CHAPTER III

MATERIALS AND METHODS

3.1 Study area:

Sonitpur district is situated in the Brahmaputra valley of upper Assam in North-East India. Sonitpur district is located between 26°21’ - 26°93’ N and 91°71’-92°37’ E. Sonitpur district is bounded by Arunachal Pradesh on the North, Lakhimpur district on the East and Darrang district on the West, Brahmaputra river on the South. Assamese, Bodo, Mishing, Nepalese, Deuri, Karbi etc. are the major communities of Sonitpur district, Assam.

3.2 The people:

Demographic Pattern

The major communities inhabiting the District are:

The Assamese

The majority people inhabiting the District are Assamese. They are among the original inhabitants of the place, and the typical culture of the place grew with them.

Language: Assamese.

Religion: The majority of Assamese people follow Hindu religions, a significant portion of them are also Muslims. A few of them are also adherents of Sikhism, Christianity, and Buddhism.

Caste: Prominent castes among the Assamese include Brahmans, Kalitas, Baishya, Koch, Ahoms, Yogi, the Scheduled Castes, etc.

The Nepalis

The Nepali community is fairly dominant in the central and southern part of the District, especially in the Biswanath Chariali and Gohpur sub-divisions.
Chapter III. Materials and methods

Language: They speak Nepali, a language of the Indo-Aryan family. Of course, they use Assamese as a lingua franca.

Religion: While a majority of them follow Hindu religion, a significant portion of them, especially the tribes, are Buddhists.

The Bengalis

The Bengali community came from erstwhile undivided Bengal, as officials and clerks of the British administration and the Tea Industry; and stayed back. Later, on account of the partition of India, people coming as refugees added significantly to the community.

Language: They speak Bengali, a language of the Indo-Aryan family. Of course, they use Assamese as a lingua franca.

Religion: They follow the Hindu religion.

The Adivasis (Tea-Tribes)

The Adivasi people were brought by the British from Chotanagpur area and Orissa to serve as labourers in the Tea Gardens. In course of time, they assimilated themselves in the greater Assamese society, while retaining their basic cultural traits. The Jhumur dance is their contribution to the culture of Assam.

Language: They have adopted Assamese as their language, but have retained the language of their forefathers.

Religion: They are Hindus and Christians.

Muslims

The district has a considerable Muslim population. Majority of Muslims have emigrated from former East Bengal (present Bangladesh). They fill up a large portion of riverine areas of the district.

Language: Over the years they have adopted Assamese language.
Tribes:

Mishings

The Mishing people live in a scattered manner in the northern part of the Naduar and Biswanath Circles, i.e. east of the Bhorali River; and form a significant portion of the population of Gohpur sub-division.

Language: They generally speak the Mishing language, belonging to the Sino-Tibetan family. But a lot of them also speak the Assamese language as a lingua franca.

Religion: A majority of them follows the Hindu religion along with its variant forms. They have their own distinct culture, but many of them have also adopted Assamese ways.

The Bodos

The Bodo dominated areas include the northern part of Dhekiajuli, Chariduar, Naduar, Helem and Gohpur (Sadar) Circles.

Language: They generally speak the Bodo language, belonging to the Sino-Tibetan family. But the Assamese language is the lingua franca for them.

Religion: A majority of them follows Hindu religion along with its variant forms, a certain portion of them follow Christianity. The Bodos perform Bathow puja. They have their own distinct culture, but many of them have also adopted Assamese ways.

Other Tribes

Other major tribes of the District include the Rabhas, Mechs, Nyishis, Garos, Adis, Apatanis, Lamas etc. Their population is sparse, and mainly confined to the foothills of the Himalayas near Arunachal Pradesh.
Chapter III. Materials and methods

Language: They speak either their tribal language, or their variant of Assamese. Of course, they use Assamese as a lingua franca.

Religion: The Rabhas and Mechs follow Hindu religion; Lamas and Nyishis are Buddhists; Garos are Christians; Adis and Apatanis either follow their traditional form of religion

According to 1991 census on the basis of religion the Hindus are found 1141928, Muslim 189859, Christian 85427, Buddhist 3199, Jain 1512, Sikh 1046 and Other 777 (Fig 3) in amongst the total people of Sonitpur district.

Fig 3: Classification on basis of religion in Sonitpur district.

3.3 Survey and collection:

The survey work was carried out during the year 2006-2008 in different areas of Sonitpur district namely Tezpur, Balipara, Jamuguri, Biswanath Chariali, Gohpur and boarder areas of Arunachal Pradesh at four times at three months intervals. The field studies were accomplished according to the methodologies of Jain & Rao [1977].
Participatory interview tools including group discussions, informal consultation, questionnaire surveys and field observations were used for primary data collection. Questionnaire and personal interview with the old people, the herbalists and experts in the field were initiated for getting first hand information. In some informal discussion taken place with the villagers where women actively participated. Schedule surveys were designed separately for different respondents. Respondents were requested to furnish for each plant: vernacular names, folk use and mode of preparation. The ethnobotanical information was collected from the informers of various ethnic people of Sonitpur district with following methods proposed by Jain [1987]. The informer views are compared amongst the information collected from the different respondent for same plants and uses. Amongst the informer Mr. Petel Pegu of Rajabari from Mishing and Mr. Daniol Borgayari of Rajgarh near Sonapur from Bodo were provided enormous information. Further validity of all the information checked and documented by common response.

Voucher specimens were collected with field notes and codes. Most of the species were identified in field and the remaining was identified in Itanagar with comparing deposited specimens in Boatny department of Rajiv Gandhi University, Arunachal Pradesh. The identified voucher specimens had been deposited in the department of Botany, Chaiduar College, Gohpur, Assam.

Species for enumeration were selected based on data/information availability. The criteria for selection were information of medicinal uses, chemical constituents, phytochemical screening, observations from earlier studies and present study. Comparative analysis was made only for the selected plant species that contained all the requisite information of the ethnomedicinal, phytochemical findings, earlier
observations and present survey. The observations of the present survey were compared to earlier observations and common uses of the people of this district.

3.4 Assessment of Material other than folklore

The identified plant species were further undertaken for assessment of their medicinal values other than folklore uses. Medicinal properties of the plant species considered for the purpose were traditional use (Ayurvedic and Siddha) chemical constituents and biological activities of the plant extracts and constituents etc. These information were gathered based on review of medicinal plant literature. (Kirtikar and Basu, 1935; Chopra et al, 1956; Ambast, 1986; Hussain et al 1992) and journal and periodicals (Phytomedicine, Current Science, Ethnobotany, Indian J Pharmacol, Journal of Ethnopharmacology 1988-2008.)

3.5 Preliminary chemical investigation

Based on the resource of analgesic and anti-inflammatory properties of different Plants and mixture contains group of plants with specific formulation I selected this formulation for detail study. This specific plant mixture was examined for presence or absence of alkaloids, flavonoids, reducing sugar, tannins, saponins, anthraquinones, steroids and triterpenoids.

3.5.1 Experimental study

3.5.1.1 Collection and processing of plant samples

The collection of sample related with anti rheumatic formulation was cleaned with water and external moisture wiped out with tissue paper. The individual used plant parts were separated and dried in a hot air oven at 50 °C for 1 h. The dried samples were then powdered in a blender for further study. Some plant parts like leaves were dried under shade to prevent the decomposition of chemical compounds present in them (Makkar 2003; Orech et al 2007).
3.5.1.2 Preparation of testing reagent

The reagents were prepared following methods described by Kapoor et al (1971, 1975) and Herborne (1973). Details of preparations of reagent were as follows.

Dragendroff's reagent

8g 4BiNO₃(OH)₂ BiO(OH) (Bismuth subnitrite) was dissolved in 20 ml concentrated Nitric acid( solution A) and 27.2g Potassium iodide in 50 ml of distilled water( solution B). The two solutions were mixed and allowed to stand when KNO₃ precipitates out. The supernatant solution discarded and made up to 100ml with distilled water.

Mayer’s reagent

This reagent was prepared as described by Kapoor et al (1971). 5g of KI was dissolved in 10 ml of water. 1.358g of Mercury chloride (HgCl₂) was dissolved in 60ml of water. The two solutions were mixed and added water to make the mixture 100 ml.

Wagner's reagent (Iodo-potasium iodide).

Dissolve 2g of iodine and 6g of Potassium Iodide in 100ml of water.

3.5.1.3 Preparation of extracts:

The extract were prepared following the methods as described by Farnsworth (1966, 1988) and Wall et al (1954). Details about the extraction are as follows:

25g powdered material of each plant samples was extracted separately with 200 ml (95%) ethanol in soxhlet apparatus for 8hours. Another 25g powder material with mixed in equal amount of plant samples was also extracted with ethanol. About 10 ml of this extract was kept separately for
flavonoids test and the remaining portion of ethanol extract was evaporated to
dryness under reduced pressure on water bath. This residue was used for
alkaloid, reducing sugar, tannins, saponins, anthraquinones, steroids and
triterpenoids test.

On the other hand for animal experiment the dried plant parts are
mixed as (Table 4) formulation used for ant rheumatic pain inflammation were
then powdered in a blender. This powdered material was extracted with 250ml
of Ethanol (95%) (SD Fine Chemicals) in soxhlet apparatus for 6 hours. The
total extract was distilled under reduced pressure to remove the solvent. A
dark brownish semisolid mass obtained was stored and used for evaluation of
anti-inflammatory activity.

3.6 Testing of extracts

3.6.1 Test of alkaloids

3.6.1.1 1.2ml solution of extract was taken in a test tube; 0.2ml dilute hydrochloric
acid and 0.1ml of Mayer’s reagent were added. Formation of yellowish buff
colouration indicates the presence of alkaloids.

3.6.1.2 2ml solution of the extract was taken in a test tube, 0.2ml of dilute
hydrochloric acid and 0.1ml of Dragendorff’s reagent were added. Formation
of orange brown precipitated indicates the presence of alkaloids.

3.6.1.3 2 ml solution of the extract was taken in a test tube, 0.2ml of dilute
hydrochloric acid and 0.1ml of wagner’s reagent were added. Formation of
reddish brown precipitated indicated the presence of alkaloids.
3.6.2 **Test for Flavonoids**

3.6.2.1 1.5ml of extract solution was hydrolyzed with 10% v/v sulphuric acid and cooled. Then it was extracted with diethyl ether and divided into three portions in three separate test tubes. 1ml of dilute sodium carbonate, 1ml of 0.1 N sodium hydroxide and 1ml of dilute ammonia solution were added to the first, second and third test tubes respectively. In each tube the formation of yellow colour demonstrated the presence of flavonoids.

3.6.2.2 Shinoids test: The extract was dissolved in alcohol, to that one piece of magnesium followed by conc. Hydrochloric acid were added drop wise and heated. Appearance of magenta colour shows the presence of flavonoids.

3.6.3 **Test for steroids and triterpenoids:**

3.6.3.1 *Liebermann-Burchard test:* 10mg of extract was dissolved in 1ml of chloroform and 1ml of acetic anhydride was added followed by 2ml of concentrated sulphuric acid. Formation of reddish violet colour indicated the presence of steroids or triterpenoids.

3.6.3.2 *Salkowski test:* when concentrated sulphuric acid was added to a chloroform solution of the extract (10mg of extract in 1ml of chloroform), a reddish-blue colour was produced in the chloroform layer and green fluorescence in the acid layer suggested the presence of steroids.

3.6.3.3 *Noller test:* 5mg of the extract dissolved in 2ml of 0.01% anhydrous stanic chloride in pure thionyl chloride. A purple colour, formed, then changed to deep red after few minutes, indicated the presence of triterpenoids.
3.6.4 Test for reducing sugar:

3.6.4.1 5ml of extract solution, 5ml Benedict’s reagent were mixed in a test tube and heated for few minutes. Formation of brick red precipitate confirmed the presence of reducing sugar.

3.6.4.2 5ml of extract was boiled with 5ml of Fehling’s solutions in a test tube for five minutes. Development of brick red colour demonstrated the presence of reducing sugar.

3.6.5 Test for tannins

3.6.5.1 5ml of extract was treated with 1ml of 10% aqueous potassium dichromate solution. Development of yellowish brown precipitate demonstrated the presence of tannins.

3.6.5.2 5ml of the extract was allowed to react with 1ml 10% aqueous lead acetate solution and the yellow colour precipitate formation indicated the positive test for tannins.

3.6.5.3 5ml of the extract was allowed to react with 1ml 5% ferric chloride solution. Formation of greenish black precipitate indicated the positive test of tannins.

3.6.6 Test of saponin

3.6.6.1 1ml solution of the extract was diluted with distilled water to 20ml and shaken in a graduated cylinder for 15 minutes. Formation of stable foam suggested the presence of saponins.

3.6.6.2 1ml of the extract solution was treated with 1% lead acetate solution. Development of white precipitate indicated the presence of saponin.
3.6.7 **Test for anthraquinones:** 10ml of the extract was extracted with benzene. It was then filtered and made alkaline with ammonia, aqueous layer shown pink or violae colour indicates the positive test for anthraquinones.

3.7 **Quantitative estimation of Phytochemical by standard methods of Biochemical analysis:**

This estimation based on the resource of analgesic and anti-inflammatory property containing few Plants individually which were used singly or in mixture of plants with specific formulation. I was selected some common vegetables which were recorded to have with analgesic and anti-inflammatory properties and one formulation of seven plants for detail study.

3.7.1 **Estimate of moisture content:** The moisture of the sample represents the richest constituent of the total chemical composition. It is also expressed as percentage and determined by AOAC methods. (AOAC, 1990). Here samples are weighed and placed in covered dishes in an oven (105°C) for overnight. Removing from dish and after cooling the sample again weighed for the difference.

\[
\text{Moisture content (\%) = } \frac{\text{Weight of fresh samples} - \text{weight of dry samples}}{\text{Weight of fresh sample}} \times 100
\]

3.7.2 **Estimate of Ash content:** The sample is ignited at 620°C to burn off by placing it to silica crucible in Muffle furnace for 3 hour to determine the ash content (AOAC 1990). The ash is used for determination of mineral contents in the sample.

\[
\text{Ash content (\%) = } \frac{\text{Weight of Ash}}{\text{Weight of sample}} \times 100
\]
3.7.3. **Estimate of Total soluble solid content:** Total soluble solid of the plant samples were determined by the following method: 5 g of oven dried plant sample was refluxed with 400 mL water for 1 h. The extract was filtered and made up to 500 mL. One hundred mL of this extract was pipetted into a glass basin containing about 20 g of purified sand. The contents of the basin were evaporated at first on a steam bath and finally in an oven at 100 °C. Drying in the oven was continued until the weight of the basin remained constant (Raghuramulu et al, 1983).

3.7.4. **Estimate of Carbohydrate content:** The amount of carbohydrate can be estimated using anthrone method (Thimmaiah, 1999). Weighted 100 mg sample into boiling tube and hydrolyze by keeping it in a boiling water bath for 3 hours with 5 ml of 2.5N HCl and cool to room temperature. It is then neutralized with sodium carbonate. Make up the volume to 100 ml and centrifuged it. The supernatant is the collected and take 0.5 and 1 ml aliquote for analysis. Prepare the standards and taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. ‘0’ serves as blank. Making the volume to 1 ml in all the tubes by adding distilled water. Here added 4 ml of anthrone reagent and heated for 8 min in a boiling water bath. Cool rapidly and read the green to dark green colour at 630 nm and absorbance putting on Y- axis where standard on X- axis on graph.

**Calculation:**

\[
\text{Amount of carbohydrate} = \frac{\text{Sugar value from graph (mg)}}{\text{Aliquot sample used (0.5 ml)}} \times \frac{\text{Total vol. of extract (ml)}}{\text{Wt. of sample (mg)}}
\]
3.7.5. **Estimate of Crude protein content**: The Protein is the major sources of Nitrogen. In most proteins, nitrogen constitutes nearly 16% of the total composition and hence, the total nitrogen content of the sample is multiplied by 6.25 to calculate the crude protein content (Raghuramulu et al, 1983).

About 0.5g of powder sample has to be grinded with water in a pestle and mortar. It is then centrifuged and supernatant used for protein estimation. From the stock standard (bovine serum albumin) solution pipetted out 0.2, 0.4, 0.6, 0.8 and 1ml working standard solution into series of test tube. From the sample supernatant has to pipette out 0.1ml and 0.2ml in two other test tube and with one blank test tube where volume makes up 1ml with water. 5ml of alkaline copper solution has to be added and 0.5ml folin-ciocalteu reagents to be added in each tube. After 30min incubation in dark the absorbance of all tube has to be taken at 660nm against the blank. From the standard curve drawing with result from the absorbance protein can be calculated as mg/ 100g sample.

3.7.6. **Estimate of Crude fat**: The dried sample weighing 2-3g in a thrimble and placed it in the Soxhlet apparatus which have to connect with pre-weighed dry solvent flask (‘a’ g). Solvent petroleum ether has to be added in required volume and connected to condenser. Condensation of the solvent by adjusting heating rate of 2-3 drops is done for 16 hours. Remove the thrimble and evaporate the excess of P. ether by heating in water bath. Cool the flask in a desiccatior and weigh(‘b’g) (Sadasivam & Manickam, 1992)

**Calculation:**

\[
\text{Crude fat content in sample (\% dry wt basis)} = \frac{(b-a) \times 100}{\text{Wt. of sample (g)}}
\]
3.7.7. **Estimate of Crude fiber content:** The crude fiber consists largely of cellulose and lignin and some nutritional matter.

Extract 2g of sample with petroleum ether has to boil in low temperature to remove fat. 2g of dried sample again to boil with H₂SO₄ for 30 min. Then filter it through muslin cloth and wash with boiling water to free of acid. The residue is then boiling with 200ml of NaOH for 30 min. Filter through muslin cloth again and wash with H₂SO₄. Remove the residue to pre-weighed ashing dish (\(W₁\), g). Dry the residue for 2h at 130 \(±\) 2⁰C, cool in a desicator and weigh (\(W₂\), g). Ignite for 30 min at 600 \(±\) 15⁰C. Then cooled in a desiccator and reweigh (\(W₃\) g) (Sadasivam and Manikam 1992).

Calculation: Loss in weight X (\(W₂ - W₁\)) – (\(W₃ - W₁\))

\[
\text{% Crude fiber content} = \frac{\text{On ignition}}{\text{Weight of sample}} \times 100
\]

3.7.8. **Estimate of Vitamin C content:** The estimation ascorbic acid or vitamin C from a sample as follows.

Grind 0.5 - 5g sample using pestle and mortar in 25- 50ml 4% oxalic acid solution. It is then centrifuged and collected the liquid in 10ml where to add bromine water to remove enolic hydrogen atoms in ascorbic acid. Here 4% oxalic acid volume has to make up to 25 or 50ml and convert 10ml stock ascorbic acid solution into dehydro form by bromination.

**Estimation:** Standard dehydro-ascorbic solution has to be arranging by pipetting out 10-100\(\mu\)g solution and arranged different aliquots (0.1ml – 2ml) brominated sample solution. Making volume 3ml in each tube by distilled water 1ml DNPH reagent has to be added to each tube where one tube should only have water as blank. After incubation at 37⁰C for 3 hours 7ml of 80%
H2SO4 has to be added to each tube and absorbance is measured at 540nm for plotting result in a graph. From the graph can be calculated the ascorbic acid content (Sadaasivam and Manikam 1992).

3.7.9. **Estimate of Total Phenolics content:** The powdered sample exactly 0.5g to 1g has to mix with 10 time volume of 80 % ethanol. It has been centrifuged and separated the supernatant. The supernatant has to be dried and dissolved in 5ml distilled water. This solution has to be pipetted out (0.2 to 2ml) into test tubes with equalized volume of 3ml each with water where 0.5ml of Folin-ciocalteu reagent to be added. Then has to add 2ml of 20% Na₂CO₃ solution and placed in water bath for one min. The absorbance has to be measured after cooling at 650 nm against reagent blank with catechol standard (Malik & Singh, 1980).

**Calculation:** From the standard curve the concentration of phenols in test sample has to be calculated as mg phenols/100g material.

3.7.10. **Estimate of Total alkaloids content:** The Extraction and estimation of Total alkaloid from the plant samples as follows.

Leaf powder 5g is extracted twice with 90 % ethanol at temperature 26°C for 2 hours and kept overnight in 30ml 90 % ethanol. The ethanolic extract is filtered and concentrated in vacuum at 40°C to 10ml, diluted with water (10ml) and then acidified with 3% hydrochloric acid (10ml). The acidic aqueous layer is then extracted twice with hexane (2 X 30ml). The hexane layer is discarded and aqueous layer then basified to pH 8.5. It is then extracted with chloroform and washed with water. It is then dried over anhydrous sodium sulfate and then evaporated to dryness. The residue
obtained from different leaf samples are dried to constant weight to get % of alkaloid content (Baruah and Borah 1998).

3.7.11. **Estimate of Total flavonoids content**: The Flavonoids, one of the main groups of phenolic compounds in all plants which are chemically divided into subgroups of flavones, flavanones, flavonols.

Flavones and flavonols are expressed as quercetin equivalent. Quercetin (Sigma, Germany) was used to make the calibration curve standard solutions of 12.5, 25.0, 50.0, 80.0 and 100.0 \( \mu \text{g mL}^{-1} \) in 80% ethanol \( (V/V) \). The standard solutions (0.5 mL) were mixed with 1.5 mL 95% ethanol \( (V/V) \), 0.1 mL 10% aluminum chloride \( (m/V) \), 0.1 mL of 1 mol L\(^{-1}\) potassium acetate and 2.8 mL water. The volume of 10% \( (m/V) \) aluminum chloride was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. The coefficient of determination was \( r^2 = 0.998 \) (Christ and Muller, 1960).

Flavanones have to be expressed as (±) naringenin equivalent. (±)naringenin (Sigma, Germany) was used to make the calibration curve (standard solution of 0.25, 0.30, 0.50, 1.00 and 2.00 mg mL\(^{-1}\) in methanol). One mL of standard solution was separately mixed with 2 mL of 1% 2, 4-dinitrophenylhydrazine \( (m/V) \) and 2 mL of methanol at 50 °C for 50 min. After cooling at room temperature, the solution was mixed with 5 mL of 1% potassium hydroxide \( (m/V) \) in 70% ethanol \( (V/V) \). Then, 1 mL of the mixture was taken and centrifuged at 1000xg for 10 min and the supernatant was filtered through Whatman No. 1 filter paper. The filtrate was adjusted to 25
mL. The absorbance of the filtrate was measured at 495 nm. The coefficient of
determination was $r^2 = 0.996$.

Estimation of flavonoids: flavones, flavonols + flavanones

3.7.12. **Estimate of Calcium content:** The prepared plant ash solution has to be
pipette out to 250ml beaker and acidified with 5-10 ml HCL. It is again
neutralized by diluted ammonia (1:5). This solution again acidified with 10ml
dilute HCL (1:20) and 10ml of oxalic acid then boiled to 1-2min. After cool
down the solution has to be neutralized to pH 5.0 by adding sodium acetate
stranded for overnight. Then the solution has to be filtered by Whatman No.
44 filter paper and precipitate has to wash to free from chlorine. Again has to
be washed the calcium oxalate from filter paper to a beaker by dilute $H_2SO_4$
(1:5) and warm water alternatively to collection all oxalate to solution. This
solution has to be titrated with 0.1 N standard potassium permanganate
solutions to calculate calcium amount (Baruah and Borah 1998).

1ml of 0.1 N KMnO$_4$ = 0.002g Ca or 0.0028g CaO.

3.7.13. **Estimate of Magnesium content:** The prepared plant ash solution has to
be pipetted out to 250 ml beaker and acidified with 5-10ml HCL the
neutralized by adding ammonia solution. This solution again has to be
acidified by adding 10ml dilute HCL (1:20) and 10ml of oxalic acid. It is
again neutralized by adding sodium acetate to calcium oxalate precipitation.
This calcium oxalate has to be separated by Whatman filter paper. The filtrate
then has to be concentrated to about 100ml and acidified to add HCL. This
solution has to be boiled and to be added 10ml of sodium citrate, 10-15mo of
sodium phosphate and neutralized with ammonia. It is then to kept overnight
for filtration through Whatman filter paper and has to be washed with dilute
ammonia (1:50) to free chlorine. It is then ignited the filter paper and precipitated and weighed as \( \text{Mg}_2\text{P}_2\text{O}_7 \).

The weighed of \( \text{Mg}_2\text{P}_2\text{O}_7 \) has to be multiply by 0.2184 to obtain amount of magnesium. The result could be express as mg/100g of sample (Piper 1950).

3.7.14. **Estimate of Iron content:** The powdered material of known amount (3g) has to be taken in to a 300 ml Kjeldhal flask warm with 10ml HNO\(_3\) then to be added 2ml Conc. H\(_2\)SO\(_4\). In this solution Conc. HNO\(_3\) has to be added until evolved H\(_2\)SO\(_4\) fumes. Then here 10 ml Perchloric acid to be added with warming to straw colour. After cooling this 40ml of water to be added and filter it with Whatman No.1 paper then to make volume 200ml with water.

**Estimation:** Pipetted out 0, 1, 2, 3, 4 and 5ml of diluted iron solution used as standard where ‘0’ tube serve as the blank. Each tube has to be added 1ml of thioglycollic acid and 8ml of ammonium hydroxide solution and mixing well. After 10 min. reading has to be taken at 535 nm against the blank. With help of standard curve can be calculated iron as mg/100g of sample (Thimmaiah 1999).

3.7.15. **Estimate of Potassium content:** The prepared plant ash solution has to be pipetted out to 250 ml beaker and acidified with 5-10ml HCL the neutralized by adding ammonia solution. This solution again has to be added 1% phenolphthalein and 10% NaOH drop-wise till colour turn red and then to be added 2ml of 1N HCL. It is then to be evaporated to drying HCL and to extract K completely with addition 15ml of Potassium solvent. The 15ml of filtrate sample (containing about 0.5 to 20mg of K/10 ml) has to be cooled in an ice bath to 3\(^\circ\)C for 20 min. Here 10ml of test solution has to mix and after
Chapter III. Materials and methods

overnight it has to be filtrated. Then precipitate has to be collected in a glass crucible and to be washed for 3 times with 70 % ethanol. After drying the precipitate has to be dissolved in 3- 4 drops of 6 N HCL with little heat. It is then to be added hot water for completely dissolve. The solution has to be washed into the colorimeter tube with up to known volume. Here to be added 1.5 ml of KOH and 0.5ml of 3% H₂O₂ with few drops of 6 N HCL. Then to be added 15 ml of saturated KHCO₃ and water to a volume 40 ml. After few minute colour intensity has to be calculated at 620 nm against blank with standard curve of KCL solution (0, 3.75, 7.5, 15, 30, 45ml) of 2mgK/ml (Piper 1950).

3.7.16. **Estimate of Phosphorus content:** The powdered dry material has to be mixed with 3 times its weight of fusion mixture (1:1 of Na₂CO₃ and NaNO₃) and heated up-to ash formation. This ash has to be extracted with 10% TCA (trichloroacetic acid) and after filter has to make volume 200 ml with TCA.

**Estimation:** The TCA mixed solution has to pipetted out 2ml and volume to make 4.2ml with water. Again has to pipetted out 0, 0.2, 0.4, 0.6, 0.8 and 1.0ml of dilute standard phosphate solution to make volume 4.2ml where ‘0’ tube tested as blank. Here each tube has to mix with 0.6ml of acid molybdate and 0.2ml ANSA (A1-amino 2-Naphthol 4-Sulphonic Acid Reagent. After 10min % of Phosphorous present in sample can be calculated by absorbance 660nm against blank (Thimmaiah 1999).

3.7.17. **Estimate of Copper content:** The 2g or less dried powder sample with 5 times conc. HNO₃ has to dried and after cooling again to add 5ml ternary acid for each g. of tissue. It is then to be digested with H₂SO₄ to remove HCLO₄ and again to add 5ml HCL. After centrifugation volume has to be making
Chapter III. Materials and methods

25ml or 50 ml with 6N HCL decant the supernatant to 10ml volumetric centrifuged tube where to add 5ml of 25 % NH₄CL and neutralized by conc. NH₄OH. The solution has to dilute with 15ml with water and centrifuge at about 2000 rpm for 5min.Supernatant has to transfer into 25ml volumetric flask where to add 5ml of conc. NH₄OH. After dilution with 22ml of water and addition 1ml of 1% carbamate solution to be taken absorbance at 440nm which to have compared standard curve of copper solution to estimate copper (Thimmaiah 1999).

3.7.18. **Estimate of Manganese content:** The weighted oven dried powder sample has to be digested with H₂SO₄, HClO₄, and HNO₃ in a 300 ml Kjeldahl flask to dense brown fumes. Cooling after full digestion here to add 5ml of ammonium persulphate solution with digestion for 5min. 2ml of phosphoric acid and 35-50ml warm water here to add after cool. This solution has to filter by Whatman No. 44 filter paper and reduced the volume about 25ml. Here to add with 0.3g potassium periodate crystels and boil until permanganate colour appears. It is then diluted with water and has to boil in water bath after making volume 100ml maintained in volumatic flask. The absorbance has to be measured with 540nm with standard curve of standard manganese solution (Piper 1950).

3.7.19. **Estimate of Zinc content:** 1g weighted powdered sample with 10ml of HNO₃, 2ml of mixture equal volumes of H₂SO₄ and HClO₄ has to digested till the solution is yellow green. After adding 15ml water and 25ml of ammonium citrate buffer solution it is then boil and cool with pH 9.8. Here to add 5ml of chloroform to a 100ml separating funnel with about 10ml of water. In order to reduce concentration of ammonia in chloroform it is then washed
with 50ml of water and 50ml of 0.02N HCL and finally chloroform has to be discarded. The aqua’s solution mixed with 50ml of mixed reagent A (ammonia- ammonium citrate solution) with 10ml of dithizone-tetrachloride reagent. When tetrachloride phase is clear 5ml of carbon tetrachloride diluted with 25ml volumetric flask to take absorbance 535nm against blank. Here zinc can be calculated with comparing with standard curve of Zinc sulphate solution (Thimmaiah 1999).

3.8 Phenol estimation by HPLC method: Phenolics were extracted from 0.2gm finely powdered plant sample with 10 ml of 70 % methanol over 10 mins with intermittent shaking. 1ml of the extract was diluted to 5ml with stabilizing solution.

The stabilizing solution used for the dilution of the extract was prepared by dissolving 0.02g EDTA (Ethylenediaminetetraacetic acid) and 0.02g ascorbic acid in water in 100ml volumetric flask with the addition of 10ml acetonitrile.

10µL of the diluted extract was injected into a phenomenex Luna 5µm phenylhexyl column of dimensions 250mm X 4.6mm, fitted with a phenomenex guard column 4mmX 3.0mm phenylhexyl cartridge in Waters High Performance Liquid Chromatograph equipped to perform binary gradient elution methods (Wang et al., 2002, Zhu and Chen, 2004), with a thermostatically controlled column compartment and ultraviolet detector set a 278nm.

A Merck Hitachi HPLC (Darmstad, Germany) equipped with vacuum degasser, a quaternary pump programmable for making gradients, thermostatic controlled column chamber, rheodyne injection valve with a 20 mL sample
loop and diode array detector was employed for the study. All the modules were controlled by PC with interface and HPLC System Manager Window-based software. The gradient elution was at the rate of 1mL/min with the following programme. The initial 10min with 100% (A) and in next 15 min to 68 % (A) and 32 % (B) with another 10 min in this condition. The total elution time was 35 min. the mobile phases were as follows:

(A) 9% (v/v) acetonitril, 2% (v/v) acetic acid with 20 µg/mL EDTA.
(B) 80% (v/v) acetonitril, 2% (v/v) acetic acid with 20 µg/mL EDTA.

All standards viz. gallic acid, (+)-catechin, (-)-EC, (-)-EGC, (-)-ECG, (-)-EGCG, caffeine, were purchased from Sigma Chemical Co. Germany. Acetonitrile, methanol (both HPLC grade) and acetic acid were purchased from Merck India Ltd. All solvents were degassed and filtered through a 0.45 mm filter (Millipore filter No. HVLP 04700). Stock solutions of standards viz. five catechins, gallic acid and caffeine at 10,000 ng/mL, were prepared in 70% aqueous methanol and stored at –20_C till further use. The standard solutions were passed through 0.5 mm Millipore filter (FHLP 01300) before injecting into HPLC. Standard curves for all standards except theophylline were plotted by injecting 2–10,000 ng/mL of standard mixture and peak area responses were obtained. The peaks so obtained were identified and estimated by comparing with the standard.

3.9 Experimental animals: Albino rats 70-140g of Wistar strain purchased from M/S B.N. Ghosh & Co. Ltd., Kolkata and supplied through morning airlines to Dibrugarh were used for this investigation. Animals were kept under uniform husbandry conditions and natural light and temperature. Rats
were fed with routine diet (Bengal gram, corn) and water ad libitum. Rats were housed with both sex and weight in standard metal and plastic cages.

3.10 **Experimental design Carragenin induced rat paw edema:** 1% solution of carrageenin was prepared. 0.1 ml of this solution was injected into the right hind paw of male rats as per the procedure described by Winter et. al. (1962). Carboxymethyl cellulose (CMC) as vehicle at rate of 0.5% treated orally that animals served as control and acetylsalicylic acid (50 mg/kg, orally) was administered as standard drug.

3.11 **Administration of the crude drug:** The CMC and acetylsalicylic acid was administered orally 30 min prior to injection of carrageenin. The plants extract (150mg/kg and 300 mg/kg) the drugs were administered simultaneously with carrageenin injection. Mean increase in paw volume was measured and percentage inhibition was calculated.

3.12 **Monitoring the effect on animal:** The animal were properly weighted and marked neatly. The animals were monitored just before injecting carrageenin. The animals were monitored after 1 hr, 2hr, 3hr, 4hr and 24hr continuously. The increases of paw volume were measured plethysmometrically. The obtained values for anti-inflammatory activity were expressed as "mean increase in paw volume ± SEM". The significance of difference between means was determined by student's t-test values of p<0.05 were considered significant and p<0.01 as highly significant.