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Original Article

Physico-phytochemical Evaluation of Raw Material and CO_2 extract of Fruits of *Terminalia chebula*

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ABSTRACT

The present communication attempts to evaluate the physicochemical and preliminary phytochemical studies on the fruit of *Terminalia chebula* Retz. Combretaceae family. *Haritaki* is one of the most celebrated herbs in the Indian traditional medicine system, Ayurveda. *Terminalia chebula* is known to exhibit different properties like anticancer, anti-inflammatory, anti-protozoal, antimicrobial, antioxidant, hepato and renal protective activities, and in the management of metabolic syndrome. As there is no detailed standardisation work reported on fruit, the physicochemical parameters, preliminary phytochemical constants, heavy metals, analysis are carried out. The phytochemical screening indicated the presence Tannin, Alkaloid, Phenol, Carbohydrate, Steroids, Protein and Resin compounds in CO_2 extract of *Haritaki*. The present investigation will helpful in assessing the quality and purity of a crude drug. Thus, the study provides facts that CO_2 extract of *Haritaki* contains medicinally important bioactive phytochemical compounds which justifies the use of plant species as conventional medicine for treatment of many diseases.

Keywords Physicochemical, Phytochemical, *Terminalia chebula* Retz, Adulterations

INTRODUCTION

Haritaki (*Terminalia chebula* Retz.), Family: Combretaceae have a great therapeutic value and is widely distributed in India. *Haritaki*, which has synonyms as *Abhayaa, Pathyaa* etc. is one among the major drug in the Ayurvedic system of medicine and its dried fruit is extensively used in various types of diseases.

Types

Depending on the type of fruits, *T. chebula (Haritaki)* is classified into seven types. Of these seven types, *Vijaya* is considered to be the best.

1. *Vijaya* - in *Vindhya* - oval in shape, which used in all diseases.
2. *Rohini* - Found everywhere, round in shape commonly used in *Vrana* (wound).
3. *Pootana* - *Sindh* - small and less bulky mesocarp is less, seed is bigger, externally used.
4. *Amruta* - *Champaranya* - bulky used for *Shodhanakarma* (cleansing).
5. *Abhaya* - *Champadesha* - fruit has five lines on it mostly used in eye diseases.
6. *Jeevanti* - *Saurashtra* - yellow in colour used in all diseases.
7. *Chetaki* - Found in the Himalaya - having three lines on it used as purgative. (Bhavaprakash nighantu, 1982)

In practice, however, there are three types of *Haritaki*:

1) *Bala Haritaki* - When the fruit of *Haritaki* falls off from the tree, the seed gets hard called '*Bala Haritaki'*. Sometimes, the fruits are collected and dried while the seeds have not hardened which are also called '*Bala Haritaki'*. It is small, dark brown or black in colour.
2) *Chambhari (Rangari) Haritaki* - *Chambhari Haritaki* is an immature fruit of *Haritaki.* It is small, less wrinkled and less furrowed than the above variety; in length about an inch; the epidermis is yellow.
3) *Survari Haritaki* - fully mature fruit of *Haritaki* is called *'Survari Haritaki'*. It is Large, dense and heavy about 2 inches long, yellowish brown in colour. (Rathinamoorthy R, *et al.*, 2014.)

Dried fruit of *Haritaki* contains high quantities phenolic compounds that consist of ellagic acid, gallic acid and chebulic acid. The fruit extract of *Terminalia chebula* is known to exhibit different properties like anticancer, anti-inflammatory, anti-protozoal, antimicrobial, antioxidant, hepato and renal protective activities, and in the management of metabolic syndrome. The phenolic active compounds might play vital role in the influence of biological activity.

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Purpose of study

Globalization, commercialization, and industrialization of *Ayurveda* lead to an adulteration of drug the efficacy of an herbal drug is directly proportional to its Identity, purity, quality. Therefore, Pharmacognostical & Phytochemical standardization of the drug is the need of time, so standardization of *Haritaki* has been included in our study. Efficacy of drug is directly proportional to identity, purity & potency of drug. So, for quality assurance standardization of drug is a need of time. Establishment of standards of quality, safety and efficacy, the attempts were made to establish physio-chemical standards of the plant *Haritaki* as per API. There are various previous research has been done on different extracts of *Haritaki* but there is no work available until today about phytochemical evaluation of CO_2 extract of *Haritaki*.

MATERIAL AND METHODS

A. Collection of sample

Fruits of *Haritaki* (*Terminalia chebula* Retz.) were procured from Organic Farm of Satara district, Maharashtra (India).

B. Authentication and voucher deposition

It was done in the Botany Department of Institute of Biosciences & Technology, MGM, Aurangabad, Maharashtra (India). A voucher specimen of the collected sample was deposited in the departmental museum for future reference (Accession no. HC0024).

C. Pharmacognostical, Physicochemical and Phytochemical study

It includes:

1. Raw material standardization
2. In process standardization
3. Finished Product standardization

Pharmacognostical study

Pharmacognostical study mainly deals with a plant which gives a complete knowledge about its method of identification and determination of quality and purity of the raw drugs. To authenticate that the fruit of *Haritaki* which have been used as raw material in this study is original and not adulterated or confused with any other drug, Pharmacognostical study is undertaken. Following the above views the Pharmacognostical study of '*Terminalia chebula Retz*.' was carried out in 'Pharmacognosy department' of authorised institute. Here *Terminalia chebula Retz*. is considered as the source plant of an important drug mentioned in *Ayurvedic* classic "*Haritaki*".

The parameters selected for Pharmacognostical study were as follows:

- Macroscopic Examination
- Microscopic Examination

1. Raw material standardization

Materials - Crude Sample of Fruits of *Haritaki (Survari Haritaki).*

Equipments

i. **Magnifying glass:** The magnifying glass has optical grade 3x and 6x magnifying capacity lenses was used.

ii. **Microscope:** Microscopes are used mainly in the laboratory for the tissues and organisms which are too small to be seen clearly with the naked eye.

iii. **Dissection box:** It included Watch glasses, Microscope glass slides (3"x1"), Cover slips ¾ circles, Camel hair brush (medium size), Pair of small forceps, Blades, Dropper, etc.

A. Macroscopic examination (WHO guidelines for Quality control methods for herbal materials, 2011)

Macroscopic identity of herbal materials is based on size, shape, colour, texture, surface characteristics, fracture characteristics and appearance of the cut surface.

- **Size**

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

- **Colour**

The untreated sample was examined under diffuse daylight. The colour of the sample was compared with that of a 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 determine its specific odour. Once the material has been examined and classified according to external characteristics, inspection by microscopy can be carried out as the next step.

B. Microscopic examination (Khandelwal KR, *et al.*, 2016)

- **Step I - Section Method**

Selection of appropriate size, part and 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 25cm 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 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- Dried Fruits of *Haritaki* (*Terminalia chebula Retz.*) (Fig.1)

Equipments

i. Mortar and Pestle (*Khalva-yantra*)

A black stoned *khalva-yantra* was used with longer diameter 25", shorter diameter 12" and depth 4.9". (Fig.2)

ii. Stainless steel container

SS vessels were used of 5L capacity.

Preparation of *Choorna* (Government of India Ministry of Health and Family Welfare Department of AYUSH. A.P.I, Part 2, Vol 4, (n.d), pp. 140)

The dried Fruits were powdered according to the standard procedure mentioned in *Sharangdhar Samhita*. (Fig.3)

Fig.1 Raw fruit of *Haritaki*

Fig.2 Course powder of *Haritaki*

Fig.3 Powder of *Haritaki*

Physico-chemical Study

1) **Determination of foreign matter** (Shriradhakrushna Parashar, 2012)

The foreign matter in a sample is determined by taking accurately weighed 5g sample and cleaning it form all kind of impurities such as dust, mud, dried seed, leaves etc. if necessary the sample is rinsed carefully with distilled water and dried properly. It was examined by using 6x or 10x magnifying glass and separated the foreign matter. Then the sample is again weighted to see the weight change. The loss in weight compared to that of the initial is the amount of foreign matter present in the sample and calculated as % w/w.

2) **Powder microscopy** (Khandelwal KR, *et al.,* 2016)

For detection of Organ or part of the plant present and it's identifying Microscopical characteristics. 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 – The type of cells, the nature of cell walls present, Cell content.

3) **Determination of total ash** (Gaidhani SN, *et al.,* 2009)

About 5g of air dried powdered of *Haritaki* a fruit was taken in a weighed crucible. It was then heated over a burner until all the carbon was burnt off at a temperature not exceeding 450°C. Then it was cooled in desiccators and weighed, exhaust the burned mass with hot water, then collect the residue on ash less filter papers, and incinerate the residue and filter paper, add the filtrate, evaporate to dryness, and ignite and calculate the percentage of total ash with reference to air dried sample of the crude drug. (Fig.4)

Fig.4 Muffle furnace

4) **Acid insoluble ash**

The ash obtained in the previous experiment was boiled with 25ml of 2N hydrochloric acid. The solution was filtered using an ash less filter papers. The residue was allowed to cool in suitable desiccators for 30 minutes, and then weighed without delay. The weight of the crucible was measured to get acid insoluble ash. (Fig.5)

Fig.5 Hot plate with crucible

Calculation: Weigh 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 [100Xa / y] gm of acid insoluble ash.

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

5) **Water-soluble ash (**Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water. Add 25 ml of water to the crucible containing the total ash, and boiled for 5 minutes. The insoluble matter was collected in a sintered-glass crucible or on an ash less filter paper. Wash it with hot water and ignited in a crucible for 15 minutes at a temperature not exceeding 450°C. Subtract the weight of this residue in mg from the weight of total ash. The content of water-soluble ash in mg per g of air-dried material was calculated.

6) **Determination of moisture content (**Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

5g of powdered of *Haritaki* fruit drug was taken in a weighed flat porcelain dish and was dried for 30 min under the same conditions to be employed in the determination. The loaded dish was placed in the drying chamber. The test specimen was dried at 105^0 for 3 hours and weighed. Continued the drying and weighing at half an hour interval until difference between two successive weighing corresponds to, not more than 0.25 per cent.

7) **Determination of pH value** (Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

The pH value conventionally represents the acidity or alkalinity of a drug. This test was performed to determine the pH of the sample drug with the help of a pH meter. 5g of the experimental drug sample was weighed and taken with a call flask. Then add 100 ml of precise water and stir well for a few minutes; kept the solution for a while and then sorted it through a filter paper. Take the filtered solution to the beaker. Set the pH meter and electrodes with a known pH buffer solution i.e. pH. Clean the electrodes with clean water and apply the test solution contained in a small beaker. Read the pH value of the solution.

8) **Water-soluble extractive value** (Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

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 (chloroform water). 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, it was transferred 25ml of weighed, thin porcelain dish, as used for the ash values determinations. (fig.6) It was evaporating 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.

Fig.6 Hot plate with conical flask

9) **Alcohol-soluble extractive value** (Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

Method: 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%

3. Finished product standardization

Preparation of CO_2 extract of Fruit of *Haritaki* (Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

- **Material –** Powdered form of fruit of *Haritaki*. *Choorna* will be prepared according to the guideline mentioned in *Sharangdhar Samhita* and subjected to standardization.
- **Equipments –** CO_2 Tank, Pump, R/M input, PRV, Cyclone Separator, Collection vessel.

Preparation of CO_2 Extract

First of all CO_2 which will be stored in tank is pumped to the extractor where pressure will be maintained at 300 bar (=296.077atm) and at the temperature 304 K (31°C). In the extractor, liquid CO_2 and feed come in contact with each other and extraction of essential component from feed will be carried out. (Fig.7, 8). The product steam which contains CO_2 and essential component goes to the cyclone separator through PRV (Pressure relieving valve), due to deduction in pressure PRV liquid CO_2 is converted into gas. In cyclone separator CO_2 gas and essential component (liquid form) are separated from each other. From the top of the cyclone separator CO_2 is recycled back to the tank, before sending it to the tank, CO_2 which will be in gaseous state is condensed with the help of condenser. From the bottom of the cyclone separator essential components will be obtained. (Fig.9)

Fig.7 Core Powder

Fig.8 CO_2 Extractor Machine

Fig.9 CO_2 extract of *Haritaki*

Preparation of CO_2 extracts of Fruit of *Haritaki* (*Terminalia chebula*) were subjected to following tests

- **pH** (Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

This test is carried out to determine the pH of the test drug with the help of pH meter. 10g of the experimental drug sample was weighed and taken with call flask. Then add 50 ml of precise water and stir well for a few minutes; kept the solution for a while and then sorted it through a filter paper. The filtered solution is taken from the 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. (Fig.10)

Fig.10 pH meter

- **Specific gravity** (Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

Specific gravity of a liquid is determined by the use of a specific gravity bottle. (Fig.11) 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.

Fig.11 Pycnometer

- **Viscosity** (Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

U tube Viscometer was used to determine the viscosity. (fig.12) The test fluid is filled into the U tube viscometer according to the expected viscosity of the liquid so that the liquid level stands within 0.2 mm of the viscometer filling mark where the capillary is stationary and the specified temperature is detected. The fluid is absorbed or pumped to a set viscometer weight and the time taken for the meniscus to exceed the specified two marks is measured. The kinematic viscosity in centistokes is calculated from the following equation:

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.

Fig.12 U tube Viscometer

- **Microbial contamination** (Government of India Ministry of Health and Family Welfare Department of AYUSH, n.d)

Equipments and apparatus - Autoclave, Incubator, Hot air oven, heating mantle, UV chamber, Inoculation chamber, Colony counter, Microscope, Refrigerator, Bunsen burner, Spirit lamp, Micrometer (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

- To use Petri dishes 9 to 10 cm wide, a mixture of 1ml of pre-treated extract and 15ml of liquefied potato agar no more than 45^0 are added to each container. In addition, we spread out the previously treated remedies on the surface of the solid surface in a Petri container of the same width.
- If necessary, dilute the pre-treated extract discharge preparation as described above in anticipation of a maximum of 300 colonies. At least eight such Petri vessels were prepared using the same purification and incubation at 30^0 to 35^0 for 5 days, unless the most reliable figure was obtained in a short 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.

- **Heavy metal tests**

Analysis of the Arsenic (Meenakshi N *et al*., 2014**)**

- **Chemicals:** Sulphuric acid, nitric acid, hydrogen peroxide, Arsenic powder, deionised water.
- **Apparatus:** 50 ml standard flask, 100 ml standard flask 1000 ml standard flask, Whatman filter papers, Tissue papers, beakers, hot plate, Pipette, measuring jar, electronic weighing machine.
- **Preparation of Stock Solution (Arsenic stock solution):** 1.0g of arsenic powder was dissolved in 50ml conc. Nitric acid by constantly stirring the volumetric flask. 1 litre was diluted with deionised water. Arsenic makes arsenic acid with concentrated nitric acid, arsenious acid with dilute nitric acid.
- **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. **(***Official methods of analysis of AOAC International,* 1995).
- **Procedure:** Accurately 1.0 g of dried sample was weighed and it was placed in a beaker. 16 ml concentrated H_2SO_4 was added and the beaker was

placed on hot plate and then temperature was gradually increased to 125^{0}C at which the sample was boiled for 1 hour. 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 Arsenic (As) using hydride generation technique as per ICH guidelines. All the measurements were run in triplicate for the samples and standard solutions.

Analysis of the Mercury (Meenakshi N *et al*., 2014**)**

- **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.
- **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 weigh 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 125ºC at which the sample was boiled for 1 hour. 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 measurements were made three times in standard samples and solutions.

Analysis of the Lead and Cadmium (Singh *et al*., 2014)

- **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 are ground in a water-soluble form. 10 ml of nitric acid was added to 2 g of precisely dried sample in a 100 ml beaker and heated on a 95 ° C hot plate for 15 minutes. The mill was cooled and 5 ml of concentrated nitric acid was added and heated for another 30 minutes at 95° C. The last step was repeated and the solution was reduced to 5 ml without boiling. The sample was cooled and added 2 ml of contaminated water and 3 ml of 30% hydrogen peroxide. As the beaker covered, the sample was slightly heated to initiate the peroxide reaction. If the efficiency is too strong, the sample is removed from the hot plate and 30% hydrogen peroxide is added to the 1 ml increase, followed by a gentle warming until the power dissipates. 5 ml of concentrated hydrochloric acid and 10 ml of deionized water were added and the sample was heated for another 15 minutes without boiling. The sample was cooled and filtered through Whatman No. filter paper No. 42 and diluted in 50 ml of dirty water.
- **Sample analysis:** Samples were converted into Pb and Cd, using a fire atomic absorption spectrophotometer. All measurements were made three times in standard samples and solutions.

PHYTOCHEMICAL ANALYSIS

Qualitative analysis for active constituents:

1. **Saponins foam test -** 5ml of the test solution was taken in a graduated cylinder and distilled water was added and shaken. The stable foam formation indicates the presence of saponin.
2. **Tannin ferric chloride test -** 2 ml of test solution, a few drops of dilute solution of ferric chloride added. The dark blue colour indicates the presence of hydrolysable tannins.
3. **Alkaloids test -** Approximately 50 mg of each extract was dissolved in 5 ml of distilled water and 2M of hydrochloric acid was added until an acid reaction occurred and filtered. Filtrate tested for alkaloids:
   a. **Wagner's test:** In a few drops of extract dissolved in acetic acid, a few drops of Wagner reagent are added. The reddish-brown rain indicates the presence of alkaloids.
   b. **Mayer's test:** In a few drops of extract dissolved in acetic acid, a few drops of mayer's reagent are added. The dull white formation formed indicates the presence of alkaloids.
   c. **Hager's test:** In a few mg of extract dissolved in acetic acid, 3 ml of Hager reagent is added, the formation of a yellow precipitate indicates the presence of alkaloids.
4. **Glycoside test Legal test -** 2ml of extract with diluted hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate was subjected.
5. **Phenols test -** To the extract in alcohol, added two drops of alcoholic ferric chloride. Formation of blue to blue black indicates the presence of phenol.
6. **Carbohydrate test -**
   a. Fehling's test: A few mg extracts were combined with an equal amount of Fehling A and B solution. The mixture was heated in a water bath. The formation of red brick rain indicates the presence of carbohydrates.
   b. Benedict's test: In 5 ml of Benedict reagent, a few mg extracts were added, and boiled for two minutes and cooled. The formation of red rainfall indicates the presence of carbohydrates.

7. **Steroids test** - Salkowski test: The extract was dissolved in chloroform and an equal volume of conc. Sulphuric acid was added. The formation of blue to red cherry on the chloroform layer and green fluorescence on the acid layer indicates the presence of steroids.
8. **Test for proteins** - Biuret's test: In 1 ml of hot hydro-alcoholic drug add 5-8 drops of 10% w / v sodium hydroxide solution followed by 1 or 2 drops of 3% w / v copper solution sulphate. Available in red or violet.
9. **Test for carboxylic acid** - The ingredient dissolved in water is treated with sodium bicarbonate. Brisk effervescence indicates the presence of carboxylic acid.
10. **Test for coumarins** - In the extraction of alcohol, a few drops of 2 N sodium hydroxide solution were added. The formation of a dark yellow colour indicates the presence of coumarins.
11. **Test for Resin** - A few mg samples were mixed with water and acetone. Turbidity indicates the presence of resin.
12. **Test for Quinine** - A few mg of alcohol extraction was treated with 0.5% sodium hydroxide. Deep colours such as pink, purple, or red indicate the presence of quinine.

OBSERVATION AND RESULTS

A. Pharmacognostical study and phytochemical study

- Raw material standardization
- Authentication of Sample
  Accession No. – HC0024

Fig.13 Fruits of *Terminalia chebula*

- **Macroscopic examination**

Table 1. Results of Macroscopic examination

Sr. No.	CHARACTERS	Specifications
1.	Colour	Blackish brown
2.	Odour	Characteristic
3.	Taste	Astringent
4.	Size	Length:2-4.5 cm and Diameter: 1.2-2.5cm
5.	Shape	Oblong, ovoid or club shaped
6.	Surface	Wrinkled surface
7.	Texture	Hard to break

Microscopy of fruit:

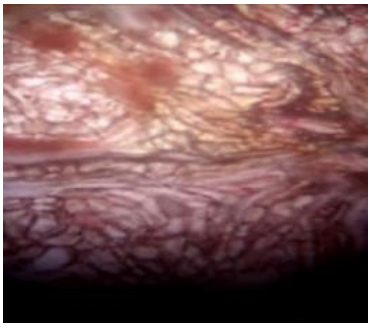
T.S fruit show somewhat wrinkled outline and following parts are seen;

1) **Epicarp**: It is single layered thick epidermal cells. It is made up of tubular or polygonal cell. Presence of cuticle. (Fig.14)
2) **Mesocarp:** It is made up of 2-3 layers of tangentially elongated collenchymatous cell. It follows broad zone of Parenchymatous cell (Fig.17). Vascular are occasionally seen. Sclerides are seen in the region presence of raphides are also seen. In addition, simply starch grain (2-7µ) and vascular fibres are also seen. (Fig.15)
3) **Endocarp:** It is innermost part which is very hard supports the Mesocarp and Epicarp.(Fig.16)

B. In-process standardization (Standardization of *Haritaki Choorna*)

Table 2. Microscopy of Powder

Character	Observation
Colour	Brownish
Sclereids	With wide lumen
Mesocarp cell (Fig.19)	Loosely arranged
Rosette crystal	Present
Tannin (Fig.21)	Moderately distributed
Stone cells	Isolated pitted stone cell with wide lumen
Fibres (Fig.20)	Less



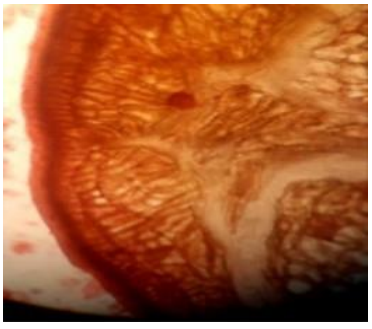


Fig.14 Epicarp, Epidermis and Parenchyme cell

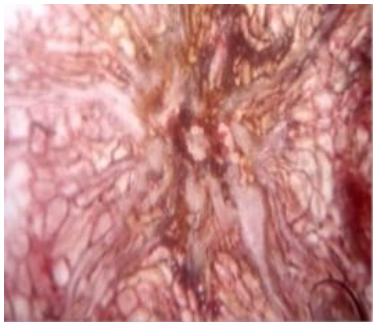


Fig.15 Mesocarp

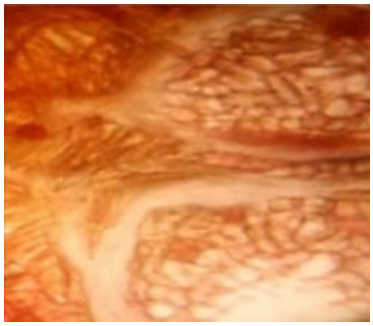


Fig.16 Endocarp

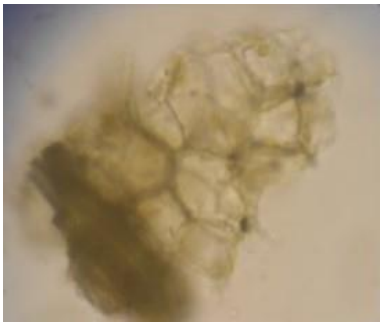


Fig.17 Parenchymal cell

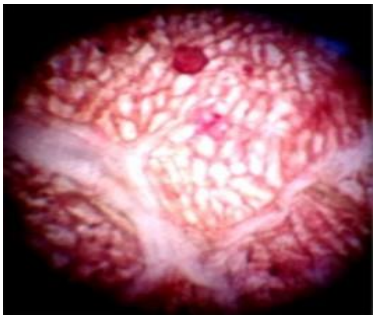


Fig.18 Oil Globules

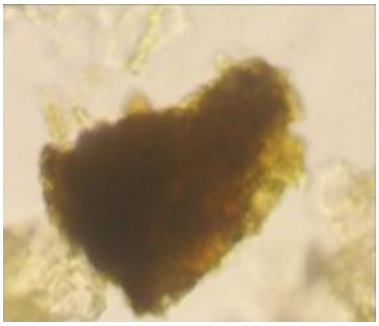


Fig.19 Mesocarp Cell

Fig.20 Fibres

Fig.21 Tannin content

Physicochemical parameters

Table shows no. 3. Physicochemical parameters test (Raw material) of *Haritaki*

Table 3. Physicochemical parameters test (Raw material) of *Haritaki*

S.No.	Parameter of crud drug	Values of *Terminalia chebula Retz.*	Standard value as per API
1.	Foreign Matter	0.9%w/w	Not more than 1%
2.	Total Ash value	3.67%	Not more than 5%
3.	Acid insoluble ash	2.85 %	Not more than 5%
4.	Water soluble ash	42.8%	Not less than 40%
5.	Moisture content	6%	-
6.	pH value	3.79	-
7.	Water soluble extractive	64.2139% w/w	Not less than 60%
8.	Alcohol soluble extractive	46.6275% w/w	Not less than 40%

Preparation of CO_2 extract of *Haritaki*

Solvent: CO_2 in liquid form

Herb (powder of *Haritaki* fruit) to Extract Ratio = 165:1

% yield of extract = 0.60 %

C. Finished product standardization (CO_2 extract of *Haritaki*)

Table 4. Shows physicochemical parameters test of CO_2 extract of *Haritaki*

S. No.	Parameter	Values of
1.	pH	3.75
2.	Specific gravity	0.537
3.	Viscosity	1.235 cp

Table 5. Shows test for Microbial Contamination (CO_2 extract)

S. No.	Parameters	Readings	Standard value
1.	Total microbial test count	<10 cfu/g	Not more than 1000 cfu/g
2.	Total yeast and mould count	<10 cfu/g	Not more than 100cfu/g
3.	Salmonella	Absent	Should be absent in 25g
4.	Escherichia coli	Absent	Should be absent in 1g
5.	Enterobacteria	Absent	Not more than 10^4cfu/g

Table 6. Shows test for heavy metals (CO_2 extract)

S.No.	Parameters	Readings	Standard value
1.	Total Heavy Metals	Below detective limit	Not More Than 10 ppm
2.	Lead	Below detective limit	Not More Than 3 ppm
3.	Arsenic	Below detective limit	Not More Than 1 ppm
4.	Mercury	Below detective limit	Not More Than 0.1 ppm
5.	Cadmium	Below detective limit	Not More Than 0.3 ppm

Preliminary Phytochemical screening

Table 7. Shows quality analysis of CO_2 extract of *Haritaki*

S.No.	Test for	CO_2 Extract
1.	Saponin test	Negative
	Foam test	
2.	Tannin test	Positive
	Ferric chloride test	
3.	Alkaloids test	Positive
	Wagner's test	
	Mayer's test	
	Hager's test	
4.	Glycoside test	Negative
	Legal test	
5.	Phenol test	Positive
	Ferric chloride test	
6.	Carbohydrate test	Positive
	Fehling's test	
	Benedict's test	Negative
7.	Steroids test	Positive
	Salkowski test	
8.	Proteins test	Positive
	Biuret's test	
9.	Carboxylic acid test	Negative
	Sodium bicarbonate test	
10.	Coumarins test	Negative
	Sodium hydroxide test	
11.	Resin test	Positive
12.	Quinine test	Negative

Fig.22 Wagner test

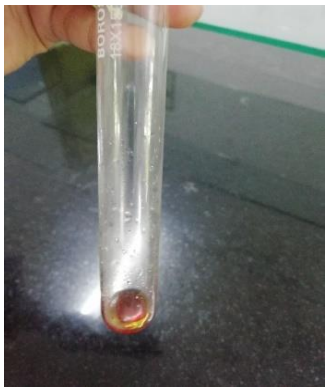


Fig.23 Hager test

Fig.24 Mayer test

Fig.25 Salkowiski test **Fig.26** Resin test **Fig.27** Tennins and Phenol

Fig.28 Fehling test **Fig.29** Biuret Test

DISCUSSION

Raw material standardization

After proper identification, Fruit of *Terminalia chebula* Retz. Was studied macroscopically, microscopically and organoleptically. Macroscopic study of fruit is blackish-brown, oblong, ovoid or club shaped, Length – 2-4.5 cm and Diameter - 1.2-2.5cm, wrinkled surface, texture is hard to break, characteristic odour and astringent in taste. Microscopic study of *Terminalia chebula* Retz. fruit Shows, Epicarp, mesocarp and endocarp which is matching with the API standards.

Organoleptically, In *Sparsha Pareeksha* the touch was rough. The sample of *Rupa* was Blackish brown colour. Prominent *Rasa* of sample was *Kshaya*. It was having characteristic odour. *Shabda Parikshan* of *Haritaki* indicated the maturity of fruit of *Haritaki*.

In process standardization

The powder of fruit of *Terminalia chebula* Retz. Is Brownish in colour, under microscope shows a Mesocarp cells, rosette crystal of calcium oxalate, few fibres, vessels with simple pits and groups of sclereids.

- **Foreign Matter -** The physico-chemical analysis exhibited foreign matter 0.9% in Fruit of *Haritaki*. According to API, the foreign matter limit for fruit not more than 1%. In that way the sample was found below allowed limit.
    - **Ash values** - Total ash value was 3.67% w/w, Acid insoluble ash value was 2.85% w/w, and Water soluble ash was 42.8% and all these ash values got match with API standard. The standard value of total ash and acid insoluble ash should not more than 5% whereas the standard value of water soluble should not less than 40%. This determines the quality and purity of crude drugs especially in powder form.
    - **Moisture content -** The test was conducted to determine for the measurement of percentage of water present in sample. In this sample moisture content value was 6% w/w. The Reported value should not more than 9.0 % at 105º C. The value indicated that the sample was free from fungi.
    - **pH value -** The pH value of sample was 3.79 %. pH is determines hydrogen ion concentration. This value clearly shows that drug is acidic in nature.
    - **Water soluble extractive -** Water soluble extractive was 64.2139% w/w while API standard for water soluble extraction not less than 60%.
    - **Alcohol soluble extractive -** Alcohol soluble extractive was 46.6275% w/w while API standard for alcohol soluble not less than 40%.

Standardization of Finished product (CO_2 extract of *Haritaki*)

The CO_2 Extract of fruit of *Haritaki* was prepared by "Supercritical carbon dioxide extraction method" also known as 'Cold Separation'. The CO_2 Extract is blackish brown in colour and in paste form.

- **pH value -** The pH value of CO_2 extract of *Haritaki* was 3.75 %. pH is determines hydrogen ion concentration. This value clearly shows that drug is acidic in nature.
- **Specific gravity -** Specific gravity is commonly used as a simple means of obtaining information about the concentration of solutions of various materials. Those with specific gravity greater than 1 are denser than water and will, disregarding surface tension effects, sink in it. Those with specific gravity 1 or less than 1 are less dense than water and will float on it. The specific gravity of CO_2 extract of fruit of *Terminalia chebula* is less than 1.
- **Viscosity -** Viscosity of a liquid is constant at a given temperature and is an index of its composition. Hence it can be used as a means of standardizing liquid drugs. Viscosity of CO_2 extract of fruit of *Terminalia chebula* is 1.235.
- **Microbial limit tests –** The following tests are designed for the estimation of the number of viable aerobic microorganism present and for detecting the presence of designated microbial species in pharmaceutical substances. The microbial contamination was within limit as mentioned in API.

- **Heavy metals –** Heavy metals like Lead, Arsenic, Mercury and Cadmium were not exceeding the normal limit in CO_2 extract of *Haritaki*.
- **Phytochemical analysis** - In CO_2 extract of fruit of *Terminalia chebula* following constituent like Tannin, Alkaloid, Phenol, Carbohydrate, Steroids, Protein, Resin were detected.

Previous research done

Qualitative phytochemical screening revealed that Alkaloids were detected in both aqueous and methanolic extracts of fruit. Saponins and glycosides were present in all extracts; however glycosides were not detected in Borntrager test. Volatile oil was only detected in the methanolic and aqueous extracts (Vemuri PK, *et al.*, 2019).

The phytochemical compositions of water, methanol, and 95% ethanol extracts of the air-dried fruit of *T. chebula* Retzius. The three extracts of *T. chebula* were the richest of phenolic compounds compared with total triterpenoid and tannin content (Chang CL *et al.*, 2012).

Acetone extract of T. chebula has been reported to contain phytochemicals with promising antimutagenic and anticarcinogenic properties (Arora S, *et al.*, 2003).

The CO_2 Extract of fruit of *Haritaki* 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. As compared to other extracts, the result shows more phytoconstituents are present in CO_2 extract of *Haritaki (T. chebula*).

CONCLUSION

The present study is concerned with the standardization of the fruit of *Haritaki*. Pharmacognostical study includes macroscopy and microscopy of drug which shows that the tested sample matched with API standards. The powder of fruit showed Moisture content 10%, total ash 3.67%, acid insoluble ash 2.85%, water soluble ash 42.8%, water soluble extractive 64.2139% and alcohol soluble extractive 46.6275% and pH value is 3.79. On the basis of the data obtained it was concluded that formulations like *Kwatha*, *Phanta* or *Hima* are the suitable forms for the drug to administer and get better results. In phyto-chemical study the qualitative tests for various functional groups with CO_2 extract of fruit of *Haritaki* (*Terminalia chebula* Retz.) was done. It is humbly submitted that this study might guide to forthcoming researchers to establish standard for fruit of *Haritaki* for strict quality control and assurance. CO_2 extracts of *Terminalia chebula* Retz. contained different types of phytochemicals such as Tannin, Alkaloid, Phenol, Carbohydrate, Steroids, Protein, Resin. Since this plant had been used in the treatment of different disease such as anticancer, anti-inflammatory, antioxidant, anti-protozoal, antimicrobial, etc, the medicinal roles of this plant can be related to such bioactive compounds. 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 unavailability of proper solvents the more desired phytochemical results could not be observed.

Further scope of study

1. Standardization of CO_2 extract of Fruit of *Terminalia chebula* Retz. should be done with the help of more precise and advanced parameters of testing.
2. CO_2 extract of fruit of *Haritaki* has contained different types of phytochemicals which suggest that it could be a potential source for treating various diseases.
3. The plant extract affecting various molecular pathways are needed to be analysed.

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None

CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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