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''Evaluation of preliminary Physico-phytochemical parameters of Raw and CO_2 extract of *Kalmegh* (*Andrographis paniculata* Nees.)''

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Abstract

Introduction: Medicinal plants are the major sources for the therapeutic remedies of various ailments from the ancient era. Their *rasa*, *guna, veerya*, *vipaka* and *prabhava* i.e. *pancha mahabhuta* are responsible for these potential medicinal effects. One of such plants known as *Kalmegh,* which is used in ancient oriental and ayurvedic medicine. The physico and phyto-chemical parameters are very significant to acquire the exact idea about the potency of *dravyas*. There is no detailed physico and phyto-chemical study work done on CO_2 extract of *panchanga* of *Kalmegh* till a date. **Materials and methods:** Here, the present communication tries to evaluate the physicochemical and phytochemical studies on the *Kalmegh* (*Andrographis paniculata* Nees.) having family Acanthaceae. *Kalmegh* plays very important role in antibacterial, antifungal, antiviral, choleretic, hypoglycemic, hypocholesterolemic, adaptogenic, anti-inflammatory, emollient, astringent, diuretic, carminative, anthelmintic, antipyretic, antioxidant, anticancer, antidiarrhoeal, gastric and liver tonic. **Results:** This study helps in assessing the presence of bioactive compounds like Alkaloids, Saponins, Sugars, Steroids and Phenols. **Conclusion:** so, this must be concluded that the CO_2 extract of *panchanga* of *Kalmegh* showed that the less phytochemicals like Alkaloids, Saponins, Sugars, Steroids and Phenols.

Keywords- Physicochemical, Phytochemical, *panchanga* of *Kalmegh,* Adulterations

INTRODUCTION

Kalmegh is the herbaceous plant, native of all over India. *Andrographis paniculata* (Burm. f.)Nees. is its Latin name with family Acanthaceae. It is widely cultivated in Southeastern Asia. It often being used before antibiotics were created to treat various infections. It is well known plant in Bengal by the name *'kalmegh' Kalamegha*, meaning "dark cloud". It is also known as *Bhui-neem*, meaning "neem of the ground", Andrographis and *Kirayat.*[1]The plant is known in north-eastern India as *Maha-tita*, literally "king of bitters" because of its bioactive component Andrographolid which is of *Tikta rasa.*[2]

Out of all Ayurvedic *Nighantu Granthas*, the *Priyanighantu* explains the *Kalmegh* in *Shatapushpadi varga.* It is of *Tikta rasa* with *Katu Vipaka* and *Ushna Veerya* and *kapha-pitta doshahara* properties. It has been also discussed that *Kalmegh* has activities like *Paachana, Swedana, Krimighna* and *Pittasaraka*. The plant has been specially used in *Yakrutroga, Krimiroga, Kushtha, and Jwara.* [3]

PURPOSE OF STUDY

Due to increase demand of ayurvedic drugs, there is increase in adulteration. So, many ayurvedic formulations are not pure. The impact of this demand is the diseases are not cure at specific time period and at specific doses. The main cause for all these conditions is Adulteration. Adulteration directly affect the identity, quality and purity of product. Therefore, there is need of Pharmacognostical & Phytochemical standardization of the drug. That's why the standardization of *Kalmegh* is explored. Here, there is made of attempt to establish the quality standardization of *Kalmegh* as per API. In our searches, there are many phytochemical standardization are done on various extracts of *Kalmegh* like water soluble, alcohol soluble, Acetone, petroleum, etc. but there is no work available on CO_2 extract of *panchanga* of *Kalmegh.*

MATERIAL AND METHODS

A. Collection of sample

Panchanga of *Kalmegh* (*Andrographis paniculata* Nees.) were collected from college campus.

B. Authentication and voucher deposition

It was done in the authorised institute. A voucher specimen of the collected sample was deposited in the departmental museum for future reference. (Accession no. 00788).

C. Pharmacognostical, Physicochemical and Phytochemical study

It includes:

1. Raw material standardization

2. In process standardization

3. Finished Product standardization

PHARMACOGNOSTICAL STUDY

Pharmacognosy describes the morphological, chemical and biological properties of drugs along with its history, cultivation, collection, quality control and preparation of crude drugs of natural origin. To avoid the confusion of identification with other drug and adulteration of *Panchanga* of *Kalmegha* which have been used as raw material in this study origin, the pharmacognostical study was undertaken. So, ensuing the above understandings pharmacognostical study of *Panchanga* of *Kalmegh* (*Andrographis paniculata* Nees.) was carried out in 'Pharmacognosy department' of authorised institute. Here Andrographis paniculata Nees. is considered as the source plant of an important drug mentioned in Ayurvedic classic "*Kalmegha*"

The parameters selected for Pharmacognostical study were as follows:

• Macroscopic Examination

• Microscopic Examination

1. Raw material standardization

Materials - Crude Sample of *Panchanga* of *Kalmegh*

Equipments

i. **Magnifying glass:** The magnifying glass has optical grade 3x and 6x magnifying capacity lenses was used.
ii. **Microscope:** Microscopes are used in the laboratory for the tissues and organisms which are too small to be seen clearly with the naked eye. For microscopic analysis Coaxial Binocular Microscope was used.
iii. **Dissection box:** It includes Watch glass, glass slide (3"x1"), Cover slips, hair brush medium of size, Pair of small forceps, Blades and Dropper

MACROSCOPIC EXAMINATION [4]

Macroscopic identification of herbal drugs is based on size, shape, colour, texture, surface characteristics, sound during fracture and appearance of the cut surface.

• Size

A ruler in millimetres was used for the measurement of the length, width and thickness of crude materials.

• Colour

The untreated sample was observed under diffuse daylight. The colour of the sample was compared with reference sample.

• Surface characteristics, texture and fracture characteristics

The untreated sample was examined with a magnifying lens (6x to 10x). The material was touched to determine if it was hard or soft; it was bend and ruptured to acquire information on brittleness and the appearance of the fracture plane - whether it is smooth, rough, fibrous, granular, etc.

• Odour

A small portion of the sample of the material was placed in the palm of the hand and air over the material was inhaled to conclude its specific odour. Once the material has been examined according to external characteristics, inspection can be carried out as the next step by microscopy.

MICROSCOPIC EXAMINATION [5] (Khandelwal KR, et al., 2016)

Step I - Section Method

Selection of appropriate size, shape of a crude drug; sample is very important in obtaining good section. In case of both stem and root, a portion of the drug having a diameter of 3 to 5 mm and a length of 25cms was selected, as a sample shorter in length will be difficult to hold and sample thicker in diameter may give rise to thick and wedge shaped sections.

Step II - Preparation of sample for sectioning

The selected sample was put in a test tube and a sufficient amount of water was added in it so that the sample remains submerged. The sample was boiled in water over a Bunsen flame for a few minutes. This would soften the hard drug sample and would help in obtaining fine sections.

For stem and root drug, a cylindrical portion which is almost straight was cut and both edges was cut-off so as to make the edge surface smooth. Hence that sample was ready for section cutting. The sample was hold vertical between the first, second finger and the thumb and the blade was moved back and forth from one end to the other, obtaining fine slices. Sufficient number of sections was taken, as all sections would not be very fine and uniform. Sections were transferred to a watch glass 84 containing water with the help of brush. Thick and oblique sections were rejected.

Step III - Staining of sections

A clean watch glass was taken and the staining solution added to it. With the help of a brush, the section was transferred from water to stain solution and kept for 2-3 minutes. After 2-3 minutes the section was picked up and transferred to watch glass containing plain water, so that excess stain was washed away. That section was ready for mounting on a slide.

Step IV - Mounting of sections

A clean glass micro slide was taken. On that slide the section to be mounted was transferred, with the help of a brush. One to two drops of water was added on the section with the dropper by ensuring that the section was submerged in the water. A clean cover slip was taken with the help of a forceps and needle. The cover slip was placed on the section gently. Whenever there was an appearance of any air bubble, the cover slip was slightly left and a drop of water was added. Excess water present outside the cover slip was wiped off with the help of blotting paper. The slide became ready for observation.

2. IN PROCESS STANDARDIZATION

- **Material-** *Panchanga* of *Kalmegh* (*Andrographis paniculata* Nees.) (Fig.01)

Equipments –

- **Mortar and Pestle (*Khalwa-yantra*)**

A black stoned *Khalwa-yantra* was used from the Pharmaceutical Department of Pharmacy College with longer diameter 25”, shorter diameter 12” and depth 4.9”.

- **Stainless steel container**

Stainless Steel vessels were used of 5L capacity from the Pharmaceutical Department of Pharmacy College.

- **Preparation of *Choorna* -** (Fig. 02)

The dried powdered separately according to the standard procedure which is mentioned in *Sharangdhara Samhita* as-

“अत्यन्तशुष्कं यद् द्रव्यं सुपिष्टं वस्त्रगालितम् ।

तत्स्याच्चूर्णं रज: क्षोदस्तन्मात्रा कर्षसंमिता ॥१॥” ....शा.सं.म.६

PHYSICO-CHEMICAL STUDY

Today Ayurvedic science is spreading its wings all over the world where the drug lore of this system has been the center of global interest. Ayurveda advocates that as the Prakriti varies from person to person similarly every drug has got its own physical and chemical characteristics which help to separate it from other closely related drug. The Physicochemical studies of these drugs done by making use of various parameters help in standardizing the drug and authenticate it. In this modern era it is expected an imminent need for a well-coordinated research plan touching physiochemical study of drug. It is essential to gratify the international standards and quality control of the drug used by convincing the drug regulatory authorities. The present study was carried out to evaluate the physicochemical parameters of test drugs.

- **FOREIGN MATTER % :**

The sample shall be free from visible signs of mould growth, sliminess, and contamination by soil, insect parts, and other animal and animal products including animal excreta or any other noxious foreign matter.

- **Determination of Foreign Matter**[6]
- 100 g of sample of kalmegh was taken and it was spread in a thin layer on a suitable platform. It was examined by using 6x or 10x magnifying glass and separated the foreign matter. Appropriate sieve can also be used to separate the foreign matter. The sorted foreign matters were weighed and calculated the foreign matter content in per cent with reference to drug sample.
- **PARTICLE SIZE (USING SIEVE METHOD):** [7]
- **Procedure:** The set of sieves was arranged in descending order. (Sieve number 10 was placed at too below which sieve 20, 40, 60, 80, 100 was respectively placed and 120 at the bottom. The set of sieves were arranged in descending order. (Sieve number 10 was placed at too below which placed sieve 20, 40, 60, 80, 100 respectively and 120 at the bottom.) The given sample (100gm) was weighed, accurately, and it was pouring on the top sieve. The lid was placed, to avoid loss during shaking. The sieve shaking machine was operated for 5 min. Fractions of sample retained on each sieve and on receiver at the bottom of sieve were collected. The sample retained on each sieve was weighed. Percent frequency of each size of particle was calculated.
- **POWDER MICROSCOPY** [5] (Fig.03)-

For detection of Organ or part of the plant present and it's identifying microscopical characteristics. Procedure:

The powder was cleared with clearing reagent (chloral hydrate). The cleared powder was stained with (staining reagents) phloroglucinol and hydrochloric acid, iodine solution. Only one staining reagent was used for each slide. Mount was made free from air bubbles to determine –

i. The type of cells,
ii. The nature of cell walls present,
iii. Cell content

• MOISTURE CONTENT % (Loss on Drying):

This method is applied to determine the amount of water, all or a part of water of crystallization, or volatile matter in the sample, which is removed during the drying.

Determination of Moisture Content (Loss on Drying): [7]

The loss on drying test is designed to measure the amount of water and volatile matter in sample, when sample is dried under specific conditions. This test method is useful in design purposes, service evaluation, regulatory statutes, manufacturing control, quality control, specification acceptance, development and research. This method determines only the mass material loss and, not its identity. Present experiment is determined by hot air oven method. The result is calculated by following formula.

Procedure: Accurately weighed 5gm coarsely powdered drugs are taken dried, weighed Petridish. Petri dish is kept in hot air oven at 105 c for 3 hours. Petridish is taken out, cooled in dessicator and weighed. Again the Petridish is kept in hot air oven at 105c for 30 min. and cooled in dessicator and weighed. This step is repeated till constant weight is obtained by 2 consecutive weighing percentage of moisture content with reference to the dried drugs are calculated using the formula, the weight loss i.e., loss on drying is calculated and expressed as %w/w.

W1=Wt of the petridish

W2= Wt of the sample

W3= Wt of the dried sample with dish

$$\frac{(W1+W2) - W3}{W2} \times 10$$

- **ASH VALUES**

1) Used to determine the quality and purity of a crude drug and to establish the identity of it.
2) Ash contains inorganic radicals like phosphates, carbonates and silicates of sodium, potassium, magnesium and calcium etc. These are present in definite amount in a particular crude drug hence, quantitative determination in terms of various ash values helps in their standardization.
3) Sometimes, inorganic variables like calcium oxalate, silica, carbonate content of the crude drug affects "Total ash value". Such variables are removed by treating with acid and acid insoluble ash value is determined. Used to determine foreign inorganic matter present as an impurity.
4) The ash remaining after ignition of medicinal plant materials is determined by 3 methods i.e., Total ash, acid insoluble ash and water soluble ash.

• **TOTAL ASH**

Determination of Total Ash[8] (Fig. 04, 05)

Total ash is designed to measure the total amount of material remaining after ignition. It includes both type of ash viz., physiological ash and non-physiological ash.

Procedure-

2 gram of the ground air-dried material (*choorna*), accurately weighed and was placed in a crucible. The material was spread in an even layer and the crucible was placed in Muffle furnace by gradually increasing the temperature to 500–600°C until it was white, indicating the absence of carbon.(Fig.6) It was cool in desiccators and weighed. Dried on a water-bath, then on a hot-plate and ignited to constant weight. The residue allowed to cool in suitable desiccators for 30 minutes and then was weighed without delay.

The content of total ash was calculated in mg per g of *choorna*,

Calculation:

Weight of the empty crucible = x

Weight of the drug taken = y

Weight of the empty crucible + Ash (after complete incineration) = z

Weight of the Ash = (z-x) gm

'y' gm of the crude drug gives (z-x) gm of the Ash

…100gm of the crude drug gives 100/y X (z-x) gm of the ash.

$$\textbf{Total ash value of sample} = \frac{100\,(z-x)}{y}\ \%$$

1. **ACID-INSOLUBLE ASH** (Fig.06)

Determination of Acid-insoluble ash:

It is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter.

Procedure-To the crucible containing the total ash, 25 ml of hydrochloric acid was added, covered with a watch-glass and boiled gently for 5 minutes. The watch-glass was rinse with 5 ml of hot water and this liquid was added to the crucible. The insoluble matter was collected on an ash less filter-paper and it was wash with hot water until the filtrate is neutral. The filter-paper containing the insoluble matter was transferred to the original crucible, dry on a hotplate and ignites to constant weight. The residue was allowed to cool in suitable desiccators for 30 minutes, and then weighed without delay. The content of acid-insoluble ash was calculated in mg per g of *choorna*.

Calculation:

Weight the residue (acid insoluble ash) = 'a' gm

'y' gm of the air-dried drug gives 'a' gm of acid insoluble ash

100gm gm of the air-dried drug gives [100/y X a] gm of acid insoluble ash.

$$\textbf{Acid-insoluble ash value of sample} = \frac{100\ X\ a}{Y}\ \%$$

2. **WATER-SOLUBLE ASH** [9] (Fig. 07)

Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water.

Procedure – To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected in a sintered-glass crucible or on an ash less filter paper. It was wash with hot water and placed it in muffle furnace for 15 minutes at a temperature not exceeding 450°C. The weight of this residue in mg was subtracted from the weight of total ash. The content of water-soluble ash in mg per g of air-dried material was calculated.

- **CHLOROFORM SOLUBLE EXTRACTIVE VALUE**
- **Procedure-**

Accurately weighted (10gm) of Kalmegh was macerated with 150ml of chloroform in a closed flask, shaken frequently during first 6 hours and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against loss of chloroform. Evaporated 25ml of filtrate to dryness in a tarred flat bottom shallow dish dried at 105 degree Celsius and weighted. Percentage soluble extractive was calculated with reference to the air-dried drug.

- **ALCOHOL-SOLUBLE EXTRACTIVE VALUE** [9]
- **Procedure-**

About 5gm of powdered drug was weighed in weighing bottle and it was transferred to a dry 250ml conical flask. A 100ml graduated flask was filled to the delivery mark with the solvent (90% alcohol). The flask was cork and it was set aside 24 hours, shaking frequently (Maceration). Then it was filtered into a 50ml cylinder. When sufficient filtrate was collected, 25ml of weighed was transferred, thin porcelain dish, as used for the ash values determinations. It was evaporated to dryness on a water-bath and completes the drying in an oven at 100^0C. Cooled in desiccators and weighed. The percentage w/w of extractive with reference to the air-dried drug was calculated.

Calculation:

25ml.of alcoholic extract gives = x gm of residue

100ml of alcoholic extract gives = 4x gm of residue

5gm of air dried drug gives – 4x gm of alcoholic (90%) soluble residue.

100gm of air dried drug gives – 80x gm of alcoholic (90%) soluble residue.

Alcohol (90%) soluble extractive value of the sample = 80x%

- **pH value** [9] (Fig. 08)

10g of test drug sample was weighted and taken in a conical flask. Then added 50 ml accurately measured water and stirred well for few minutes; kept this solution for some time and then filtered it through filter paper. The filtered solution was taken in a beaker. The pH meter was standardized and electrodes with buffer solution of known pH i.e. 7 pH. The electrodes were rinse with distilled water and introduced into the test solution contained in a small beaker. The pH value of solution was read.

3. FINISHED PRODUCT STANDARDIZATION

Preparation of CO_2 extracts of *Kalmegh* (Fig.-09)

- Material- *Choorna* of *Kalmegh*
- Equipments

a. CO_2 tank

b. Pump

c. R/M input

d. PRV

e. Cyclone separator

f. Collection vessel

Preparation of CO_2 extract

1. First of all liquid CO2 which is stored in tank will be pump to the extractor where pressure will be maintain at 300 bar (=296.077atmosphere) and temperature at 31^0c.

2. In the extractor liquid CO_2 and feed will come in contact with each other and extractor of essential component from feed will carried out. The product stream which contains CO_2 and essential component will go to the cyclone separator through PVR (pressure relieving valve); due to reduction in pressure in PVR liquid CO_2 will be converting into gas.

3. In cyclone separator CO_2 gas and essential component (liquid form) will be separated from each other.

4. From the top of cyclone, separate CO_2 will be recycled back to the tank, condense with the help of condenser.

5. From the bottom of the cyclone separator essential components will be obtained.

CO_2 extract of *Kalmegh* was subjected to following test-

• **pH** (Fig. 10):

The pH value of an aqueous liquid may be defined as the common logarithm of the reciprocal of the hydrogen ion concentration expressed in g per litre. Although this definition provides a useful practical means for the quantitative indication of the acidity or alkalinity of a solution, it is less satisfactory from a strictly theoretical point of view. No definition of pH as a measurable quantity can have a simple meaning, which is also fundamental and exact. This test is carried out to determine the pH of the test drug with the help of pH meter.

Procedure: 10g of test drug sample was weighted and taken in a conical flask. Then added 50 ml accurately measured water and stirred well for few minutes; kept this solution for some time and then filtered it through filter paper. The filtered solution was taken in a beaker. The pH meter was standardized and electrodes with buffer solution of known pH i.e. 7 pH. The electrodes were rinse with distilled water and introduced into the test solution contained in a small beaker. The pH value of solution was read.

• **SPECIFIC GRAVITY** (Fig. 11, 12):

Definition: The specific gravity of a liquid is the weight of any substance compared with that of equal volume of water at same temperature is known as specific gravity. Specific gravity of a liquid is determined by the use of a specific gravity bottle.

Method:

A clean and dry 25ml capacity pycnometer was taken and its weight was noted. It was filled with the sample, cleaned properly from outside and the weight was taken at 40°C. Then it was cleaned, rinsed and filled with distilled water, dried from outside and the weight was noted at 40°C. The weight of sample and the distilled water was calculated. Then the specific gravity was determined by dividing the weight of the sample by the weight of the water.

• **VISCOSITY** (Fig. 13):

Viscosity is a property of a liquid, which is closely related to the resistance to flow.

Procedure: The liquid under test is filled in a U tube viscometer in accordance with the expected viscosity of the liquid so that the fluid level stands within 0.2 mm of the filling mark of the viscometer when the capillary is vertical and the specified temperature is attained by the test liquid. The liquid is sucked or blown to the specified weight of the viscometer and the time taken for the meniscus to pass the two specified marks is measured.

Kinematic viscosity = kt

Where, k = the constant of the viscometer tube determined by observation on liquids of known kinematic viscosity; t = time in seconds for meniscus to pass through the two specified marks.

• **HEAVY METAL TESTS:**

The test for heavy metals is designed to determine the content of metallic impurities that are coloured by sulphide ion, under specified conditions. The limit for heavy metals is indicated in the individual monographs in terms of the parts of lead per million parts of the substance (by weight), as determined by visual comparison of the colour produced by the substance with that of a control prepared from a standard lead solution.

Analysis of the Lead and Cadmium [10] -

Chemicals: Nitric acid, hydrochloric acid, sulphuric acid, hydrogen peroxide, sodium borohydride and stannous chloride were of analytical grade (E. Merck). The water used in all experiment was ultrapure water obtained from Milli-Q-water purification system (Ranken Rion Ltd, India).

Sample preparation: Samples were digested by the wet digestion method. 10 ml of nitric acid was added to 2 g of accurately weighed dried sample in a 100 ml beaker and was heated on a hot plate at 95°C for 15 min. The digest was cooled and 5 ml of concentrated nitric acid was added and heated for additional 30 min at 95°C. The last step was repeated and the solution was reduced to about 5 ml without boiling. The sample was cooled again and 2 ml of deionized water and 3 ml of 30% hydrogen peroxide was added. With the beaker covered, the sample was heated gently to start the peroxide reaction. If effervescence becomes excessively vigorous, sample was removed from the hot plate and 30% hydrogen peroxide was added in 1 ml increments, followed by gentle heating until the effervescence was subsides. 5 ml of concentrated hydrochloric acid and 10 ml of deionized water was added and the sample was heated for additional 15 min without boiling. The sample was cooled and filtered through a Whatman No. 42 filter paper and diluted to 50 ml with deionized water.

Sample analysis: Digested samples were analysed for Pb and Cd, using flame atomic absorption spectrophotometer. All the measurements were run in triplicate for the samples and standard solutions.

Limit Test for Arsenic [7]

In the limit test for arsenic, the amount of arsenic is expressed as arsenic, as ppm.

Apparatus – A wide-mouthed bottle capable of holding about 120 ml is fitted with a rubber bung through which passes a glass tube. The latter, made from ordinary glass tubing, has a total length of 200 mm and an internal diameter of exactly 6.5 mm (external diameter about 8 mm). It is drawn out at one end to a diameter of about 1 mm and a hole not less than 2 mm in diameter is blown in the side of the tube, near the constricted part. When the bung is inserted in the bottle containing 70 ml of liquid, the constricted end of the tube is above the surface of the liquid, and the hole in the side is below the bottom of the bung. The upper end of the tube is cut off square, and is either slightly rounded or ground smooth. Two rubber bungs (about 25 mm x 25 mm), each with a hole bored centrally and true, exactly 6.5 mm in diameter, are fitted with a rubber band or spring clip for holding them tightly together. Alternatively the two bungs may be replaced by any suitable contrivance satisfying the conditions described under the General Test.

Analysis of the Mercury [11]-

Chemicals: Sulphuric acid, hydrogen peroxide, nitric acid, deionised water, Mercury metal. Apparatus: 1000 ml standard flask, 100 ml standard flask, 50 ml standard flask, Tissue papers, Whatman filter papers, Beakers, Hot plate, Electronic weighing machine, Pipette, Measuring jar. Preparation of Stock Solution (Mercury stock solution) 1.0g of mercury metal was dissolved in 20ml of conc. nitric acid by constantly stirring the volumetric flask. 1 litre was diluted in a volumetric flask with deionised water. $Hg + 4HNO_3$ (Conc.) --> $Hg(NO_3)_2 + 2NO_2 + 2H_2O$ Mercury does not react with non-oxidizing acids but does react with concentrated nitric acid, HNO_3, or concentrated sulphuric acid, H_2SO_4, to form mercury (II) compounds together with nitrogen or sulphur oxides. Mercury dissolves slowly in dilute nitric acid to form mercury (I) nitrate, mercurous nitrate, $Hg_2(NO_3)_2$.

Sample preparation

Sample preparation for analysis of Heavy metals in medicinal plants was done according Wet digestion method (AOAC 1995) for non-volatile heavy metals. Wet digestion involves the destruction of organic matter through the use of both heat and acid.

Procedure

1g of dried sample was weight accurately and it was placed in a beaker or digestion tube. 16 ml concentrated H_2SO_4 was added and the beaker was placed on hot plate and then temperature was gradually increased to 1250C at which the sample was boiled for 1hour. Beaker was removed and allowed cooling. 4 ml H_2O_2 (30%) was added and digested at the same temperature. As the reaction finished another 4 ml H_2O_2 (30%) was added. The mixture was heated till the digestion is complete. After cooling, the content was filtered into 100 ml volumetric flask using Whatman filter paper No.41 and the solution was completed to the mark using deionized water.

Sample analysis:

Digested samples were analysed for Mercury (Hg) using hydride generation technique as per ICH guidelines. Hg was analysed by cold vapour atomic absorption spectrometry. All the measurements were run in triplicate for the samples and standard solutions.

Microbial Limit Tests

Equipments and apparatus: Autoclave, Incubator, Hot air oven, heating mantle, UV chamber, Inoculation chamber, Colony counter, Microscope, Refrigerator, Bunsen burner, Spirit lamp, Micrometre (stage and ocular), Petri dishes, conical flasks, micropipette.

Reagents for examination

- Ethanol (95%) and distilled water.
- Nutrient Agar used as a general purpose agar for the culture of non-fastidious organisms.
- Potato dextrose Agar (PDA), a selective medium for the isolation of yeasts and fungi.
- **Sterilization of materials used-** All the glassware and media used were sterilized by autoclaving. The media were prepared in a conical flask plugged with cotton wool and wrapped with aluminium foil before autoclaving. Sterilization in the autoclave was carried out at 121°C for 15 minutes.
- **Plate count for bacteria:** Using Petri dishes 9 to 10 cm in diameter, a mixture of 1ml of the pre-treated extract preparation and about 15ml of liquefied potato digest agar at not more than 450 was added to each dish. Alternatively, spread the pre-treated extract preparation on the surface of the solidified medium in a Petri dish of the same diameter.
- If necessary, dilute the pre-treated extract preparation as described above so that a colony count of not more than 300 may be expected. At least eight such Petri dishes was prepared using the same dilution and incubate at 30^0 to 35^0 for 5 days, unless a more reliable count is obtained in a shorter time.
- The number of colonies was counted that are formed.

- The results was calculated using plates with the greatest number of colonies but taking 300 colonies per plate as the maximum consistent with good evaluation. Plate count for fungi:
- Proceed as described in the test for bacteria but use N. agar with antibiotics in place of potato digest agar and incubate the plates at 20^0 to 25^0 for 5 days, unless a more reliable count is obtained in a shorter time.
- The results was calculated using plates with not more than 100 colonies.

Table no. 01: Microbial contamination

Sr. No.	Parameters	Permissible limits for herbal extracts and Powders
1.	Total microbial plate count (TPC)	Not More Than 1000 cfu/gm

PHYTOCHEMICAL ANALYSIS

Qualitative analysis for active constituents:

- **Determination of Alkaloids:**

Mayer's test- Test sample substance shaken with few drops of 2N HCL. Aqueous layer formed, decanted and to which one or two drops of Meyer's reagent (Potassium Mercuric chloride solution) were added. A white precipitate was formed indicating the presence of alkaloids. (Fig. 14)

- **Determination of Tannins:** The sample mixed with basic lead acetate solution. A white precipitate was formed indicating the presence of Tannins in the extract. (Fig.15)
- **Determination of Flavonoids:**

Alkaline reagent test- When the plant extract treated with sodium hydroxide solution, there was increase in intensity of yellow colour which would colourless on addition of few drops of dilute hydrochloric acid; indicates the presence of flavonoids. (Fig. 16)

- **Determination of sugar:**

Fehling's solution test- The test sample was added with equal volume of Fehling A and Fehling B solutions and heated. There was formation of brick red precipitate of cuprous oxide; indicated the presence of reducing carbohydrate. (Fig.17)

- **Determination of Saponins:**

Frothing test- A few ml of sample was taken into a test tube and add up to 20 ml of water, shaken vigorously, then leave to stand for 10 min. A thick persistence froth was resulted; indicates that presence of saponins. (Fig. 18)

- **Determination of Phenols:**

FeCl3 test- Few drops 5% FeCl3 solution were added to 2-3ml of aqueous extract, the appearance of yellow colouration indicated the presence of Phenols. (Fig. 19)

- **Determination of Quinones:**

NaOH test- Few drops of NaOH were added to test sample; red colour indicates the presence of quinones. (Fig. 20)

- **Determination of Steroids:**

Sulphur powder test- A pinch of sulphur powder was added to the extract. The sulphur powder sinks at the bottom of the test tube; indicating the presence of steroids. (Fig. 21)

- **Test for protein:**

Biuret reagent test- To the extract, Biuret solution was added. The blue reagent turn into violet; indicates the presence of protein. (Fig. 22)

- **Test for terpenoids:**

Noller's test- Add Tin and thionyl chloride into the extract and warmed it. The orange colouration shows the presence of terpenoids. (Fig. 23)

OBSERVATION AND RESULTS

A. Pharmacognostical study and phytochemical study

- Raw material standardization

• Authentication of Sample Accession No. – 00788

- **Table no. 02: Results of Macroscopic examination of *Kalmegh*** (Fig. 24,25,26,27,28)

CHARACTERS	ROOT	STEM	LEAF	FLOWER	FRUIT
Colour	Dusty brown	Dark green	Green	White	Green capsule, with yellowish brown seeds
Odour	Characteristic bitter fumes	Characteristic bitter fumes	Characteristic bitter fumes	Not specific	Not specific
Taste	extremely Bitter	extremely Bitter	extremely Bitter	extremely Bitter	Extremely Bitter
Shape	Cylindrical, with longitudinal furrows	Quadrangular	lanceolated, pinnate, entire leaf margin	Small, solitary	linear-oblong, acute at both ends
Surface	Moderately Fine	Smooth and without hairs	Glabrous	Smooth	Not hairy
Texture	Hard to break	Fibrous	Brittle	Brittle	Fibrous

- **Microscopic examination** (Fig.-29, 30)

➢ T.S. of Stem of *Andrographis paniculata shows:*

- quadrangular outline with dense collenchyma strands at the four angles of the stem
- Epidermis is single layered of rectangular cells.
- Collenchymas cell zone is a group of 2–3 layered with secretary cavities having white coloured deposition.
- Cortex forms a narrow zone, with 5–6 layers of parenchymatous cells with chloroplast.
- Solitary sclereids and a group of sclereids of 4–6 are present in secondary phloem tissues.
- Vessels are small sized, mostly solitary and majority of them are arranged in radial rows. Vessels are circular or polygonal shaped. Wood with spiral, reticulate and pitted xylem vessels are revealed.
- Xylem occupies the major portion of the stem.
- The bulk of the xylem is constituted by fibres.
- Medullary rays are many in number. Rays are mostly uniseriate but occasionally biseriate rays are found.
- Prismatic crystals of calcium oxalate are present.

- **In-process standardization (Standardization of *Kalmegha Choorna*)**

- **Powder microscopy shows following characteristics-**

➢ Epidermal cells with stomata

- Epidermal cells with cystoliths
- Sclereids
- Reticulate vessels
- Spiral vessels
- Starch grains
- Calcium oxalate crystals

- **Physicochemical parameters**
- **Table no.03: Determination of Foreign Organic Matter %**

- **Table no.04: Determination of Physicochemical parameters**

Panchanga* of *Kalmegh	1.89%

Sr. No.	Tests	Powder of *Andrographis paniculata* Nees.
1.	**Particle size**	52µm
2.	**Moisture %**	3% at 100^0C
3.	**Total ash**	20%
4.	**Acid-insoluble ash**	5.217%
5.	**Water-soluble ash**	0.397 %
6.	**pH**	8.12
7.	**Alcohol-soluble extractive**	85%
8.	**Chloroform-soluble extractive**	90%

- **Preparation of CO_2 extract of *Andrographis paniculata* Nees.**

Amount of Powdered root drug taken = 1600gm

Weight of extract = 50gm

% yield of extract = 3%

C. Finished product standardization (CO_2 extract of *Andrographis paniculata* Nees.)

Table 4. Shows physicochemical parameters test of CO_2 extract of *Andrographis paniculata* Nees.

- **Table no. 05: Heavy Metal Tests:**

Sr.No.	Tests	Specifications	Observations
1.	**Total heavy metals**	Not more than 10 ppm	Complies
2.	**Lead**	Not more than 3 ppm	<1 ppm
3.	**Cadmium**	Not more than 0.3 ppm	<0.1 ppm
4.	**Arsenic**	Not more than 1 ppm	<5 ppb
5.	**Mercury**	Not more than 0.1 ppm	<2 ppb

- **Table no. 06- Microbial contamination:**

Sr.No.	Tests	Specification	Observations
1.	**Total plate count**	Not more than 1000 cfu/gm	20 cfu/gm
2.	**Total Yeast and Mould count**	Not more than 100 cfu/gm	< 10 cfu/gm
3.	**Salmonella**	Should be absent 25gm	Absent
4.	**E. Coli**	Should be absent 1gm	Absent
5.	**Enterobacteria**	Not more than 10^4 cfu/gm	Absent

Preliminary Phytochemical screening

Table no. 07-Shows quality analysis of CO_2 extract of *Andrographis paniculata* Nees.

Phytochemical Test	**Co_2 extract of *Andrographis paniculata* Nees.**
Alkaloids	
Mayer's test	Present
Tannins	
Lead acetate test	Absent
Flavonoids	
Alkaline reagent test	Absent
Saponins	
Froth test	Present
Quinones	
NaOH test	Absent
Sugars	
Fehling's test	Present
Steroids	
Sulphur powder test	Present
Phenols	
Fecl3 test	Present
Proteins	
Biuret test	Absent
Terpenoids	
Noller's test	Present

Fig.01- Morphology of *Kalmegh*

Fig. 02- Powder of *Kalmegh*

Fig. 03- powder microscopy of *Panchanga* of *Kalmegh*

Fig. 4- Ash with muffle furnace

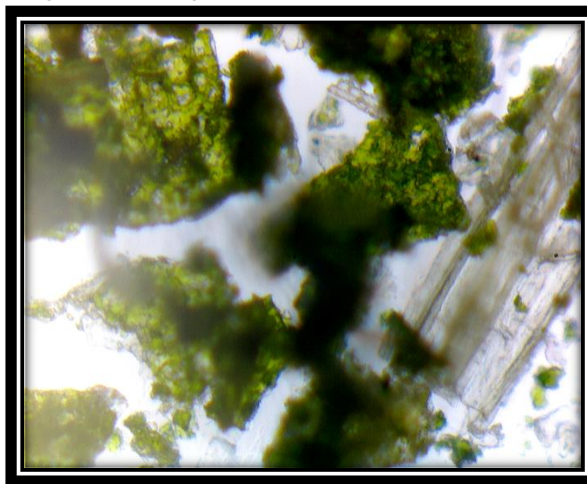

Fig. 5- Ash value with crucible

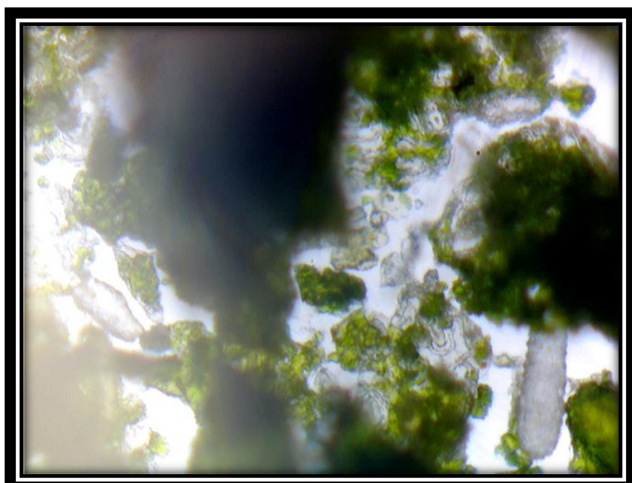

Fig.06- Acid insoluble Ash

Fig.07- Water soluble Ash

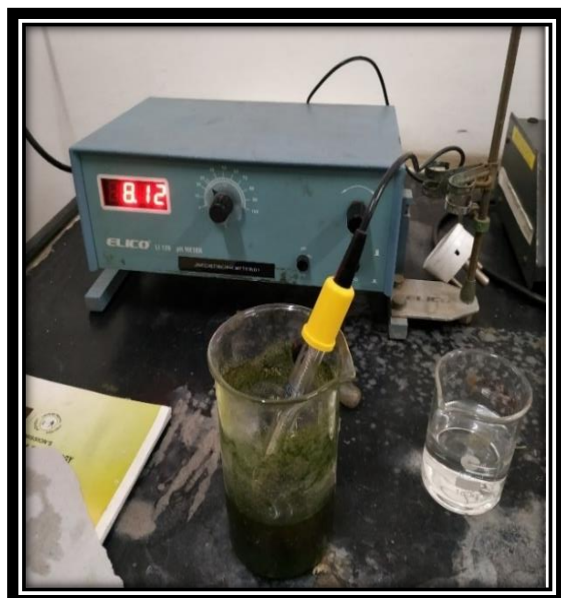

Fig. 08- pH of *Choorna* of *Kalmegh*

Fig. 09 - CO_2 Extractor Machine

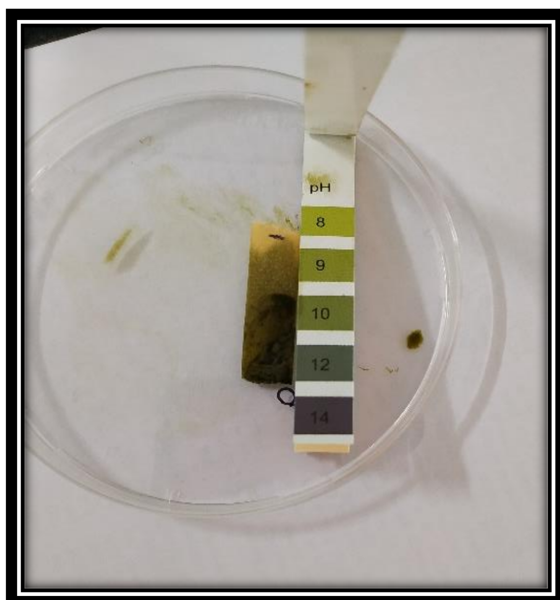

Fig.10- pH of CO_2 extract

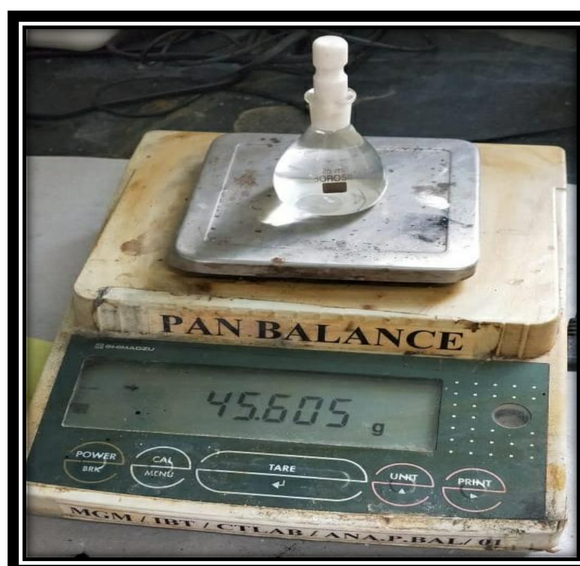

Fig.11-specific gravity of water

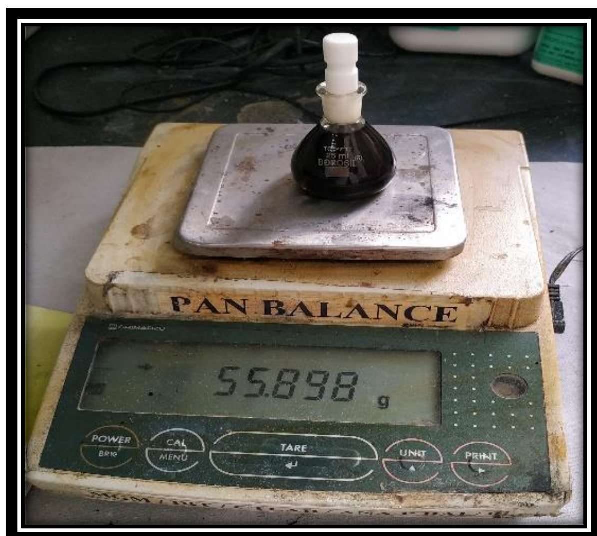

Fig.12-specific gravity of extract

Fig. 13-Viscosity of CO2 extract

Fig. 14- Mayer' test

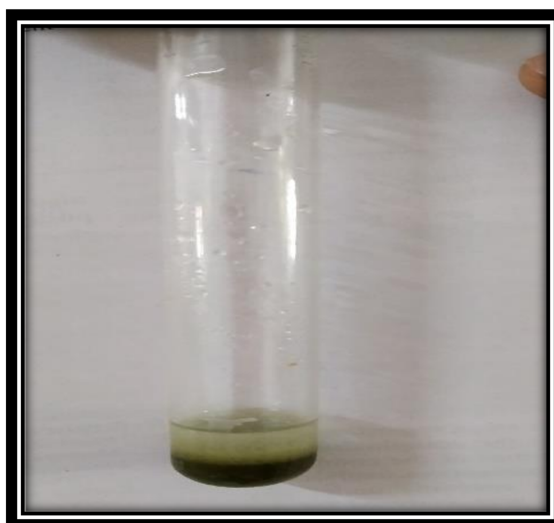

Fig. 15- test for tannins

Fig. 16- test for Flavonoids

Fig. 17- test for Sugars

Fig.18- test for Saponin

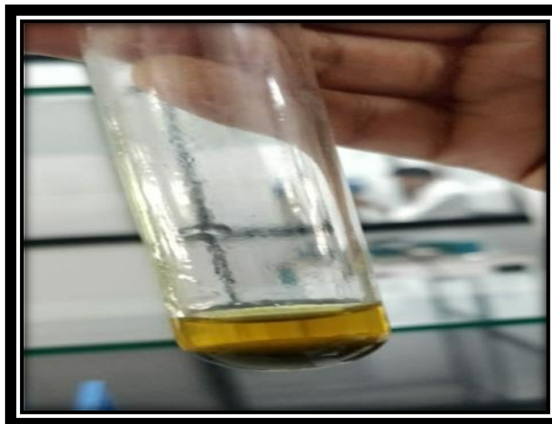

Fig.19- test for Phenols

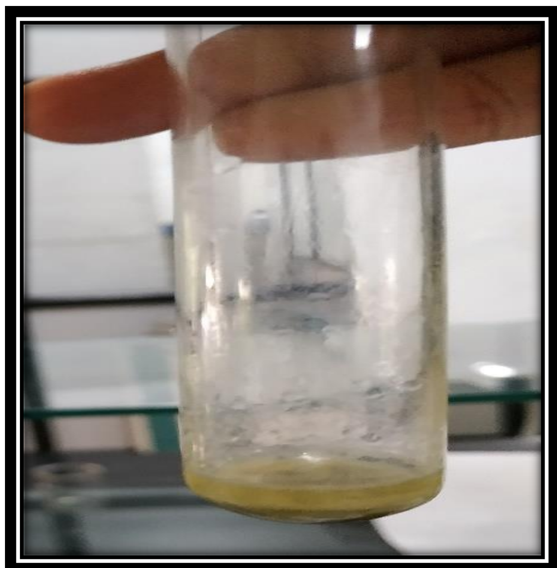

Fig. 20- Test for Quinone

Fig. 21- Test for Steroids

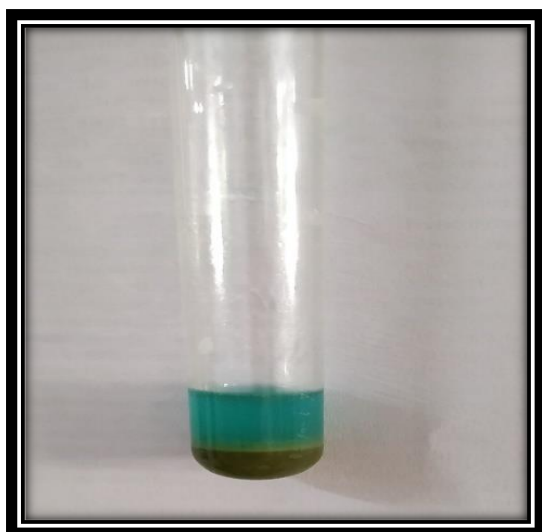

Fig. 22- test for Protein

Fig. 23- test for Triterpenids

Fig.24- Roots of *Kalmegh*

Fig. 25- Stem of *Kalmegh*

Fig. 26- Leaves of *Kalmegh*

Fig.27 - Fruits of *Kalmegh*

Fig. 28- Seeds of *Kalmegh*

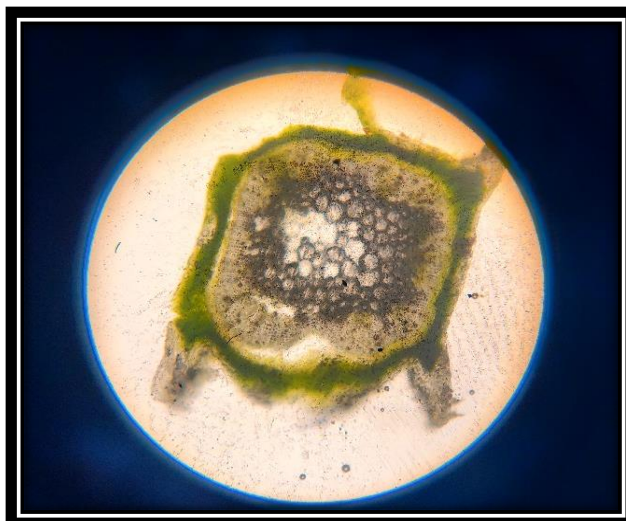

Fig.29 - T.S. of stem of *Kalmegh*

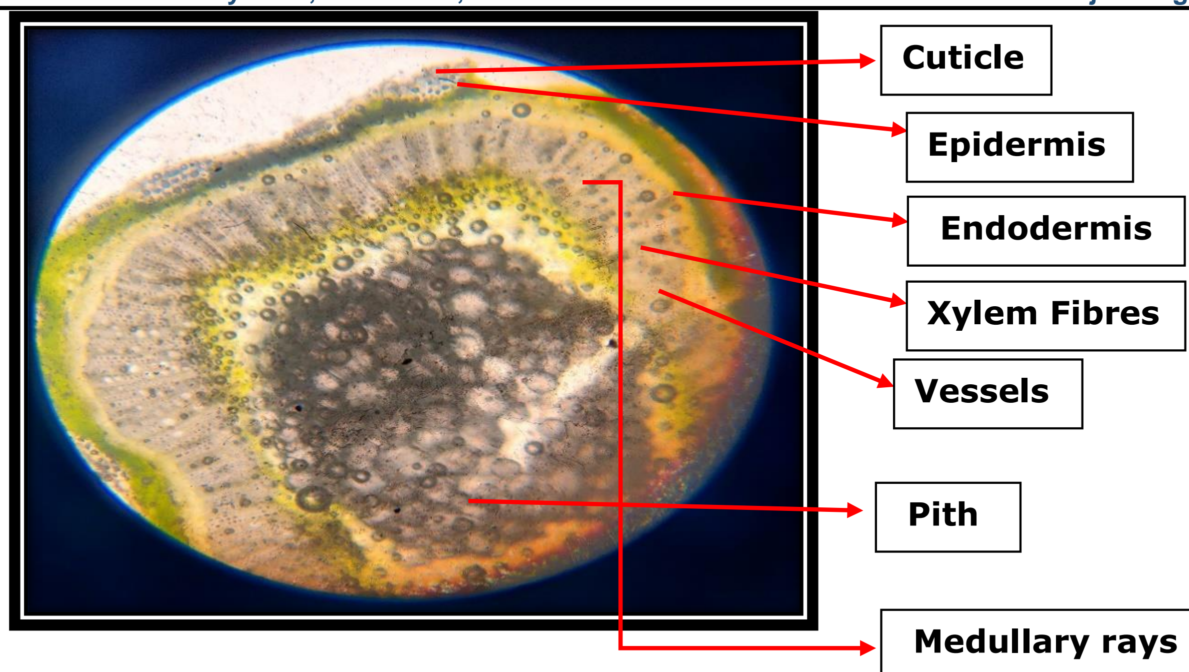

Fig. 30- T.S. of stem of *Kalmegh* details

DISCUSSION-

- **Raw material standardization**
- **Organoleptic Characters**:

Dry powder of *Andrographis paniculata* Nees.

Taste: Dry powder analysis of *rasa* follows *Tikta pradhana rasa.*

Colour: Dry powder of drug having green colour.

Odour: *Vishesha Gandha* (Characteristic bitter fumes)

Texture: rough, fibrous in Texture.

- **Microscopic Examination:**

Stem of *Kalmegh*-

Transverse section of stem shows that the quadrangular outline having dense collenchyma strands at the four edges of the stem, epidermis of rectangular cells, 2–3 layered Collenchyma cell zone with secretary cavities filled of white coloured deposition, narrow zone of Cortex, 5-6 layered parenchymatous cells with chloroplast, Solitary sclereids are present in secondary phloem tissue, Vessels are circular or polygonal in shape and arranged in a radial row. The major portion of the stem occupied by woody spiral xylem vessels, the bulk of the xylem is constituted by fibres and medullary uniseriate or occasionally biseriate rays, Prismatic crystals of calcium oxalate are also present. (Fig. 28, 29)

- **In process standardization**
- **Powder microscopy**

Under microscope following tissue elements were observed:
Epidermal cells with diacytic stomata, Sclereids cells, Calcium oxalate crystals, fragment of diacytic stomata, cystoliths are sharp at one end and blunt at the other, palisade cells of flower, group of starch grains, brown content, non-glandular multicellular trichomes and epidermal cells of leaf in surface view, fragment of pitted vessel of roots, Spiral vessels, fragment of covering Trichomes, multicellular sessile glandular trichomes. (Fig. 30)

Particle Size

Particle size through 40 mesh was 52µm in *Andrographis paniculata* Nees.

Foreign Matter - The physico-chemical analysis exhibited foreign matter 1.89% in *Panchanga* of *Kalmegh*.

Moisture content - Loss on drying of sample was 3% in *Andrographis paniculata* Nees.

pH value - The pH value of *Andrographis paniculata* Nees. sample was 8.12.

Ash values- In *Andrographis paniculata* Nees; the total ash content was 20%, acid- insoluble ash was 5.217% and the water soluble ash was 0.397%.

Alcohol soluble extractives:

The alcohol soluble extractive of *Kalmegh* was 85%.

Chloroform soluble extractives:

Chloroform soluble extractive value of *Kalmegh* is 90%.

- **Finished product (CO_2 extract) standardization-**

The CO_2 Extract of *Kalmegh* was prepared by "Supercritical carbon dioxide extraction method" also known as 'Cold Separation'. The final finished product was greenish to black in colour and sticky in nature.

pH-

The pH value indicates the potential hydrogen ions existing in particular substance. pH of CO_2 extract of *Kalmegh* was showed 10. That means, the pH of CO_2 extract of *panchanga* was alkaline in nature. **Specific gravity:**

Specific gravity is an important property of fluids being associated to density and viscosity. Knowing the specific gravity will allow determination of a fluid's characteristics compared to a standard, usually water, at a specified temperature. This will let the user to determine if the test fluid will be heavier or lighter than the standard fluid. Specific gravity of *Andrographis paniculata* Nees. was 1.225. **Viscosity:**

Viscosity is the measure of resistance of a substance to flow under an applied force. The viscosity of CO_2 extract of *Andrographis paniculata* Nees. was 0.77cp.

Microbial Limit Tests:

The following tests are designed for the assessment of the number of viable aerobic microorganisms present and for detecting the presence of designated microbial species in pharmaceutical substances. The term 'growth' is used to entitle the presence and presumed proliferation of viable micro-organisms. The microbial contamination in *Kalmegh* was found to be within limit.

Test for Heavy metals:

The heavy metal test for CO_2 extract of *Kalmegh* was within limits. i.e., Lead<1ppm, Cadmium <0.1ppm, Arsenic<5 ppb and Mercury <2 ppb. Thus, heavy metals like lead, cadmium, mercury and arsenic were not exceeding the normal limit in the extract.

Phytochemical analysis:

In CO_2 extract of *Kalmegh* Alkaloids, saponins, steroids, Terpenoids and Phenols were detected.

PREVIOUS RESEARCH DONE:

Table no. 08: Solvents used for active component extraction

Water [12]	Ethanol [13]	Methanol [14]	Chloroform[15]	Ether[15]	Acetone[16]	CO_2 Extract Of *Kalmegh*
Alkaloids Amino acids Tannins Saponins Flavonoids Glycosides	Alkaloids Glycosides Tannins phenols Flavonoids Terpenoids Saponins	Alkaloids Amino acids Saponins Phytosterols Polyphenols Tannins Flavonoids Glycosides Terpenoid	Alkaloids Saponins Terpenoids Flavonoids Protein Glycoside Phytosterols Tannin oils and fats	Alkaloids Terpenoids Glycosides Coumarins Fatty acids	Tannin Flavonoids Phenolic compound Steroids	Alkaloids Saponins Steroids Terpenoids Phenols

The CO_2 Extract of *Kalmegh* was prepared by "Supercritical carbon dioxide extraction method" involves using highly pressurised carbon dioxide at lower temperature (31ºC). The extraction is done at a low temperature ensuring all volatiles

remain in the extract. The above table shows that, the CO_2 Extract of *Kalmegh* have Alkaloids, saponins, steroids, Terpenoids and Phenols which are less as compared to methanolic and chloroform extract.

CONCLUSION

The present study is concerned with the standardization of the *Panchanga* of *Kalmegh.* The powder of *Kalmegh* showed Moisture content 03% at 100^0C, total ash 20%, acid insoluble ash 5.217%, water soluble ash 0.397%, alcohol soluble extractive 85%, chloroform soluble extractive 90% and pH value is 8.12. On the basis of the data obtained it was concluded the *Kwatha* is only form for this drug to administer and get better results. In phyto-chemical studies, the qualitative tests of CO_2 extract of *Kalmegh* (*Andrographis paniculata* Nees.) was done. It is modestly give in to that this study might guide to upcoming researchers to start standard for *Kalmegh* for strict quality control and assurance. CO_2 extracts of *Andrographis paniculata* Nees. contained different types of phytochemicals such as Alkaloids, Saponins, Steroids, Terpenoids and Phenols. In comparison with other solvents, CO_2 extract of *Kalmegh* showing less phytochemicals viz., Alkaloids, Saponins, Steroids, Terpenoids and Phenols. Since this plant must be used in the treatment of different disease such as anticancer, anti-inflammatory, antioxidant, anti-protozoal, antimicrobial, etc. We must therefore approach this plant for different therapeutic purposes on the basis of their bioactive compounds in order to be fully utilized.

LIMITATIONS OF THE CURRENT STUDY

Due to unobtainability of proper solvents the more desired phytochemical results could not be observed.

FURTHER SCOPE OF STUDY

1. Standardization of CO_2 extract of *Kalmegh* should be done with the help of more precise and advanced parameters of testing.

2. CO_2 extract of *Kalmegh* has contained different types of phytochemicals which suggest that it could be a potential source for treating various diseases.

3. The plant extract affecting several molecular pathways are needed to be analysed.

ACKNOWLEDGEMENT

None

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