/kg and 200 mg/kg) of the *LivSwasthya* extract were orally administered on a daily basis in the morning. This administration was facilitated using a 16G gavage needle. In contrast, the control group received Millipore water and was not subjected to any additional treatment. On the concluding day, prior to the implementation of euthanization, experimental cohorts 2, 3, 4, and 5 were subjected to the administration of carbon tetrachloride. Body weights were meticulously observed and recorded at regular intervals throughout the duration of the experiment.

Group 1 received Millipore water + nutrition supplement as a solvent control. (Control)

Group 2 received CCl_4 on the first day, along with a dietary supplement serving as the control for illness induction. (CCl_4 Control)

Group 3 received an oral dose of CCl_4 on the first day, followed by an oral dose of 100 mg/kg BW of *LivSwasthya* and a food supplement for 7 days. (*LivSwasthya*

100).

Group 4 received an oral dose of CCl_4 on the first day, followed by an oral dose of 200 mg/kg BW of *LivSwasthya* and a dietary supplement for 7 days. (*LivSwasthya* 200).

Group 5 received an oral dose of CCl_4 on the first day, along with 25 mg/kg BW of silymarin and a food supplement for 7 days. Positive control: Silymarin The silymarin group was administered Silybon-140 mg, manufactured by Micro Lab Limited in India.

3.15.5 Preparation of liver tissue samples:

Following the administration of the crude water extract of *LivSwasthya* for a duration of one week, the subjects underwent euthanization. Subsequently, on the 8th day, precisely 24 hours post-euthanization, blood samples were collected from the subjects using the retro-orbital puncture technique. These blood samples were obtained using EDTA blood collection tubes. In accordance with the regulations set forth by the Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA), the rats involved in the study were subjected to sedation using a combination of xylazine and ketamine. The administered doses ranged from 80 to 120 mg/kg for xylazine and 10 to 16 mg/kg for ketamine. Following sedation, the rats were subsequently placed in a CO_2 chamber for the purpose of euthanization, as documented in the studies conducted by Middha SK et al. (2019) and Conlee KM et al. (2005). The protocol for homogenate preparation was delineated with certain modifications, drawing upon a range of research articles (Ohkawa et al., 1979; Sellamuthu et al., 2013; Pilkhwal et al., 2010; Boro et al., 2022). The liver and kidney specimens from the experimental rats were surgically removed, thoroughly rinsed, perfused, and cleansed using ice-cold 1X phosphate-buffered saline (PBS) with a pH of 7.4. The precise weight of each organ was determined. A solution containing 0.1 (mM) ethylenediaminetetraacetic acid (EDTA) at a concentration of 0.2 g was combined with 10 mL of phosphate-buffered saline (PBS) at a concentration of 50 millimolar (mM). Subsequently, an organ sample weighing 200 mg was homogenised in 10 mL of a 50 mM phosphate buffer solution with a pH value of 7.0. The homogenate underwent ultra-centrifugation at a speed of 3600 rpm for a duration of 5 minutes at a temperature of 4°C. To facilitate further investigations pertaining to in-vivo enzymatic antioxidant activities, the supernatant was procured.

3.15.6 Biochemical assays in blood:

In this experiment, the activities of various enzymes were assessed using standard (Amplification diagnostic) kits from Graz, Austria. The primary function of these assays was to evaluate the liver function and damage. Specifically, the serum enzymatic levels of SGOT (Serum Glutamic Oxalo-acetic Transaminase), ALP (Alkaline-phosphatase), SGPT (Serum Glutamic Pyruvic Transaminase), Total bilirubin, HDL-cholesterol, LDL-cholesterol, total cholesterol (TC), and triglycerides (TG) were measured to ensures the effectiveness of treatments, accuracy and reliability of the results obtained.

3.15.7 Assessment of in-vivo liver enzymatic assays:

Catalase test (CAT), Glutathione peroxidase (GPx), Malonaldehyde Assay (MDA), Bilirubin Test, Creatine and Creatinine test, Protein estimation (Lowry's test), Superoxide Dismutase (SOD), Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic-Oxaloacetic Transaminase (SGOT).

3.15.7.1 Catalase:

The enzymatic Catalase assay was performed following the experimental procedure as described by Fakurazi et al. (2012) and Kaur et al. (2006), with minor adaptations. A 100 mL sample of tissue extract was combined with 10 mL of absolute alcohol and subjected to an incubation period of 30 minutes in an ice bath. Subsequently, the mixture was incubated for an additional 10 minutes at room temperature. Subsequently, a volume of 10 microliters containing a 1% concentration of Triton-x-100 was introduced into the amalgamation. The tissue extract (10 microlitre) was then combined with 240 microlitre of 50mM PO_4^{-3}, and 250 microlitre of 0.066M H_2O_2 in 50mM PBS (pH 7.4) were added. The decrease in OD was measured at 240nm for approximately three minutes at the interval of every 1 minute against the blank solution (1.25mL PBS + 1.25ML H_2O_2). The catalase activity was calculated as one mole H_2O_2 degraded per minute per milligram of protein, with one unit of catalase activity being equivalent to this amount.

3.15.7.2 Glutathione peroxidase (GPx):

The Glutathione peroxidase test was conducted in accordance with the established protocol outlined by Middha SK., et al in the year 2019. The experimental procedure was commenced by introducing 100µl of 0.24U Glutathione Reductase (GR) into a solution containing 500µl of 1X PBS (50mM, pH 7.4). Subsequently, 0.01M (100µl) of GSH (Reduced Glutathione), 100µl (1.5mM) of NADPH, and 100µl of tissue

extract were sequentially added to the mixture. The concoction was subsequently subjected to incubation at a temperature of 37°C for an approximate duration of 10 minutes. Subsequently, a precise quantity of tert-butyl (50µl) was introduced into the experimental setup, followed by the addition of 450µl of the reaction mixture. The ensuing reactivity was then assessed by quantifying the absorbance at a wavelength of 340nm.This method was utilized to measure the levels of Glutathione peroxidase in the sample under investigation. The procedure was conducted in a professional manner to ensure accurate and reliable results.

3.15.7.3 Malondialdehyde (MDA):

The present investigation involved the evaluation of the enzymatic assay of malondialdehyde (MDA) through the implementation of a methodology inspired by the seminal research conducted by Lim et al. (2016), albeit with certain adaptations made to the original protocol. The objective of this assay was to quantify the extent of lipid peroxidation in liver and kidney tissue samples procured from Wistar rats. To prepare the homogenate, 0.1mM EDTA (0.2g) was added to 10mL PBS (50mM). Next, 200mg of organ samples was homogenized in 10mL of PBS and centrifuged at 3600rpm. This method allowed for the accurate assessment of MDA levels, which is an important indicator of oxidative stress and cellular damage. In its entirety, this study offers significant insights into the underlying mechanisms of liver and kidney diseases, shedding light on their pathophysiology. These findings hold potential significance in the realm of therapeutic advancements, potentially paving the way for the development of novel interventions.

3.15.7.4 Bilirubin:

The present investigation involved the evaluation of the enzymatic assay of malondialdehyde (MDA) through the implementation of a methodology inspired by the seminal research conducted by Lim et al. (2016), albeit with certain adaptations made to the original protocol. The objective of this assay was to quantify the extent of lipid peroxidation in liver and kidney tissue samples procured from Wistar rats. To prepare the homogenate, 0.1mM EDTA (0.2g) was added to 10mL PBS (50mM). Next, 200mg of organ samples was homogenized in 10mL of PBS and centrifuged at 3600rpm. This method allowed for the accurate assessment of MDA levels, which is an important indicator of oxidative stress and cellular damage. In its entirety, this study offers significant insights into the underlying mechanisms of liver and kidney diseases, shedding light on their pathophysiology. These findings hold potential

significance in the realm of therapeutic advancements, potentially paving the way for the development of novel interventions.

a. Total Bilirubin:

A test sample was prepared by mixing R2 (0.2 mL), R1 (0.05 mL), R3 (1 mL), and R4 (0.2 mL). And to account any potential interference, a blank solution was also prepared by mixing R2 (0.2 mL), R3 (1mL), and the sample (0.2 mL). Subsequently, both solution mixtures were subjected to incubation at a temperature of 20℃ for a duration of 10 minutes, thereby ensuring the attainment of complete reaction. After that, a volume of R4 (1mL) was introduced into each solution and thoroughly homogenised. Next, the reaction mixtures underwent an additional incubation period of 5 minutes at a temperature range of 20-25℃. In order to assess the overall concentration of total bilirubin, the absorbance was meticulously measured at a specific wavelength of 546 nm.

b. Direct Bilirubin:

The direct bilirubin test, the method was carried out on both the blank solution (0.2 mL sample, 0.2 mL Sodium nitrite, and 1mL Caffeine reagent) and the test sample (0.05 mL Sulphanilic acid, 0.2 mL Sodium nitrite, and 1 mL Caffeine reagent). However, R4 (Artificial Standard) was eliminated from the procedure for this particular test. The measurement of absorbance was subsequently conducted at a specific wavelength of 546 nm in order to ascertain the existence of direct bilirubin.

3.15.7.5 Creatine and Creatinin test:

The estimation of Creatine and Creatinine in urine samples was carried out using Jaffe's method. The experimental rats were subjected to individual housing within metabolic cages for a duration of approximately 24 hours. During this period, the rats were provided with unrestricted access to both food and water. Urine specimens were obtained and subjected to processing procedures in accordance with a modified iteration of the protocol outlined by Toora and Rajagopal (2002).

To prepare a stock solution of Creatinine, 100 milligrams of the Creatinine were added to 100 milliliters of distilled water and diluted 10-times. The quantification of Creatinine test sample was conducted using standard solutions, which were prepared by combining aliquotes ranging from 0.2 to 1.0 mL of the stock solution with varying volumes of distilled water (ranging from 2.0 to 1.0 mL), 0.1% picric acid (2 mL), and 10% NaOH (2 mL). Following a period of incubation at ambient

temperature for a duration of 15 minutes, a volume of 5 millilitres of distilled water was introduced into the experimental setup. Subsequently, the absorbance of the resulting solution was quantified utilising a spectrophotometer, specifically at a wavelength of 540 nm.

The test samples were meticulously prepared through the precise amalgamation of 1 mL of urine sample with distilled water, a solution containing 0.1% picric acid, and a solution containing 10% NaOH. This meticulous process was undertaken to facilitate the formation of a chromophore, a crucial component for subsequent analysis. The solution underwent an incubation period of 15 minutes at ambient temperature prior to the quantification of absorbance at a wavelength of 540 nm.

3.15.7.6 Superoxide Dismutase (SOD):

The determination of Superoxide Dismutase (SOD) activity in tissue extracts was conducted in accordance with the experimental procedure established by Middha et al. in 2016. The experimental protocol entailed the utilisation of 0.05 M Carbonate buffer (880 µl) possessing a pH of 10.2. This buffer solution was supplemented with 0.1 mmol EDTA. Subsequently, 30 mmol epinephrine (20 µl) was introduced into the solution, which was further diluted in 0.05% acetic acid. Subsequently, the aforementioned concoction was introduced into a 100 µl sample of tissue extract. The ensuing alterations in activity were meticulously assessed over a duration of 4 minutes, employing a wavelength of 480 nm. The establishment of a standardised measurement for SOD has been undertaken with the aim of assessing the enzyme's activity. The quantification of enzyme necessary to achieve a 50% inhibition of epinephrine oxidation was determined to be equal to 1 unit (U). This measurement was then converted into units per milligram of protein, providing a standardized means of expressing the enzyme's activity (Middha SK et al., 2016).

3.15.7.7 Serum Glutamic Oxaloacetic Transaminase (SGOT):

The assessment of SGOT (Serum Glutamic Oxaloacetic Transaminase), also known as AST (Aspartate-Transaminase), was conducted following the protocol delineated by AGAPPE Diagnostics limited. (https://www.agappe.com/media/catalog/product/file/11408003_1.pdf). The procedure used Reagent 1, which comprises Tris Buffer at a concentration of 88 mmol/L and a pH of 7.8, L-Aspartate (260mmol/L), Lactate Dehydrogenase (>1500 U/L), and Malate Dehydrogenase (>900 U/L). Reagent 2, on

the other hand, contains alpha keto-glutarate (12 mmol/L) and NADH (Nicotinamide Adenine Di-nucleotide hydrogen) at a concentration of 0.24 mmol/L.

The working solution R3 was obtained by combining R1 and R2 in a ratio of 4:1. The measurement of AST concentration in rat blood serum was performed by combining 1000µl of R3 (working solution) with 100µl of the blood serum sample. Next, the mixture was subjected to incubation at a temperature of 37℃ for a duration of 1 minute. The change in absorbance was then measured at a wavelength of 340nm, with readings taken at 1-minute intervals for a period of 3 minutes.

The SGOT activity (U/L) was calculated using the formula:

$$SGOT\ activity = \frac{OD}{Minute} \times 1745$$

This procedure is a reliable and accurate method for determining the SGOT level in rat blood serum.

3.15.7.8 Serum Glutamic Pyruvic Transaminase (SGPT):

The Serum Glutamic Pyruvic Transaminase or Alanine-Transaminase (ALT) levels were measured using the SGPT (S.L) AGAPPE Diagnostics Limited (https://www.agappe.com/media/catalog/product/file/11409003.pdf). According to the procedure, Reagent 1 is composed of Tris buffer with a concentration of 110 mmol/L and a pH of 7.5, L-Alanine with a concentration of 600 mmol/L, and LDH more than 1500 U/L. Reagent 2 is composed of alpha keto-glutarate at a concentration of 16 mmol/L, along with NADH (Nicotinamide Adenine Di-nucleotide Hydrogen) at a concentration of 0.24 mmol/L. Achieving a viable solution, R3, required the amalgamation of R1 and R2 in a 4:1 proportion. The Alanine-Transaminase level in rat blood serum was tested by combining 1000µl of R3 (working solution) with 100µl of the blood serum sample.

Following a 1-minute incubation period at a temperature of 37℃, the change in absorbance was measured at a wavelength of 340nm for a duration of 3 minutes, with readings taken at 1-minute intervals.

The SGPT activity (U/L) was then calculated by using the formula:

$$SGPT\ activity = \frac{OD}{Minute} \times 1745$$

3.15.8 Histopathological study

A minute segment of the kidney and liver was excised, subsequently immersed in a solution of physiologically balanced saline, and subjected to individual processing protocols to facilitate subsequent histopathological evaluation. The liver tissues underwent an initial fixation process using a 10% buffered neutral formalin solution for a minimum duration of 48 hours. Subsequently, the tissues were subjected to gradual dehydration using ethanol ranging from 50% to 100%. Following dehydration, the tissues were cleansed using xylene and finally embedded in paraffin for further analysis. The 4 µm slices were sectioned using a microtome. The liver slices underwent a dewaxing process using xylene, followed by rehydration using a sequential series of alcohol solutions of varying grades. Subsequently, the slices were subjected to a five-minute washing step using distilled water. The tissue samples were meticulously processed and subjected to a staining protocol involving hematoxylin for a duration of 40 seconds, followed by eosin staining for a period of 20 seconds. The tissue sections were subjected to examination under an Olympus microscope at a magnification of 40x. This was done to observe and document any histopathological changes, including but not limited to fatty changes, cell necrosis, and vacuolation (Boro et al., 2022).