CHAPTER

4

METHODOLOGY

4.1 STUDY AREA

The study was conducted at Vadodara city which lies between 22°18'00''N and 73°12'01''E, a second largest city in Gujarat state, India. With population of more than 0.17 million and area of approx. 220.33 square kilometer, it is among top largest cities of India (Vadodara Municipal Corporation, 2022a). The study area was selected conveniently for the present study. As per census of India data, Vadodara had sex ratio of 920 female for 1000 male which is consistent with Gujarat state data. Percentage of the literacy in Vadodara was 90.48%, which was high compared to 79.31% & 74.04% for Gujarat state and India respectively. Considering religious practices, 85.39% population practice Hinduism, followed by 11.4% Islam, 1.52% Jains, 1.12% Christians and 0.45% Sikhs (*Government of India*, 2011). Further, 71.37% speak Gujarati language, followed by 14.83% Hindi, 7.6% Marathi and 1.91% Sindhi.

4.2 HUMAN ETHICAL CLEARANCE

The study protocol and questionnaire were approved by Institutional Ethical Committee for Human Research, The Maharaja Sayajirao University of Baroda wide No. IECHR/2018/20 (certificate is attached as annexure 1).

4.3 INFORMED CONSENT

As per IECHR guidelines, all participants were briefed about the study purpose and protocol. After providing information, their queries were addressed satisfactorily. Once subjects

agreed-upon study objective, an informed consent was taken in either Gujarati (local language) or English consent form (Annexture 2).

4.4 ETHNOBOTANICAL SURVEY OF THE TRADITIONAL PRACTICES

The ethnobotanical study was conducted at the study area with an objective of documenting the demographics, knowledge, attitude and practices concerning gestation in mothers.

4.4.1 STUDY DESIGN

For enrolment of women participants, Maternity clinic and Anganwadis located in Vadodara city area were visited conveniently. Women visiting these places were asked if they are willing to participate in the study.

With an objective of working out the prevalence of ethnobotanical & traditional herbal medicine practices, and to document such practices, the study was designed. Here, descriptive cross-sectional study design was adopted, also known as prevalence study. As per descriptive design, the sample was selected and interviewed for once at a time during the study. The informants were divided as either traditional practices user or non-user and their health outcomes were recorded using a questionnaire (Annexure 3).

4.4.2 SAMPLE SIZE

The sampling for the present objective of descriptive study was done as convenience sampling. The estimate of the sample was presented by considering 31523 birth registration for Vadodara city in 2018 (Vadodara Municipal Corporation, 2022b). With expectation of 10% sample representation of new mothers, we sought to study 315 women. However, considering the prevalence of the herbal or traditional practices non-user in study area, quarter additional informants were included in the study. For this objective, total 387 informant women were interviewed, and their responses were recorded.

4.4.3 INCLUSION AND EXCLUSION CRITERIA

Women of any age between 20 to 50 years, who had delivered baby any time before the date of an interview, was included for the study. Any religion, of any socio-economic status mothers were included for the study.

However, participants with history of any major systemic diseases or major surgeries or physical or mental disability during their gestation period, was excluded from the study.

4.4.4 DATA COLLECTION

For this cross-sectional study, participants were selected and enrolled for interviews conveniently. In brief, the semi-structured questionnaire was prepared with experience of pilot study and necessary changes were included in the final questionnaire. The questionnaire was Interviewer Administered and hence was prepared in English language only. The participants were interviewed only once, and face-to-face, for about 15 minutes in single sitting.

The questions in demographic details included age, religion, birthplace, Marital status, size of the family, educational qualification, Occupation status, Income, parity, and period of their residence in the state. Further, their present systemic health problems, Diet, diet restrictions followed during gestation and classes of medicines taken during gestation were recorded. They were asked if they have used protein foods, Jeera, saunth, ganthoda, gokhru, ajwain, castor oil, specific grains, Herbal lep/bath, Churna or decoction, Methi/vasanu laddus, or none of the above. Those participants responded 'none of the above' in the question were asked again whether any ayurveda or plant based, or traditional knowledge-based medicines were used. Such participants were classified as non-user for further analysis. Non-users were asked the reason behind not using any herbal or traditional practices. Users were asked for their reason and belief behind using herbal or traditional practices, as well as their source of knowledge. For non-users, the interview was finished with this information.

Users group however were continued for the further questions on herbal usage. Users were asked details about the traditional practices followed. Period of gestation where herbal medicines were used, was noted down. For each of the separate practices, a purpose was noted. It was either antenatal, parturition or postpartum practices, which included newborn

related practices as well. The local names of the herbs and plant part used along with its recipe and dosage, wherever possible was noted. Participants were asked about their satisfaction level using traditional practices. Source of knowledge and side effects (if any) were also recorded in the questionnaire.

4.4.5 DATA ANALYSIS

Data recorded in the questionnaire were entered on a Microsoft Excel (Windows Office 365) spreadsheet. The questionnaire with subjective information about various practices were maintained separately for its proper documentation. The demographic details, traditional practices' usage, knowledge, and attitude was treated as two groups of 'users' and 'non-users' for further analysis. Quantitative data were analyzed using R Studio (R Core Team, 2022). Descriptive statistics includes frequency, percentage, and summary. Demographic variables were tested using chi-square test for checking odd of the usage of traditional practices, with 95% confidence interval (CI). Further, the odds ratio for variables with ≤ 0.05 p-value were chosen to build models for logistic regression analysis. Using multiple logistic regression models, factors associated with usage of traditional practices were tested.

Descriptive data regarding names of traditional practices related to plants' usage, utility period during gestation, dosage or frequency, its ingredients information and purpose were listed serially. The plant names, part used, and its form (whether fresh or dried) were separated on another sheet. Results of this ethnobotanical survey were evaluated using Relative Frequency of Citation (RFC^a) calculation. It was determined using formula as described earlier (Kankara et al., 2015).

$$Relative\ Frequency\ of\ Citation\ (RFC^a) = \frac{F_c}{N}$$

Where, F_c = Number of informants citing plant species; and N = Total Number of informants

Further, for all plant species, their scientific name and family were listed using published literature (Khare, 2007; Pade & Vyas, 2004). Plant names were confirmed using International Plant Names Index (IPNI) and the World Flora Online (WFO) plant list.

4.5 PROSPECTIVE CLINICAL STUDY OF WOMEN USING HERBS

The prospective clinical study was conducted among women using herbal practices with an objective of documenting the outcome.

4.5.1 STUDY DESIGN

For enrolment of women participants in this objective, A Maternity clinic located within Vadodara city at GPS location 22°34'40''N, 73°17'62''E was selected conveniently. The clinic is a licensed maternity clinic and is visited by more than ten patients a day. Objective of the study was to evaluate the resultant clinical differences among traditional practices users and non-users. With an objective of working out the clinical relevance of traditional herbal medicine practices, and to document such practices, the study was designed. Study design employed is prospective study design (Kadir et al., 2014).

As per prospective study design, the participants were asked if they are aware about Methi-laddu/ Methipak and Batrisu vasanu. Participants were classified in three groups of Methi-laddu users (Exposed group 1), Batrisu vasanu users (Exposed group 2) and non-users (Unexposed). The participants were interviewed for twice during the study, once at the time of enrolment and once at their postpartum visit. Throughout the period clinical details were obtained from clinic at antenatal visit, hospitalization during delivery and at postpartum visit. The health outcomes were recorded using a clinical proforma. During the study, no intervention was done, but outcomes were observed among the cohorts. And hence, we call the study design as Prospective cohort study more appropriately. The questionnaire used at antenatal (annexture 4) and postpartum (annexture 5) along with clinical proforma (annexture 6) are included in this document as annextures.

4.5.2 SAMPLE SIZE

The sampling was done as convenience sampling. Considering the limitations of the participants availability as the study requires follow-up, the limited number of representations was proposed as 50. For this objective, total 50 informant women were

interviewed and enrolled for the study. To begin with, 80 participants were enrolled at the beginning of the study, considering the dropouts and exclusion criteria. However, only N=38 participants remained at the end of study and were analyzed for the outcome. The cohorts were made as non-users N=14 (Unexposed), Methi-laddu users N=7 (Exposed group 1) and Batrisu vasanu users N=17 (Exposed group 2).

4.5.3 INCLUSION AND EXCLUSION CRITERIA

Healthy women of any age between 20 to 35 years, who reported to the maternity clinic in last trimester were asked whether they are aware about methi-laddu or Batrisu vasanu or ethnobotanical traditions. Women who planned using Methi-laddu or Batrisu vasanu postpartum were recruited for the study. Non-users were ensured through interview, not taking any postpartum herbal drugs. Any religion, and of any socio-economic status mothers were included for the study.

However, participants with history of any major systemic diseases or major surgeries or physical or mental disability during their gestation period, and high-risk pregnancies were excluded from the study. Mothers giving still birth, IUGR and pre-term birth were excluded from the further follow-up.

4.5.4 CLINICAL DATA COLLECTION

Participants were selected and enrolled for interviews conveniently. In brief, the structured questionnaire and clinical proforma was used for data collection. The questionnaire & proforma was in English language as it was Interviewer Administered. The participants were interviewed once, and face-to-face, for about 5 minutes at the time of enrollment. Participants were asked primary questions and enrolled to the study if they commit using Methi-laddu or Batrisu vasanu or none. In initial questionnaire, socio-demographic details including age, religion, birthplace, marital status, size of the family, educational qualification, occupation, income, smoking and alcohol status, diet, parity, and month of pregnancy were noted. Their knowledge, attitude, and usage of any traditional practices during gestation period were also noted.

Clinical proforma about the participants current examination were filled in assistance with health practitioner. The details of appetite, bowel, bladder, physical activity, build, nutrition, height, weight, edema, fetal growth, breast status, pelvimetry etc. were noted. During parturition, the patient clinical status was obtained again from health practitioner about modes of delivery, intrapartum blood loss, APGAR score of child and birth weight. During postnatal visit, vitals of the mother and child were recorded. It includes colostrum, appetite, bowel, bladder, edema, episiotomy stitches, breast examination, galactagogue & hematinic supplements, lactation inhibition etc. For newborn, weight, urination, stool passing, and health complaints were registered in clinical details.

During postnatal visit, the participants were again asked for whether they have used or are using methi-laddu or batrisu vasanu or none to ensure the study sample. The recipe contents, dosage and period of intake was noted.

4.5.5 DATA ANALYSIS

Data recorded in the questionnaire were entered on a Microsoft Excel (Windows Office 365) spreadsheet. Data were treated as cohorts namely control group, Exposed group 1, and Exposed group 2. Control or Unexposed group (N=14) here is clinically a no-treatment concurrent control. Exposed group 1 (N=7) is the participants taking Methi-laddu postpartum and Exposed group 2 (N=17) is participants taking batrisu vasanu postpartum.

The questionnaire with socio-demographic details, and knowledge and attitude towards herbal supplements were analyzed for descriptive statistics using Median, range, frequency, and percentage (Al-Ghamdi et al., 2017). Quantitative data were analyzed using R Studio (R Core Team, 2022). The weight velocity (g/day) and growth velocity (g/kg/day) were computed for newborn. To compare among the groups, non-parametric test Kruskal-Wallis test was performed. Contingency tables were tested using chi-square test for checking odds of the event, with 95% confidence interval (CI). Further, the odds ratio for variables with ≤ 0.05 p-value were chosen to build models for logistic regression analysis. Using multiple logistic regression models, factors associated with usage of traditional practices were tested (de Wet & Ngubane, 2014; Jayawardana et al., 2017; Sperandei, 2014).

4.6 HERBAL INGREDIENT ANALYSIS OF BATRISU VASANU PRODUCTS

Batrisu vasanu, a popular traditional practice among postpartum women was found to be less explored. The marketed products were analyzed for its constituent ingredients.

4.6.1 SELECTION OF A TRADITIONAL PRACTICE – BATRISU VASANU

Batrisu vasanu is a popular polyherbal galactagogue and traditional supplementary food in Gujarat state, India (Charola et al., 2021). However, its herbal formulation is not reported in any authentic literature. For the objective of identifying variations among the marketed Batrisu vasanu samples, the marketed products were studied.

4.6.2 COLLECTION OF SAMPLES

Purposively seven districts of Gujarat state were selected and ten crude herbs/ condiment shops among each district were randomly visited. Each shop was asked about marketed product Batrisu vasanu or Kaltu. The samples were collected if the product was having on-pack information regarding herbal ingredients. Loose products without any ingredients mentioned, were excluded.

4.6.3 ANALYSIS OF INGREDIENTS FROM ON-PACK INFO

The herbs mentioned on pack were noted on Microsoft Excel spreadsheet. The names of plants mentioned were either common name or vernacular (Gujarati language) names. The details of the plants were analyzed using volumes I to IX of Ayurvedic Pharmacopoeia of India (API) – Part I (Government of India, 2016). The data was analyzed using R Studio (R Core Team, 2022). Results were expressed as frequency, percentage and mean±SD. Relative frequency of citation (RFC^b) for each ingredient was calculated as per following equation (Kankara et al., 2015).

$$Relative\ Frequency\ of\ Citation\ (RFC^b) = \frac{F_h}{N}$$

Where, F_h= Frequency of ingredient herb in samples; N= Total number of samples

To analyze the variability of ingredient herbs in each sample, Jaccard similarity coefficient for each sample pairs and mean Jaccard coefficient for all samples were calculated. Using Food pairing principle (Ahn et al., 2011), mean number of herbs shared (N_h) among all samples were calculated as mentioned below.

$$N_h = \frac{2}{n(n-1)} \sum_{i \neq j} |H_i \cap H_j|$$

For n number of samples, each sample *i* and *j* has *H* set of herbs. Statistically, if no sample pairs share any herbs, then N_h would be zero (Charola et al., 2021).

4.7 PHYTOCHEMICAL SCREENING OF BATRISU VASANU SAMPLES

The phytochemicals namely total phenols, total flavonoids, total flavonols and antioxidant property assays were performed.

4.7.1 SAMPLE PREPARATION

Collected Batrisu vasanu samples were powdered and 10% (w/v) methanolic extract was prepared (Kumar et al., 2011). 10 g samples were added to 100 ml methanol and were occasionally shaken for 24 hours. Then samples were filtered using Whatman Filter paper No. 1. The filtrates were then concentrated using Rotary evaporator. The extracts were allowed to air dry and then stored at 4 deg C till further analysis. For all biochemical analysis extracts of concentration 1 mg/ml was used, unless specified.

4.7.2 BIOCHEMICAL ESTIMATIONS FOR TOTAL PHENOLS

Total phenols were determined using Gallic acid as standard and was expressed as Gallic acid equivalent (GAE) (McDonald et al., 2001). Gallic acid stock was prepared at 100 μg/ml and aliquoted in a test tube as 0.1 to 1.0ml. To each test tube, 2.5 ml Folin-Ciocalteu reagent (1:1 dilution with water) and 2 ml of 7.5% sodium carbonate was added. The absorbance was

measured at 765nm in visible spectrophotometer after 30 min incubation. All samples were tested similarly with 0.5 ml aliquots of extract stock.

4.7.3 ESTIMATION OF TOTAL FLAVONOIDS

Total Flavonoids were determined using Aluminum chloride colorimetric test with Rutin as standard (Chang et al., 2020). The data are expressed as Rutin equivalent (RE). From working solution of 0.1 mg/ml Rutin, 0.1 to 0.8 ml aliquots were made in test tubes. Each test tube was added with 2.3 ml methanol and then 0.1 ml 10% Aluminum chloride, 0.1 ml of 1 M Potassium acetate and 2.8 ml distilled water. The reaction mixture was read at 415 nm in visible spectrophotometer after 30 min incubation. Samples were tested similarly with 0.5 ml aliquots of extracts.

4.7.4 ESTIMATION OF TOTAL FLAVONOLS

Total flavonols were determined using Rutin as standard (Kumaran & Joel Karunakaran, 2007). Aliquots of 0.5 to 2.0 ml of 1 mg/ml Rutin and the volume was made upto 2 ml using methanol. To each test tube, 2.0 ml of $AlCl_3$ (2% in ethanol) and 3 ml of sodium acetate (50 g/l) was added. The reaction mixture was allowed to incubate in dark for 2.5 hours. Procedure was repeated with sample extracts as 0.5 ml aliquots. Absorbance was read at 440 nm using visible spectrophotometer.

4.7.5 DPPH RADICAL SCAVENGING ASSAY

For testing DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay (Blois, 1958), 0.135 mM DPPH solution in methanol, and standard 55 μM Ascorbic acid was prepared with distilled water. Different aliquots of ascorbic acid as well as all the samples ranging from 0.1 to 1.0 ml were made and 1 ml of 0.135 mM DPPH was added to it. After mixing thoroughly, reaction mixture was allowed to incubate in dark for 30 minutes. The reaction mixture was read at 517 nm in Visible spectrophotometer.

DPPH radical scavenging activity percentage was calculated using following formula.

$$\%\ Radical\ scavanging\ activity = \frac{(Absorbance\ (Control)\ -\ Absorbance\ (Test))}{Absorbance\ (Control)}\ X\ 100\ \%$$

Further, IC_{50} value (concentration of sample where 50% of DPPH radical are scavenged) was determined from the graph plotted for different concentrations of sample and Ascorbic acid standard against % Radical scavenging activity (Kumar et al., 2011).

4.7.6 FERRIC ION REDUCING ANTIOXIDANT POTENTIAL (FRAP) ASSAY

For FRAP assay, FRAP solution was prepared by mixing three solutions A, B and C. Solution A was prepared by taking 1.55 g of sodium acetate and 8 ml glacial acetic acid to 500 ml distilled water. Solution B was prepared by adding 1.23 mg of TPTZ to 10 ml of 40 mM HCl solution. Solution C was prepared as 20 mM $FeCl_3$ in distilled water. A mixture of 100 ml of solution A, 10ml solution B and 10 ml solution C was made, and after 10 minutes incubation at room temperature, FRAP reagent was ready to use. Make aliquots of 0.5 mM FeSO4 solution as 0.1 to 0.3 ml as standard and add 5.7 ml of FRAP reagent. Repeat the procedure with 0.3 ml extracts for all samples. After 30 minutes of incubation in dark, the absorbance was measured at 593 nm in visible spectrophotometer. Express the results as μM Fe (II) per gram of extract (Benzie & Strain, 1996).

4.7.7 TOTAL ANTIOXIDANT CAPACITY ASSAY

Total antioxidant capacity was tested in samples on the basis of its capacity to reduce Mo (VI) to Mo (V) at acidic pH (Prieto et al., 1999). Mo reagent was prepared by mixing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate to a final volume of 500 ml. 0.1 mg / ml Ascorbic acid solution was taken as standard. Aliquots of 0.05 ml to 0.3 ml for Ascorbic acid were added with 3 ml of Mo reagent. After 1.5 hours of incubation at 95 deg C in boiling water bath, samples were cooled down and read at 695 nm in visible spectrophotometer. Samples were also taken as 0.3 ml aliquots and antioxidant capacity was expressed as mg Ascorbic acid equivalent (AAE) / g of extract.

4.7 ANIMAL STUDIES FOR BATRISU VASANU

The Batrisu vasanu sample was tested in female rat model for validation of its efficacy and safety in humans.

4.7.1 ANIMAL ETHICS

The study protocol and number of animals to be used was duly passed through Institutional Animal Ethics Committee Wide No. MSU-Z/ IAEC04/ 16-2020 (revalidation), of The Maharaja Sayajirao University of Baroda (Annexture 7).

4.7.2 MAINTENANCE OF THE ANIMALS

Nulliparous, 12 Wistar female rats ranging from 180 to 220 g weight were housed in Animal house facility, Department of Zoology, The Maharaja Sayajirao University of Baroda as per guidelines of animal ethics. The animals were housed in a well-ventilated environment, provided with 12-hour light and 12-hour dark (12:12 L:D) cycle and ambient temperature. The animals were allowed for one week of acquaintance before intervention and were provided food and water access ad libitum.

4.7.3 DOSAGE AND TREATMENT

Four animals each were then randomly assigned to three groups – Control group, Treatment group 1 and Treatment group 2. Batrisu vasanu was powdered and was added to standard chow diet, 1 g per day human equivalent for Treated 1 and 10 g per day human equivalent for Treated 2 (Tsoukalas et al., 2021). The chow diet was powdered and mixed with batrisu vasanu, and then was dried as pellets. Animals were provided with 200 g food pellets of respective treatments every day. Daily leftover food pellets were weighed and topped up again to 200 g. Water was also filled in bottle as 200 ml per day and every day intake was measured by counting leftover. Every third day the animals were weighed for body weight and their food intake, water intake was noted down group-wise. The treatments were conducted for three weeks. Fecal material from the cages were collected intermittently and weighed.

4.7.4 HEMATOLOGICAL AND SEROLOGICAL TESTING

At the end of the third week, the rats were humanly sacrificed, and blood was collected by heart puncture. The blood was collected in a plain tube, EDTA tube, and sodium fluoride for serology, hematology, and glucose test. The collected samples were processed to derive CBC parameters based on the counting and sizing, in combination with an automatic diluting and mixing device for sample processing and subjected to an automated haematology analyzer (Siemen's ADVIA). The results obtained included a red blood cell (RBC) count, white blood cell (WBC) count, platelet count, hemoglobin concentration, hematocrit, RBC indices, and a leukocyte differential count.

Hormone profiling was done for follicle-stimulating hormone (FSH), prolactin, progesterone, estradiol, and serum luteinizing hormone (LH), T3 and T4 were done by CLIA method (Ganga et al., 2016). The hormone profiles were assayed by CLIA test kits based on the principle of sandwich-CLIA.

i. The micro sandwich-CLIA plates provided in kits were pre-coated with rat-specific primary antibodies. Individual coated plates for anti-follicle stimulating hormone, anti-luteinizing hormone, anti-prolactin, anti-progesterone, anti-estradiol, anti-T3, and anti-T4 were used for assaying the hormone levels.
ii. 50 µl standard or sample was added to the appropriate micro CLIA plate wells, respectively. Then volume makeup was done to 100 µl using phosphate buffer saline.
iii. The reagents were mixed well using micro-stirrers and incubated for 2 hours at room temperature to allow proper antigen-antibody interaction.
iv. The mixture was then subjected to wash with PBS.
v. The next step was the addition of 50 µl biotinylated secondary antibody specific for rat follicle-stimulating hormone, luteinizing hormone, prolactin, progesterone, estradiol, T3, and T4 was added to each well, followed by incubation for 2 hours at room temperature.
vi. The mixture was then subjected to wash with PBS.

vii. Lastly, horseradish peroxidase (HRP) conjugate is added to each microplate well successively and incubated for 1 hour at room temperature.

viii. The free components were washed off using PBS.

ix. Then, 50 µl substrate solution was added to each well.

Only those wells that had an interaction amongst primary antibody (rat follicle-stimulating hormone, luteinizing hormone, prolactin, progesterone, estradiol, T3 and T4, respectively), biotinylated secondary antibody, and HRP conjugate relayed fluorescence. The fluorescence was measured in the relative light unit (RLU) value spectrophotometrically by the chemiluminescence immunoassay analyzer (Norman S7, China). The RLU value was associated with the concentration of rat follicle-stimulating hormone, luteinizing hormone, prolactin, progesterone, estradiol, T3, and T4. Finally, the concentration of rat serum hormone levels was calculated in the samples by comparing the RLU value of the samples to the standard curve and determining the hormone levels in ng/ml.

Estimations of blood glucose, uric acid, triglycerides, albumin, and globulin were assayed using Reckon diagnostics kits as per the manufacturer's protocol (Reckon diagnostics pvt ltd, India). Creatinine, ALT, and AST were analyzed using coral diagnostic kits (Erba pharmaceuticals, India).

I. For blood glucose estimation, end point enzyme assay was used where 10 µl standard or sample respectively was added to 1 ml working reagent. Volume makeup to 1.5ml was done using sterile distilled water. The reagents were mixed well and incubated at room temperature for 5 mins. Then, the test solution was subjected to spectrophotometric analysis at 505 nm.

II. For estimation of triglycerides, end point enzyme assay was used where 20 µl standard or sample respectively was added to 1 ml working reagent. Volume makeup to 1.5ml was done using sterile distilled water. The reagents were mixed well and incubated at room temperature for 5 mins. Then, the test solution was subjected to spectrophotometric analysis at 520 nm.

III. For estimation of uric acid, end point enzyme assay was used where 20 µl standard or sample respectively was added to 1 ml working reagent. Volume makeup to 1.5ml was done using sterile distilled water. The reagents were mixed well and

incubated at room temperature for 5 mins. Then, the test solution was subjected to spectrophotometric analysis at 520 nm.

IV. For estimation of albumin, end point enzyme assay was used where 10 µl standard or sample respectively was added to 1 ml working reagent. Volume makeup to 1.5ml was done using sterile distilled water. The reagents were mixed well and incubated at room temperature for 10 mins. Then, the test solution was subjected to spectrophotometric analysis at 630 nm.

V. For estimation of globulin, end point enzyme assay was used where 30 µl standard or sample respectively was added to 1 ml working reagent. Volume makeup to 1.5ml was done using sterile distilled water. The reagents were mixed well and incubated at room temperature for 10 mins. Then, the test solution was subjected to spectrophotometric analysis at 630 nm.

VI. For estimation of creatinine, kinetic enzyme assay was used where 10 µl standard or sample respectively was added to 1 ml working reagent. Volume makeup to 1.5ml was done using sterile distilled water. The reagents were mixed well and incubated at room temperature. Then, the test solution was subjected to spectrophotometric analysis at 510 nm. first at 10 seconds after incubation, followed by a second reading at 80 seconds after incubation. The ratio of the product formed at the end of these time points was considered for determining the creatinine levels.

VII. For estimation of ALT, kinetic enzyme assay was used where 10 µl standard or sample respectively was added to 1 ml working reagent. Volume makeup to 1.5ml was done using sterile distilled water. The reagents were mixed well and incubated at room temperature for 60 seconds. Then, the test solution was subjected to spectrophotometric analysis at 340 nm. after every 30 seconds. This relay was followed till 2 mins. The ratio of the product formed at the end of these time points was considered for determining the ALT levels.

VIII. For estimation of AST, kinetic enzyme assay was used where 10 µl standard or sample respectively was added to 1 ml working reagent. Volume makeup to 1.5ml was done using sterile distilled water. The reagents were mixed well and incubated at room temperature for 60 seconds. Then, the test solution was subjected to spectrophotometric analysis at 340 nm. after every 30 seconds. This relay was

followed till 2 mins. The ratio of the product formed at the end of these time points was considered for determining the AST levels.

4.7.5 STATISTICAL ANALYSIS

For botanical assessment of the Batrisu vasanu samples collected, RFC^b values, N_h and Jaccard similarity coefficient were calculated. Mean Jaccard coefficient was also calculated. A hierarchical cluster analysis was performed for preparing a cluster dendrogram using binary distance of ingredient herbs in each sample.

The biochemical analysis performed like total Phenolic content, total flavonoids and flavonols were presented as Mean$\pm$SD for each sample. The ANOVA (Tukey's test) was performed to determine the statistical differences among the samples.

For animal studies, the data are mentioned as Mean$\pm$SEM for each group. The hematological and serological data are analyzed using t-test, one-way ANOVA, and multiple comparisons by Tukey's test. The p-value of ≤ 0.05 was considered significant.