CHAPTER V

5. PHYSICOCHEMICAL, PHYTOCHEMICAL ANALYSIS AND IN VITRO ANTIDIABETIC STUDIES Of Myxopyrum serratum
A. W. Hill AND Nilgirianthus ciliatus Nees

5.1 INTRODUCTION

India has an ancient heritage of traditional medicines. An Indian traditional medicine is based on various systems including Ayurveda, Siddha, Unani and Homeopathy. The demand on plant based therapeutics shows an increasing trend in both developing and developed countries, since they are non-narcotic, easily biodegradable producing minimum environmental hazards with minimal adverse effects and also easily available at affordable prices (Ghosh A et al., 2008). Correct knowledge of such crude drugs is very important aspect in preparation, safety and efficacy of the herbal product. The process of standardization can be achieved by stepwise pharmacognostic studies (Niveditha devi et al., 2012). Determination of extractive values, ash residues and active components (saponin, alkaloids & essential oil content) plays a significant role for standardization of the indigenous crude drugs (Hima fazal et al., 2011). Nutritionally important mineral elements accumulate in the plants which are used as food supplements. Elements like Lead, cobalt, Chromium, Cadmium etc which accumulate in the plants and are harmful to human health when consumed (Lasisi A et al., 2005). Therefore, the world health organization (WHO) recommends that medicinal plants which form the raw materials for most herbal remedies, should be checked for the presence of heavy metals. Much scientific documentation are available on crude drug extracts, but in promoting these herbal drugs in international/national markets is difficult due to lack of reproducible biological reports, selection of wrong plants, lack of data on time, area of collection
and confusion in the identity of the source material. However lack of proper standards of medicinal plants result in the improper usage of plant drugs. So scientific validation for identification and standardization of plant drugs are essential especially for the traditional system of medicine.

5.2. MATERIAL AND METHODS

5.2.1. Collection of Plant materials

The plant specimens for the proposed study *Nilgirianthus ciliatus* Nees (NC) was collected from Malapuram district, Kerala and *Myxopyrum Serratulum* A.W.Hill (MS) was collected from Trivandrum district, Kerala and authenticated by Prof. P. Jayaraman, Director, Institute of Herbal Botany, Plant Anatomy Research Centre, Chennai.

5.2.2. Preparation of Plant Extracts

The ethanolic extract of *Nilgirianthus ciliatus* Nees and *Myxopyrum Serratulum* A.W.Hill was prepared by cold maceration method (Mukerjee PM 2005) (figure 5.1). 1000 g of the coarsely powdered plant sample was macerated with required quantity of ethanol for 72 h with occasional shaking. Ethanol is useful for preventing the microbial growth. After 72 h, the ethanolic extract was filtered through Whatman filter paper (No.1). The plant material was then macerated again with fresh ethanol for 48 h and 24 h respectively and the combined filtrate obtained from the first and the second maceration was then distilled under vacuum, the temperature of distillation being in the range of 40-50°C by means of rotary evaporator. After the distillation of 80% alcohol, the extract was then evaporated to dryness. The concentrated dried extracts were separately preserved in a desiccator;
percentage yield of the dried extracts was calculated with respect to the dried plant materials. The extracts were reconstituted with ethanol for further analysis.

**Figure 5.1: Flowchart for the preparation of ethanolic extracts of NC and MS**

The fresh whole plant of NC and MS were washed with distilled water

Dried under shade at room temperature

Coarsely powered by using mechanical device

Powdered plant materials of MS and NC were separately extracted by cold maceration method using ethanol

All the extracts were filtered through a cotton plug followed by whatmann filter paper (No 1) and then concentrated by using a rotary evaporator at low temperature (40-50°C) under reduced pressure

Extracts were preserved in air tight containers and kept at 4°C until further use

5.2.3. *Physico chemical Evaluation*

**Ash values**

The ash remaining following ignition of plant materials is determined by three different methods which measure total ash, acid insoluble ash and water soluble ash (WHO, 2007 & Wallis TE, 2011). The ash content of a crude drug is generally taken to be the residue remaining after incineration. It usually represents the inorganic salt naturally occurring in the drug and adhering to it, but it may also involve the
inorganic matter added for the purpose of adulteration. There is a considerable
difference which varies within narrow limits in the case of some individual drug.
Hence an ash determination furnishes a basis for judging the identity and cleanliness
of a drug and gives information related to its adulteration with inorganic matter. In
most cases the inorganic matter is present in small amount which are difficult to
remove in the purification process and which are not objectionable if only traces are
present. Ash values are helpful in determining the quality and purity of the crude drug
in powdered form.

**Total Ash**

The total ash method is designed to measure the total amount of material
remaining after ignition. This includes both “physiological ash”, which is derived
from the plant tissue itself, and “non-physiological” ash, which is the residue of the
extraneous matter (e.g. sand and soil) adhering to the plant surface.

Accurately weighed 3 g of the air-dried material in a silica crucible which
was previously ignited and cooled. The materials were spreaded in an even layer and
ignited in a muffle furnace by gradually increasing the heat to 500–600 °C until it is
white, indicating the absence of carbon. The ignition was repeated till a constant
weight was obtained. After complete ignition, it was cooled in a desiccator and
weighed. The percentage of total ash was calculated with reference to air-dried
material.

**Acid-insoluble ash**

Acid-insoluble ash is the residue obtained after boiling the total ash with
dilute hydrochloric acid and igniting the remaining insoluble matter. This measures
the amount of silica present, especially as sand and siliceous earth.

The ash obtained by the above method was boiled with 25 ml of 2N hydrochloric acid. Filtered the insoluble matter on an ashless filter-paper (Whatman No.42) and washed with hot water until the filtrate is neutral. Insoluble matter in the filter paper was ignited to ash, cooled in a desiccator and weighed without delay. The percentage of acid-insoluble ash was calculated with reference to the air-dried material.

**Water soluble ash**

Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water. The ash obtained by the above method was boiled with 25 ml of water and boiled for 5 min. Filter the insoluble matter on an ashless filter-paper (Whatman No 42). The residue was washed with hot water twice and ignited to ash in a crucible at a temperature not exceeding 450 °C, cooled in a desiccator and weighed without delay. The percentage of water-soluble ash was calculated with reference to the air-dried drug material.

**Sulphated Ash**

One gram of the plant material was weighed in a silica crucible which was previously ignited and cooled. The crucible was ignited gently at first, until the substances was thoroughly charred. The residue was cooled, moistened with 1 ml of sulphuric acid and heated gently until the white fumes were no longer being evolved. Then it was ignited at $800^0 \pm 25^0$ C until all the black particles were disappeared. The crucible was cooled, few drops of sulphuric acid were again added and ignition was done as earlier. The percentage of sulphated ash was calculated on the air-dried material.
Loss on drying

Loss on drying is the loss of weight expressed as percentage w/w resulting from water and volatile matter of any kind that can be driven off under specified conditions. The test is carried out on a well mixed sample of the substances.

One gram of the air-dried material was transferred into previously dried and tared flat weighing bottle and the contents were distributed evenly. Dried the sample in an oven at 100-105°C for 2h, until the constant weight was obtained. After the completion of drying, it was cooled in a desiccator and weighed without delay. The loss of weight was calculated in mg per g of air-dried material. The results were tabulated in table no.5.1

Ether soluble extractive

About 5.0 g of coarsely powdered air-dried material was macerated with 100 ml of ether for 24 h in a glass-stoppered conical flask, shaking frequently for the period of first 6 h and then allowed to stand for 18 h. It was filtered immediately; taking care against the loss of ether, 25 ml of the filtrate was transferred to a tared flat-bottomed shallow dish, the solvent was evaporated on a water-bath and dried in an oven at 105 °C for 6 h. It was cooled in a desiccator and weighed without delay. The percentage of ether soluble extractive value was calculated with reference to the air dried material.

Ethanol soluble extractive

Ethanol is an ideal solvent for extraction of various chemicals like tannins and resins. Therefore this method is frequently employed to determine the approximate resin content of drug. Generally 95% ethyl alcohol is used for determination of
alcohol soluble extracts. Alcohol soluble extracts are one of the tools for standardization of crude drug.

About 5.0 g of coarsely powdered air-dried material was weighed in a glass-stoppered conical flask which was macerated with 100 ml of the ethanol for 24 h, shaking frequently for the period of first 6 h and then allowed to stand for 18 h. Filtered immediately, taking care against the loss of ethanol, 25 ml of the filtrate was evaporated in a flat bottomed shallow dish on a water-bath which was dried at 105 °C for 6 h, Cooled in a desiccator and weighed without delay. The percentage of ethanol soluble extractive value was calculated with reference to the air dried material.

**Water soluble extractive**

Determination of water soluble extractive value is used for evaluating crude drugs which are not readily estimated by other means. This method is applied to drugs which contain water soluble active constituents of crude drugs such as tannins, sugars, plant acids, mucilage and glycosides. The water soluble extractive value can be used to indicate poor quality, adulteration with any unwanted material or incorrect processing of the crude drug during the process of drying and storage.

About 5.0 g of coarsely powdered air-dried material was weighed in a glass-stoppered conical flask and macerated with 100 ml of the distilled water for 24 h, shaking frequently for the period of first 6 h and then allowed to stand for 18 h. It was filtered rapidly, taking care against the loss of water, transferred 25 ml of the filtrate to a tared flat-bottomed shallow dish and evaporated the solvent on a water-bath which were dried at 105 °C for 6 h. Cooled in a desiccator and weighed without
delay. The percentage of water soluble extractive value was calculated with reference to the air dried material. The results were depicted in table no.5.1

**Determination of crude fiber content**

Crude fiber contents in the sample were determined by acid base digestion method (Ali Aberoumand, 2010). In a 500 ml conical flask, 2 g of dried plant powder and 200 ml of 1.25% H₂SO₄ (7.2 ml of 94% concentrated acid per 1000 ml distilled water) was added. The mixture was boiled for 30 min with bumping chips. The flask was allowed to cool; after cooling it was filtered via muslin cloth. The residue was washed several times with boiled distilled water. The obtained residue was boiled with 200 ml of 1.25% sodium hydroxide solution (1.25 g of NaOH dissolved in 100 ml distilled water). The contents were filtered and washed with 25 ml of 1.25% H₂SO₄, three times with 50 ml of water and finally with 25 ml of alcohol. The residue was transferred into pre-weighed porcelain crucible (W₁) and dried in an oven at 130 ± 2°C for 2 h to get constant weight (W₂). The residue was again ignited at 600 ± 15°C; cooled in desiccator and weighed (W₃). Crude fibre content was expressed as percentage loss in weight on ignition. The Crude fiber content in the samples was calculated by using following formula

\[
\text{Loss in weight on ignition} = \left( \frac{(W₂ - W₁) - (W₃ - W₁)}{\text{Weight of the sample}} \right) \times 100
\]

**5.2.4. Fluorescent analysis**

Fluorescence characters of medicinal plants were determined under ordinary and ultraviolet light. The fluorescence analysis of drug extract helps to identify the drug with specific fluorescent colors and to find out the fluorescent impurities. The study of fluorescence analysis can be used as a diagnostic tool for testing adulteration.
The plant powder and extracts were treated with various chemical reagents and changes in the color were viewed under day light, near and far UV (Chase CR and Pratt R, 1949).

5.2.5. Heavy metal analysis

Atomic absorption spectroscopy is the analytical technique mostly employed for heavy metal analysis because of its low interference level and reasonable sensitivity. Atomic absorption spectroscopy method was capable of determining metal concentration ranging from trace to major constituent levels. From each herbal powder, a sample weighing 2 g was taken in a crucible and was ignited in a muffle furnace at 550°C for 6 h. The ash produced was digested in 5 ml of concentrated nitric acid followed by evaporation on hot plate. Small amount of distilled water was added to the digested residue, filtered and volume was made to 30 ml using distilled water. This digestion was achieved according to standard procedure recommended by the Royal Committee of Experts (Kumar sukender et al., 2012). The solutions formed were quantitatively analyzed using atomic absorption spectrophotometer (Varian Atomic absorption spectrum) for the heavy metals including Arsenic (As), lead (Pb), cadmium (Cd), Mercury (Hg) and chromium (Cr).

5.2.6. Phytochemical screening of plant extracts

Plants used in traditional medicine contain a wide range of bioactive compounds that can be used to treat infectious diseases. The most important bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds. The extracts of NC and MS obtained were subjected to qualitative tests for their identification of various plant constituents like alkaloids, carbohydrates, glycosides,

**Test for alkaloids**

Few quantity of the each extract was stirred separately with 5 ml of 1% aqueous Hydrochloric acid on water bath and filtered. The filtrate was used to perform following tests.

- **Dragendorff’s test**
  
  To 1 ml of filtrate, few drops of Dragendorff’s reagent (potassium bismuth iodide solution) was added. An orange-red precipitate indicates the presence of alkaloids.

- **Mayer’s test**
  
  Few drops of Mayer’s reagent (potassium mercuric iodide solution) was added to 1 ml of filtrate. Whitish or cream colored precipitate indicates the presence of alkaloids.

- **Hager’s test:**
  
  Few drops of Hager’s reagent (saturated aqueous solution of picric acid) was added to 1 ml of filtrate. Yellow colored precipitate indicates the presence of alkaloids.

- **Wagner’s test:**
  
  Few drops of Wagner’s reagent (iodine in potassium iodide) was added to 1 ml of filtrate. Reddish brown colored precipitate indicates the presence of alkaloids.

- **Muroxide’s test**
  
  Few drops of conc. HNO₃ were added to 3 – 4 ml of filtrate solution and it was evaporated to dryness. The content was cooled and 2 drops of NH₃OH
solution was added. Formation of purple color indicates the presence of alkaloids.

**Test for flavonoids**

About 0.1g of each extract was boiled with distilled water and filtered. The filtrate was used for the analysis of following tests.

- **Ferric chloride test for flavonoids**
  
  Few drops of 10% ferric chloride solution was added to 2 ml of the filtrate. Green-blue or violet coloration indicates the presence of a phenolic hydroxyl group.

- **Lead acetate test for flavonoids**
  
  To 2 ml of each of the filtrate, 3 ml of lead acetate solution was added. Appearance of a yellow colored precipitate indicates the presence of flavonoids.

- **Alkaline reagent test for flavonoids**
  
  To 2 ml of the filtrate, 10% aqueous sodium hydroxide was added. Color changes from yellow to colorless upon the addition of dilute hydrochloric acid indicates the presence of flavonoids.

- **Shinoda's test for flavonoids**
  
  About 0.5g of each extract was dissolved in ethanol, warmed and then filtered. Three pieces of magnesium metal was added to the filtrate followed by few drops of conc. HCl. Pink, orange, or red to purple coloration indicates the presence of flavonoids.
Test for Tannins

About 0.5 g of extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate. Appearance of a blue-black, green or blue-green precipitate indicates the presence of tannins.

Test for Anthraquinone glycosides

Borntrager's Test

About 0.2 g of extract was shaken with 10 ml of benzene and then filtered. 5 ml of 10% ammonia solution was added to the filtrate and shaken well. Appearance of a pink, red or violet color in the ammoniacal (lower) phase indicates the presence of free anthraquinone.

Test for steroids

For testing the presence of steroids, 1g of the air-dried plant powder was extracted with few ml of ethanol then filtrated and the filtrate was evaporated till dryness. The residue was dissolved in 10 ml chloroform, filtered and the filtrate was divided into two equal portions for the analysis following tests.

- **Libermann-Burchard test**

  To the first portion of chloroform filtrate, 1 ml of acetic acid anhydride was added, followed by 2 ml of sulphuric acid in the wall of the test tube. The appearance of a reddish violet color at the junction of the two layers and a bluish green color in the acetic acid layer indicates the presence of steroids.

- **Salkowiski's test**

  To the second portion of chloroform filtrate, an equal volume of dil. sulphuric acid was added. The appearance of a red color in the chloroform layer and
greenish yellow fluorescence in the acid layer indicates the presence of steroids.

**Test for Protein and Amino Acids**

- **Biuret test**
  One ml of 40% sodium hydroxide solution was mixed with 2 drops of 1% copper sulphate solution till the formation of blue color, the mixture was then added to the 1 ml of the extract. Formation of pinkish or purple violet color indicates the presence of proteins.

- **Ninhydrin test**
  Two ml of the extract was mixed with 1 ml of 0.2 g of ninhydrin (1,2,3indanetrione monohydrate) in 50 mL of water. A blue to blue-violet color indicates the presence of amino acid.

- **Xanthoproteic test**
  To 1 ml of the extract, 1 ml of concentrated nitric acid was added. Formation of yellow color indicates the presence of proteins.

- **Million’s test**
  1 ml of test extract mixed with sulphuric acid and Million’s reagent. Formation of white precipitate indicates the presence of protein.

**Test for Saponins**

**Foam or frothing test**

Few grams of extracts were diluted with 20 ml of distilled water and shaken in a graduated cylinder for 15 min. Formation of 1 cm layer of foam indicated the presence of saponins.
Test for carbohydrates

One gram of the extract was boiled with 20 ml distilled water. The aqueous solution was filtered and the filtrate was tested for the presence of carbohydrates.

- **Molisch’s test**
  
  Two ml of the prepared filtrate was mixed with 0.2 ml of alcoholic solution of 10% α-naphthol, to this mixture 2 ml of concentrated sulphuric acid was added along the sides of the test tube, formation of bluish violet zone indicates the presence of carbohydrates.

- **Fehling’s test**
  
  One ml of Fehling’s A and 1 ml of Fehling’s B solutions were mixed and boiled for 1 min, then equal volume of extract was added. It was heated in a boiling water bath for 5 - 10 min. Formation of brick red precipitate indicates the presence of carbohydrates.

- **Benedict’s test**
  
  To 1 ml of the filtrate, 5 ml of Benedict’s reagent were added. The mixture was heated; appearance of red precipitate indicates the presence of reducing sugars.

- **Barfoed’s test**
  
  Equal volume of Barfoed’s reagent and extract was mixed and it was heated for 1 - 2 min in a boiling water bath and then cooled. Formation of red colour indicates the presence of carbohydrate.
Detection of glycosides

- **Legal’s test**

  Extracts were treated with sodium nitro prusside in pyridine and methanolic alkali. Formation of pink to blood red color indicates the presence of glycosides.

- **Keller-Killiani test**

  One drop of 5% ferric chloride solution was added to 2 ml of ethanolic extract and 3 ml of glacial acetic acid and this solution was transferred carefully to the surface of 2 ml of conc. H$_2$SO$_4$. Formation of reddish brown color at the junction of the two liquid layers and bluish green color appeared in upper layer indicates the presence of glycosides.

### 5.2.7. Quantification of active constituents

Phenols, alkaloids and flavonoids are amazing group of compounds virtually found in all plants. They are present in edible and non-edible plant parts and are responsible for many biological activities in both the plant and animal systems (Kahkonen MP *et al.*, 1999). Over 5000 naturally occurring phenols and flavonoids have been identified from various plants. These are known to possess antioxidant, antibacterial, antitumor and antiviral properties and their effects on human nutrition and health was also important. Hence it was essential to calculate the total amounts of important active constituents in specific extract.

**Determination of alkaloids**

Alkaloids were quantitatively determined by the Harborne method (Mbaebie BO *et al.*, 2012). 10% Acetic acid in ethanol (200ml) was added to powdered sample (5g), covered tightly and allowed to stand for 4 h. The solution was filtered and
concentrated to ¼ of its original volume on a water bath. Concentrated ammonium hydroxide was added drop wisely to the extract until precipitation was completed and the solution was allowed to settle. The precipitate was collected and washed with dilute ammonium hydroxide. After washing, the residue was collected, dried and weighed.

The alkaloid content was determined by

\[
\text{% of Alkaloid} = \frac{\text{Final weight of the sample}}{\text{Initial weight of the extract}} \times 100
\]

**Determination of Phenolic content**

Total phenolic content of the plant extracts were determined using Folin-Ciocalteu method (Singleton V *et al.*, 1999). Standard solution of gallic acid was prepared in the concentration ranging from 20 to 80 µg/ml. The plant extract solution was prepared at concentration range of 1 mg/ml. 100 µl of plant extracts and standard was mixed with 1.9 ml of Folin-Ciocalteu reagent (diluted previously 10 fold with distilled water). The mixture was incubated in dark for 5 min at room temperature and thus 1 ml of sodium carbonate (6% w/v) was added and mixed gently. The resulting mixture was again incubated for 90 min at room temperature for color development. The absorbance of blue color sample was measured at 725 nm using UV/Vis spectrophotometer.

The results were compared to gallic acid calibration curve and the total phenolic content of extracts was expressed as mg of gallic acid equivalents (GAE) per g of dry extracts.
Determination of Flavonoids

Total flavonoids assay was carried out using the colorimetric method (Chang C et al., 2002). One mg/ml of the plant extract was prepared, the specified volume of extract and standard solution of quercetin (0.1 ml) was mixed with 4 ml of distilled water and 0.3 ml of 5% NaNO₂ and incubated for 5 min. After 5 min 0.3 ml of 10% AlCl₃ was added and incubated for 6 min. After the incubation, 2 ml of 1 M NaOH was added. The volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured at 420 nm using UV-VIS spectrophotometer.

The results were compared to quercetin calibration curve and the total flavanoid content of extracts was expressed as mg of quercetin equivalents per g of dry extracts.

Determination of lipid content

One gram of the dried and powdered test sample was macerated with 10 ml distilled water. To this, 30 ml of chloroform-methanol mixture in the ratio (2: 1, v/v) was added and mixed thoroughly. The mixture was left overnight at room temperature; 20 ml of chloroform and 20 ml of distilled water was added and centrifuged. Three layers were formed, out of the three layers, a clear lower chloroform layer containing all lipids was collected in pre-weighed beaker, evaporated completely and weighed, which was taken as the weight of total lipids/g of the dried sample (Jayaraman J, 1981).
5.3. *In vitro* anti diabetic activity

5.3.1. Alpha Amylase Inhibition Assay

Alpha-amylase activity can be measured by hydrolysis of starch in presence of α-amylase enzyme. The starch is hydrolysed on the catalytic action of amylase to fragments, which can be determined with 3, 5-dinitrosalicylic acid, due to their semiacetelic reducer groups. The formed nitroaminosalycilic acid concentration is corresponded to the enzymatic activity of amylase.

The α-amylase inhibition assay was determined according to Miller (1959) using DNS method. Various concentrations of plant extracts (1.95, 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 μg/ml) and 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) containing 0.04 units of α-amylase solution were incubated for 10 min at 37°C, followed by 1% starch solution (500 μl) in 0.02 M sodium phosphate buffer (pH6.9) was added to all the test tubes. The reaction was stopped by adding 3, 5-dinitro salicylic acid (1.0 ml) color reagent. The tubes were then incubated in a boiling water bath and cooled at room temperature. The reaction mixture was diluted to 10 ml with distilled water and the absorbance was read at 540 nm. The control samples representing 100% enzyme activity were also prepared without plant extracts and compared with the samples containing plant extracts. The results were expressed as percentage of inhibition.

5.3.2. Alpha glucosidase inhibition assay

It is a simple and direct procedure for measuring α-glucosidase activity in biological samples. α-glucosidase activity is determined by a reaction in which α-glucosidase hydrolyzes p-nitrophenyl- α-D-glucopyranoside to a colorimetric product, which is proportional to the α-glucosidase activity.
The α-glucosidase inhibitory assay was determined according to Artanti et al., (2012). 100 µl of sample at different concentrations, 100 µl of 20 mM pNPG (p-Nitrophenyl α-D-glucopyranoside) and 2.2 ml of phosphate buffer (100 mM) at pH 7.0 were well mixed and incubated for 5 min at 37°C. 100 µl of enzyme solution (1 mg/0.1 ml) was added to initiate the reaction followed by further incubation for 15 min at 37°C. The reaction was stopped by addition of 2.5 ml of sodium carbonate (200 mM). Alpha glucosidase activity was measured spectrophotometrically at 405 nm. The results were expressed as percentage of inhibition. Percentage of inhibition for α-amylase and α-glucosidase inhibitory activity was calculated using the formula

\[
\text{Percentage of inhibition} = \frac{\text{Abs (Control)} - \text{Abs (Extract)}}{\text{Abs (Control)}} \times 100
\]

5.4. RESULTS AND DISCUSSION

With the global increase in the demand for plant-derived medicine as an alternative to synthetic medicine, there is a need to ensure the quality of the herbal drugs using modern analytical techniques for therapeutic efficacy and safety. Standardization methods should taken into consideration all aspects that contribute to the quality of the herbal drugs, namely correct identity of the sample, organoleptic evaluation, pharmacognostic evaluation, volatile matter, quantitative evaluation (ash values, extractive values), phytochemical evaluation, test for the presence of xenobiotics, microbial load testing, toxicity testing and biological activity. Of these, the phytochemical profile is of special significance since it has a direct bearing on the activity of the herbal drugs. The purity and quality of the drug was calculated by determining the ash values and moisture content of the herbal drug because it contains earthy matters and some inorganic radicals like phosphates, carbonates and silicates of potassium, sodium and calcium. Physicochemical parameters of plant powder as
shown in the Table 5.1 were in compliance with those mentioned in Ayurvedic Pharmacopoeia of India. The percentage of loss of weight on drying, total ash, acid insoluble-ash, water-soluble ash and sulphated ash were obtained by employing standard methods of analysis. Extractive values were also calculated, it is useful for the determination of exhausted or adulterated drug. Moreover the total ash of a crude drug also reflects the purity of crude and the prepared drug (Purohit et al., 2005). Acid insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth. Water soluble ash is the water soluble portion of the total ash. The total ash content of NC is 6.3 % and MS is 5.6 %. Acid insoluble ash for NC and MS are 2.5 % and 2.4 % respectively. Percent weight of moisture content was found to be 4.03 % for NC and 3.70 % for MS.

Extractive values were calculated using ether, water and alcohol which were recorded in Table 5.1. It is useful for the evaluation of a crude drug as it gives an idea about the nature of chemical constituents present in it and is useful for estimation of chemical constituents soluble in that particular solvent used for extraction (Joseph C & George M, 2011). The water soluble extractive value was indicating the presence of sugar, acids and inorganic compounds and the alcohol soluble extractive values indicated the presence of polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids and secondary metabolites present in the NC and MS plant sample. The total moisture content of the crude drug was also determined and the results were given in table 5.1. In fluorescent analysis, the plant powder and extract were treated with various solvents like nitric acid, sulphuric acid, sodium hydroxide, ethanol and water; the color developed was visualized under ordinary light, short UV (254nm) and long UV (366nm) in UV chamber. The results were recorded in table 5.2 and 5.3. Heavy metals are ubiquitous in trace concentrations in soils and the plants
grown in these soils face the heavy metal stress and causes changes in production of secondary metabolites. High levels of heavy metal contamination in medicinal plants may suppress secondary metabolite production. Heavy metals like arsenic, cadmium, lead, mercury which are non essential elements were found in low levels when compared to other findings. Based on these results, all the metals are found below the WHO permissible limits. The results of the present study supports the safety of this plant to use as raw material in herbal formulation since the heavy metal concentration is within the limits (Table 5.4). Preliminary phytochemical studies indicates the most of the active constituents such as flavonoids, phenols, tannins, glycosides, steroids, triterpenoids are present (Table 5.5). Preliminary phytochemical studies of MS indicate the presence of alkaloids, flavonoids, phenols, saponins, tannins, terpenoids, steroids and glycosides. Preliminary phytochemical studies of MS indicate the presence of flavonoids, phenols, saponins, tannins, terpenoids, steroids and glycosides.

**Table 5.1: Physicochemical parameters results of MS and NC**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameters</th>
<th><em>Myxopyrum serratulum</em> (%w/w)</th>
<th><em>Nilgirianthus ciliatus</em> (%w/w)</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Percentage yield</td>
<td>12.64</td>
<td>8.29</td>
</tr>
<tr>
<td>2.</td>
<td>Ash values</td>
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<td></td>
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<td></td>
<td>Total Ash</td>
<td>5.6±0.16</td>
<td>6.3±0.49</td>
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<td></td>
<td>Acid insoluble ash</td>
<td>2.4±0.53</td>
<td>2.5±0.57</td>
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<td></td>
<td>Water soluble ash</td>
<td>2.0±0.58</td>
<td>3.1±0.89</td>
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<td></td>
<td>Sulphated ash</td>
<td>3.65±0.26</td>
<td>4.75±0.71</td>
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<td>3.</td>
<td>Loss on drying</td>
<td>3.70±0.08</td>
<td>4.03±0.47</td>
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<tr>
<td>4.</td>
<td>Extractive values</td>
<td></td>
<td></td>
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<td></td>
<td>Ether soluble extract</td>
<td>0.24±0.12</td>
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<td>Ethanol soluble extract</td>
<td>3.92±0.56</td>
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<td>Water soluble extract</td>
<td>5.68±0.85</td>
<td>4.36±0.24</td>
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<td>5.</td>
<td>Crude fiber content</td>
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<td>17.61±1.85</td>
</tr>
</tbody>
</table>
### Table 5.2: Fluorescent analysis report of *Myxopyrum serratum* A.W. Hill

<table>
<thead>
<tr>
<th>Reagents</th>
<th><em>Myxopyrum serratum</em> powder</th>
<th><em>Myxopyrum serratum</em> extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day light</td>
<td>UV light</td>
</tr>
<tr>
<td></td>
<td>At 254</td>
<td>At 366</td>
</tr>
<tr>
<td>Sample as such</td>
<td>Green</td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td>Brown</td>
<td>Green</td>
</tr>
<tr>
<td>Sample+ methanol</td>
<td>Greenish Yellow</td>
<td>Dark Green</td>
</tr>
<tr>
<td></td>
<td>Dark Green</td>
<td>Light Green</td>
</tr>
<tr>
<td>Sample+50% HNO3</td>
<td>Reddish orange</td>
<td>Dark brown</td>
</tr>
<tr>
<td></td>
<td>Yellowish green</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Sample+1N NaoH</td>
<td>Fluorescent Green</td>
<td>Dark Green</td>
</tr>
<tr>
<td></td>
<td>Dark brown</td>
<td>Green</td>
</tr>
<tr>
<td>Sample+50% H2SO4</td>
<td>Light Green</td>
<td>Dark Green</td>
</tr>
<tr>
<td></td>
<td>Dark Green</td>
<td>Yellow</td>
</tr>
<tr>
<td>Sample+80% H2SO4</td>
<td>Brown</td>
<td>Greenish Black</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>Dark Green</td>
</tr>
<tr>
<td>Sample + Conc. H2SO4</td>
<td>Brown</td>
<td>Black</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>Dark Green</td>
</tr>
<tr>
<td>Sample +H2O</td>
<td>Yellowish brown</td>
<td>Dark Brown</td>
</tr>
<tr>
<td></td>
<td>Dark Brown</td>
<td>Yellowish green</td>
</tr>
<tr>
<td></td>
<td>Brown</td>
<td>Dark brown</td>
</tr>
</tbody>
</table>

### Table 5.3: Fluorescent analysis report of *Nilgirianthus ciliatus* Nees

<table>
<thead>
<tr>
<th>Reagents</th>
<th><em>Nilgirianthus ciliatus</em> powder</th>
<th><em>Nilgirianthus ciliatus</em> ethanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day light</td>
<td>UV light</td>
</tr>
<tr>
<td></td>
<td>At 254</td>
<td>At 365</td>
</tr>
<tr>
<td>Powder as such</td>
<td>Dark Green</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>Greenish Yellow</td>
</tr>
<tr>
<td>Powder+ methanol</td>
<td>Green</td>
<td>Greenish yellow</td>
</tr>
<tr>
<td>Powder+50% HNO3</td>
<td>Orange</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Powder+1N NaoH</td>
<td>Green</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Powder+50% H2SO4</td>
<td>Green</td>
<td>Green</td>
</tr>
<tr>
<td>Powder +80% H2SO4</td>
<td>Brown</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Powder+ Conc. H2SO4</td>
<td>Black</td>
<td>Dark Green</td>
</tr>
<tr>
<td>Powder+H2O</td>
<td>Light green</td>
<td>Brown</td>
</tr>
<tr>
<td></td>
<td>Light green</td>
<td>Light green</td>
</tr>
</tbody>
</table>
Important active constituents were quantified by specific procedures and results were mentioned in Table 5.6. Measurement of total phenolic content by Folin-Ciocalteu assay in ethanolic extract of NC and MS was found to be 8.86 mg/g and 78.09 mg/g of dry weight of extract, expressed as gallic acid equivalents. The total flavonoids concentrations in NC and MS extract was found to be 1.63 and 51.2 mg/g respectively, expressed as quercetin equivalents (Figure 5.2). The high contents of phenolic compounds and flavonoids indicated that these compounds contribute to the pharmacological activity. The analysis reports will help the future investigators for proper identification of the plant. The findings will also enable pharmacognostical standardization of the plant materials.

**Table 5.4: Heavy metal Analysis of NC and MS**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Heavy Metals</th>
<th>NC</th>
<th>MS</th>
<th>Permissible limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arsenic</td>
<td>0.56 ppm</td>
<td>0.31 ppm</td>
<td>NMT 5 ppm</td>
</tr>
<tr>
<td>2</td>
<td>Chromium</td>
<td>0.12 ppm</td>
<td>0.14 ppm</td>
<td>NMT 2 ppm</td>
</tr>
<tr>
<td>3</td>
<td>Cadmium</td>
<td>0.05 ppm</td>
<td>0.03 ppm</td>
<td>NMT 0.2 ppm</td>
</tr>
<tr>
<td>4</td>
<td>Lead</td>
<td>1.25 ppm</td>
<td>0.95 ppm</td>
<td>NMT 5 ppm</td>
</tr>
<tr>
<td>5</td>
<td>Mercury</td>
<td>0.02 ppm</td>
<td>0.01 ppm</td>
<td>NMT 0.1 ppm</td>
</tr>
</tbody>
</table>

**Table 5.5: Preliminary phytochemical screening of MS and NC**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Phytochemical constituents</th>
<th><em>Myxopyrum serratulum</em> (MS)</th>
<th><em>Nilgirianthus ciliatus</em> (NC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Carbohydrates</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>3</td>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Phenols</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Proteins And Amino acids</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>6</td>
<td>Saponins</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Triterpenoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Steroids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 5.6: Quantitative determination of active constituents

<table>
<thead>
<tr>
<th>S. No</th>
<th>Content</th>
<th><em>Myxopyrum serratum</em> (MS)</th>
<th><em>Nilgirianthus ciliatus</em> (NC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phenolic Content (mg GAE/g of extract)</td>
<td>78.09</td>
<td>8.86</td>
</tr>
<tr>
<td>2.</td>
<td>Flavonoid (mg QE/g of extract)</td>
<td>51.2</td>
<td>1.635</td>
</tr>
<tr>
<td>3.</td>
<td>Lipid</td>
<td>3.63%</td>
<td>2.20%</td>
</tr>
<tr>
<td>4.</td>
<td>Alkaloid</td>
<td>1.56%</td>
<td>0.12%</td>
</tr>
</tbody>
</table>

Figure 5.2: Calibration curve of Gallic acid (A) and Quercetin (B)

Antidiabetic activity

Postprandial hyperglycemia is the major problem in diabetes mellitus, it increases the risk of diabetes associated with secondary diseases and cause death (Salma et al., 2006). Carbohydrate rich diets cause the rapid absorption in the intestine by means of hydrolyzing enzymes (α-amylase and α-glucosidase). These enzymes break complex carbohydrates into absorbable monosaccharides (Kwon YI et al., 2007). Suppression of these enzyme activities would delay the degradation of starch and oligosaccharides, which leads to decreases in the absorption of glucose and as a result the reduction of post prandial blood glucose rise. This can be achieved by using α-amylase and α-glucosidase inhibitors. Natural α-amylase and α-glucosidase
inhibitors from medicinal plants give dietary strategies to control postprandial hyperglycemia and the natural form of these inhibitors could be used in therapies with minimum side effects (Pinto M & Shetty K, 2010).

*In vitro* antidiabetic α-amylase and α-glucosidase inhibition assay was carried out in both plants with the concentration range from 1.95 µg to 1000 µg/ml. Alpha amylase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide such as glycogen and starch to yield glucose and maltose. Alpha amylase inhibitors bind to alpha- bond of polysaccharide and prevent break down of polysaccharide in mono and disaccharide. The percentage inhibition at various concentrations of ethanolic extract of NC, MS and standard drug acarbose showed a concentration-dependent reduction (Figure 5.3). The highest concentration 1000 µg/ml of NC showed a maximum percentage inhibition of 83.41±1.41 and 62.98±3.51 for α-glucosidase and α-amylase respectively. MS at 1000 µg/ml concentration showed maximum percentage inhibition of 56.96±0.63 and 70.14±0.51 for α-glucosidase and α-amylase respectively (Table 5.7, 5.8). For α-glucosidase, the percentage inhibition varied from 83.41- 9.97 % from the highest concentration to the lowest concentration of 1.95 µg /ml and for α-amylase the percentage inhibition varied from 62.98 - 5.34 % from the highest concentration to the lowest concentration of 1.95 µg /ml. The IC₅₀ values of ethanolic extract of NC for α-amylase and α-glucosidase inhibition was found to be 462.49 µg/ml and 21.90 µg/ml respectively. The IC₅₀ values of ethanolic extract of MS was 435.02 µg and 688.61 µg for alpha amylase and alpha glucosidase respectively. The results showed that the NC has more α-glucosidase inhibitory activity and MS has more α-amylase inhibitory effect. The ethanolic extracts of NC and MS plants have high quantity of Phenolic compounds.
and flavonoids; these active constituents may be responsible for these inhibitory activities.

Table 5.7: *In vitro* antidiabetic activity of ethanolic extract of NC and MS

<table>
<thead>
<tr>
<th>Conc µg/ml</th>
<th>NC α-glucosidase IC₅₀</th>
<th>α-amylase IC₅₀</th>
<th>MS α-glucosidase IC₅₀</th>
<th>α-amylase IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.95</td>
<td>31.59±0.31</td>
<td>5.34±0.11</td>
<td>9.97±3.47</td>
<td>6.52±3.25</td>
</tr>
<tr>
<td>3.9</td>
<td>42.48±1.25</td>
<td>12.21±0.78</td>
<td>14.43±0.11</td>
<td>10.87±0.51</td>
</tr>
<tr>
<td>7.81</td>
<td>44.03±2.03</td>
<td>14.12±0.47</td>
<td>15.03±0.32</td>
<td>18.84±0.34</td>
</tr>
<tr>
<td>15.62</td>
<td>49.56±2.50</td>
<td>16.03±0.54</td>
<td>16.52±0.74</td>
<td>25.85±0.51</td>
</tr>
<tr>
<td>31.25</td>
<td>53.32±0.47</td>
<td>23.66±1.08</td>
<td>29.17±1.05</td>
<td>33.57±2.05</td>
</tr>
<tr>
<td>62.5</td>
<td>57.96±5.63</td>
<td>38.93±0.81</td>
<td>33.76±0.42</td>
<td>38.78±0.34</td>
</tr>
<tr>
<td>125</td>
<td>58.85±0.31</td>
<td>41.03±0.54</td>
<td>36.95±0.21</td>
<td>42.58±0.34</td>
</tr>
<tr>
<td>250</td>
<td>59.73±0.00</td>
<td>45.67±0.54</td>
<td>40.74±0.42</td>
<td>48.44±0.34</td>
</tr>
<tr>
<td>500</td>
<td>73.67±1.41</td>
<td>51.94±0.54</td>
<td>45.93±0.00</td>
<td>56.14±1.20</td>
</tr>
<tr>
<td>1000</td>
<td>83.41±1.41</td>
<td>62.98±3.51</td>
<td>56.96±0.63</td>
<td>70.14±0.51</td>
</tr>
</tbody>
</table>

Results expressed as Mean± SEM triplicates

Table 5.8. *In vitro* antidiabetic activity of Acarbose

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (µg/ml)</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-glucosidse</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>38.45±0.00</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>46.75±0.48</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>59.78±0.56</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>72.48±0.68</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>89.32±0.56</td>
</tr>
</tbody>
</table>

Results expressed as Mean ± SEM
Figure 5.3. *In vitro* antidiabetic activity of plant extracts and Acarbose

*In vitro* antidiabetic activity of *Myxopyrum serratulum*

*In vitro* antidiabetic activity of acarbose

*In vitro* antidiabetic activity of *Nilgirianthus ciliatus*
5.5. REFERENCES


