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COMPARATIVE EVALUATION OF SOXHLET AND MACERATION EXTRACTS OF TERMINALIA CHEBULA RETZ PHYTOCHEMICAL PROFILING, PHYSICOCHEMICAL PARAMETERS, TOTAL PHENOLIC CONTENT BY DOUBLE-BEAM UV-VISIBLE SPECTROSCOPY, CHROMATOGRAPHIC CHERACTREIZATION (TLC FINGERPRINTING) AND ANTIMICROBIAL ACTIVITY

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ABSTRACT

Terminalia chebula Retz. (Haritaki), of family Combretaceae, is an important medicinal plant and its fruits are used in traditional medicine for various pharmacological activities. Preliminary phytochemical screening was carried out to determine the major phytoconstituents present in both the extracts. Chromatographic fingerprinting was conducted by thin layer chromatography. Total phenolic content was determined by Folin - ciocalteu method using double beam uv- visible spectrophotometry. The antimicrobial activity was assessed by the agar well diffusion method against selected bacterial strains. Both extraction method produced extracts with different phytochemical constituents such as carbohydrates, protein, alkaloids, amino acid, saponins. Etc. the chromatographic profiles differed among the extracts, indicating variation in the extracted phytoconstituents. The Soxhlet extract showed relatively higher phenolic content and antimicrobial activity than the maceration extract under the experimental conditions. To summarize, the results demonstrate that the extraction method affect the phytochemical compassion, phenolic content, chromatographic profile and antimicrobial properties of terminalia chebula fruit. The study provides useful preliminary information for selection an appropriate extraction method for further phytochemical and pharmacological investigation of Haritaki.[1-8]

KEYWORDS: Haritaki, Soxhlet extraction, Maceration, secondary metabolites, phenolic compounds, Terminalia chebula.

INTRODUCTION



(figure1.1)

Haritaki (*terminalia chebula Retz*) is very much appreciated in ayuvada for its ability to prevent as well as cure diseases. It is the most important of medicinal herbs in India from ancient times. It is called as the "king of medicines" and always listed as the first ingredient in ayuvada for its extraordinary therapeutic properties. In ayuvada seven varieties of Haritaki fruits are described. Belong to the family Combretaceae, number of species in the world. it is found in sub-Himalayan region from raw Eastwood to west Bengal and assam ascending to altitude of 1500m in Himalayas. Leaves, fruit are used in treatment of many diseases like inflammation, constipation, ulcer, diabetes, wount, work as hepatoprotective ..., etc.[1-5]

PROFILE OF HARITAKI

COMMON NAME: Haritaki / chebulic myrobalan
SCIENTIFIC NAME: terminalia chebula Retz
FAMILY: Combretaceae

HISTORICAL BACKGROUND OF HARITAKI:

Haritaki belongs to the family Combretaceae and has a long history of use in traditional medicine. It has been traditionally used in ayurveda and folk medicine systems for treating various ailments. The plant is commonly known as "king of medicines" because of its traditional use in constipation, ulcer, and hepatoprotective. In Indian traditional medicine, it has been used for diarrhoea, skin infection, ulcer and as a hepatoprotective. These long-standing traditional applications have contributed to growing scientific interest in Haritaki and encouraged investigations into its phytochemical composition, pharmacological activities, and therapeutic potential.[1-5]

TAXONOMICAL CLASSIFICATION:

Common names: Haritaki / chebulic myrobalan **Scientific name:** terminalia chebula Retz **Kingdom:** plantae **Sub-kingdom:** Tracheobionata **Infrakingdom:** streptophyta **Division:** Magnoliophyta **Subdivision:** viridiplantae **Infradivision:** spermatophytina **Class:** Magnoliopsida dicotyledons **Subclass:** Rosidae **Order:** Myrtales **Family:** Combretaceae **Genus:** Terminalia **Species:** chebula

GEOGRAPHICAL DISTRIBUTION

Haritaki is widely distributed in hotter parts of India, Sri Lanka, Bhutan, Napal, Bangladesh, Myanmar, Cambodia, Laos, Vietnam, and Thailand, often in the forest. In (south and southeast Asia) India, it is native in Tamilnadan, Karnataka, Kerala, Maharashtra, Bihar, and west Bengal. It prefers dry and humid conditions, from sea leave up to 1500-1800meters altitude. It commonly grows in tropical and subtropical regions, particularly in dry deciduous and mixed forests of south and southeast Asia.[40-43]

PLANT DESCRIPTION

Seeds: globose, 2-6 cm long
Stem: bark is usually 6 mm
Leaves: sub-opposite ovate or oblong ovate, 8-20cm long, deciduous in the cold season.
Flowers: the flower is yellowish white and fragrant, produced in large compound inflorescences.
Fruits: ovoid, 25-38 mm long the unripe fruits are brittle bodies, black and ovoid, and shrive led.

PHYTOCHEMICAL CONSTUENTS

(Table 1.1)

phenolic acids	Caffeic acid, ferulic acid, protocatechuic acid, syringic acid.
Flavonoids	Quercetin**,** luteolin, rutin, catechin, and kaempferol.
Hydrolysable tannins	Chebulic acid, Chebulinic acid, chebulagic acid, gallic acid, ellagic acid, Corilagin, punicalagin, and terchebulin.
Triterpenes and glycosides	Arjun Olic acid, maslinic acid, arjungcoside-1, and chebulosides1and two.
Sterols and volatile compounds	beta-sitosterol, daucosterol, palmitic acid, and various essential oils.

MOTHEDOLOGY

COLLEACTION AND AUTHENTICATION OF PLANT MATERIAL

Fresh and disease- free fruit (HARITAKI) were collected from a tree(forest) and the plant thoroughly to remove dust and foreign matter. The authenticated by a from the department of botany, (government arts college). the botanist examined the fruit and confirmed it as Haritaki. Based on its morphological characteristics. After authentication, the plant material was used for further processing.[10-17]

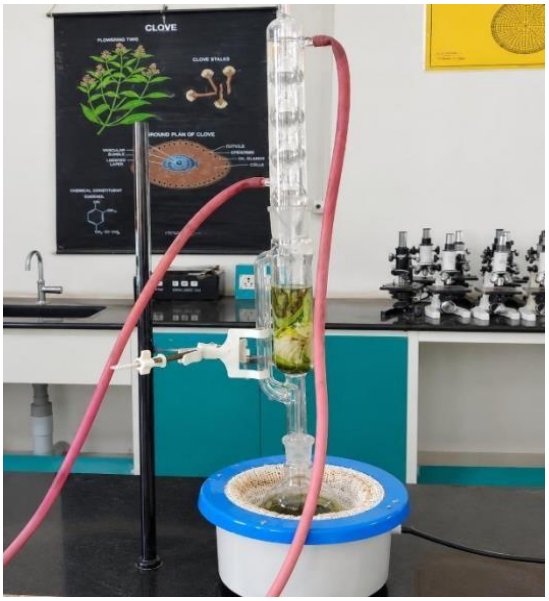
Drying and sieving

The cleaned fruit were shade-dried at room temperature for 10-15 days to prevent degradation of heat- sensitive phytoconstituents. After complete drying, the fruit were powdered using a mechanical grinder. The powder material was sieved with **mesh size72** to obtain a uniform particle size and stored in airtight containers until extraction.[5]



(figure1.2)

Extraction of fruit (powder) material: Soxhlet extraction



(fig 1.3)

Maceration extraction

The dried powder of Haritaki was subjected to extraction using ethanol as the solvent. The extraction was collected using a Soxhlet apparatus. The obtained extract was concentrated using a rotary evaporator under reduced pressure and controlled temperature. The extract was stored in an airtight container and kept under refrigeration for further analysis.[5-15]



(fig1.4)

The dried powder of Haritaki was subjected to extraction using ethanol as the solvent. The extraction was collected using a conical flask or glass container. Seal the container tightly to prevent evaporation and let it stand at room temperature for 5-7 days, shaking and swirling the mixture intermittently each day. The obtained extract was concentrated using a rotary evaporator or water bath under reduced pressure and controlled temperature.to yield a concentrated, semisolid crude extract. The extract was stored in an airtight container and kept under refrigeration for further analysis.[5-10]

PHYTOCHEMICAL EVALUVATION OF TWO EXTRACTIONS: CARBOHYDRATE

Molisch's test

Take few ml of sample in a test tube. Add 2-3 drops of Molisch's reagent and mix well. Then add cons H2SO4 along the side of the test tube. Formation of a violet/purple ring at the junction indicates carbohydrates.[5]

Benedict test

Take few ml of sample in a test tube. add 2-3 drops of benedict's reagent and mix well. Heat gently in a water Both for a few minutes in the result development of a ppt formed indicates reducing sugars.[21]

Fehling test

Take few ml of sample in a test tube. add 2-3 drops of Fehling A and B reagent and mix well. Heat gently in a water Both for a few minutes in the result development of a brick red ppt formed indicates reducing sugars.[5-21]

ALKALOIDS

Dragendorff test

Take few ml of sample in a test tube. add 2-3 drops of Dragendorff reagent and mix well. Formation of an orange red ppt indicates alkaloids.

Wagner test

Take few ml of sample in a test tube. add 2-3 drops of Wagner reagent and mix well. Wait for few minutes. Formation of an orange red ppt indicates alkaloids.

Hager test

Take few ml of sample in a test tube. add 2-3 drops of Hager reagent and mix well. Then add picric acid along the side of the test tube. Wait for few minutes. Formation of an orange red ppt indicates alkaloids.

AMINO ACID

Ninhydrin test

Take few ml of sample in a test tube. add 2-3 drops of Ninhydrin reagent and mix well. Heat gently in a few minutes in the result development of a violet/blue colour indicates amino acids.[21]

PROTEINS

Biuret test

Take few ml of sample in a test tube. add 2-3 drops of sodium hydroxide and mix well. Then add CUSO4 along the side of the test tube. Wait for few minutes. Formation of a violet/purple colour indicates proteins.

Xanthoproteic test

Take few ml of sample in a test tube. add 2-3 drops of conc. HNO3 reagent and mix well. Heat gently in a few minutes in the result development of a yellow colour indicates proteins.[5-21]

Millon test

Take few ml of sample in a test tube. add 2-3 drops of conc. HNO3 reagent and mix well. Heat gently in a few minutes in the result development of a yellow colour indicates proteins.[5]

SAPONIN

Foam test

Take few ml of sample in a test tube. Add 8-10ml of water and mix well. Shake gently in a few minutes in the result development of a foam (stays for above 10-15 minutes) indicates saponin.[5

GLYCOSIDES

Borntrager Test

Take few ml of sample in a test tube. add 2-3 drops of Dil.Hcl and mix well. Then add NH3 along the side of the test tube. Wait for few minutes. Formation of a red colour indicates glycosides.

PHENOLIC COMPOUNDS

Phenol test

Take few ml of sample in a test tube. add 2-3 drops of Fecl3 and mix well. Wait for few minutes. Formation of a violet colour indicates phenol.

STEROIDS

Salkowski test

Take few ml of sample in a test tube. add 2-3 drops of Conc.H2SO4 and mix well. Then add chloroform along the side of the test tube. Wait for few minutes. Formation of a reddish-brown colour indicates steroids.

TANNINS

Lead Acetate Test

Take few ml of sample in a test tube. add 2-3 drops of lead acetate solution and mix well. Wait for few minutes. Formation of a cream ppt indicates tannins.

Ferric chloride test

Take few ml of sample in a test tube. add 2-3 drops of Ferric chloride solution and mix well. Wait for few minutes. Formation of a green colour indicates tannins.

Potassium dichromate test

Take few ml of sample in a test tube. add 2-3 drops of Potassium dichromate solution and mix well. Wait for few minutes. Formation of a deep brown ppt indicates tannins.

PHYSICOCHEMICAL ANALYSIS

MOISTURE CONTENT AND TOTAL SOLUBLE SOLID

Accurately weigh 5 g of the powder sample. Keep the sample in a hot air oven maintained at 105°c. dry the sample for approximately 5 hours. Remove the sample and weigh it at 30- minute intervals. Continue drying and weighing until a constant weight is obtained. Allow the dried sample to cool to room temperature in a desiccator. Record the final weight of the dried sample. Calculate the moisture content using the weight loss before and after drying.

Calculate formula

- Weight of empty petri dish = W1(g)
- Weight of drug sample =X(g)
- Weight before drying (petri dish +sample) =W3=W1+X
- Weight after drying = W2 (g)
- Loss on drying (%) =((W3-W2) ×100)/X

DETERMINATION OF PH

The PH of an extract of Haritaki. was determined using a calibrated digital pH meter. This analysis given an indication of the acidity or alkalinity of the solution.

Procedure

Calibrate the PH meter using standard buffer solution. Switch on the PH meter and allow it to stabilize. Immerse the electrode in the buffer solutions for calibration. Prepare a 10% aqueous solution of the sample. Dip the electrode into the sample solution. Record the pH value.

DETERMINATION OF ASH VALUES

Total ash

Accurately weigh 5g of powdered Haritaki. Transfer the sample uniformly into a silica crucible. Incinerate the sample in a muffle furnace at 450°c. continue heating for approximately 6 hours or until carbon-free ash is obtained. Remove the crucible and allow it to cool in a desiccator. Weight the crucible containing the ash and record the final weight.

- Weight of empty crucible = W1(g)
- Weight of the sample =X(g)
- Weight of crucible with ash=W2(g)
- Total ash (%) =((W2-W1) /X) ×100

ACID- INSOLUBLE ASH

Boil the total ash with 25 ml of 2 M hydrochloric acid for 5 minutes. Filter the insoluble matter through a Gooch crucible. Wash the residue thoroughly with hot water. Ignite at a temperature not exceeding 450 °c for 15 minutes. Cool the crucible in a desiccator. Weigh the crucible and record the final weight.

- Empty Gooch crucible = W1(g)
- Weight of the sample =X(g)
- Acid-insoluble ash =W3=W2-W1
- Crucible with insoluble ash = W2 (g)
- Acid-insoluble ash (%) =W3/X ×100

WATER SOLUBLE ASH

Boil the total ash with15ml of distilled water for 5 minutes. Filter the insoluble residue through a Gooch crucible. Wash with hot water and ignite at ≤450°c for 15 minutes. Cool in a desiccator and weigh the insoluble residue. Calculate water- soluble ash by subtracting the insoluble residue weight from the total ash.

- Empty crucible = W1(g)
- Weight of the sample =X(g)
- Total ash=A (g)
- Acid-insoluble ash =W3=W2-W1
- Crucible with insoluble ash = W2 (g)
- Weight of insoluble ash =W3=W2-W1
- Water-soluble ash=W4-W3
- Water soluble ash (%) =A-[(W3)/X] ×100

ESTIMATION OF TERPENOIDS BY CHROMATOGRAPHIC TECHNIQUE:

PRINCIPLE

TLC is based on the differential absorption and partition of the individual components of a mixture between a stationary phase and a mobile phase. The stationary phase generally silica gel or alumina is coated as a thin layer on a suitable support, while the mobile phase consists of an appropriate solvent or solvent mixture. this differential migration results in the of the components into distinct spots on the TLC plate, which can be visual.

The component with more affinity towards stationary phase-travels slower The component with less affinity towards stationary phase-travels faster.

METHODOLOGY

Mobile phase

Prepare mobile phase by the mixture of

Stationary phase (Absorbent)

Prepare stationary phase by using silica G as an absorbent.

Preparation

Prepared slurry is poured on the microplate uniformly without any fluctuation. Allow the coated plate to dry. Pre- coated TLC plates may also be used.

Activation of TLC plates

Activate the TLC plates by removing moisture through air drying or an over at 100-110°c for about 30minutes.

Preparation and application of sample

Dissolve the sample in suitable volatile solvent and apply a small spot on the origin line (1-2 cm from the bottom) using a capillary tube.

Development of chromatograph
Place the TLC plate in a saturated development chamber containing the selected mobile phase. The solvent moves upward by capillary action, separating the sample components according to their affinities.

$$RF = \frac{\text{(Distance travelled by solute)}}{\text{(Distance travelled by solvent)}}$$

ESTIMATION OF PHENOL CONTENT IN TWO EXTRACTS OF HARITAKI

Procedure

- Take 0.1 ml of fruit extract
- Add one drop of folin-ciocalteu reagent(10%v/v)
- To stand for 15 minutes.
- Add 2 ml of saturated sodium carbonate solution.
- Mix well and incubate for 30 minutes at room temperature. Measure the absorbance at 760 nm using a UV-visible spectrophotometer.
- Prepare a calibration cure using gallic acid standard solutions (2,4,6,8, and 10 ug/ml). calculate the TPC of both extracts from the calibration curve and compare the result.

Formula
Total phenolic content = C×V/M Where:
TPC=total phenolic content (mg TAE/g extract)

C=concentration obtained from the Gallic acid calibration curve (mg GAE/ml) V=volume of extract used (ml)
M=Weight of the plant extract(g)

EVALUVATION OF ANTIMICROBIAL ACTIVITY ON VARIANTS OF HARITAKI:

Methodology
Antimicrobial activity of the microbial extract was determined by well-cut method. The microbial extraction was used in different concentration such as 5ul, 10ul, 15ul, 20ul and gentamycin (20ul) used as control. The well was filled with microbial extract on MHA (muller-Hindon agar) medium. The MHA medium inoculated with isolated bacterial pathogens. The inoculated plates were then incubated for about 18-24hours at 37Oc. the diameter of the circular zones of inhibition were measured after incubation.[22]

Procedure
Prepare microbial cultures and adjust turbidity Speare cultures on sterile agar plates.
Place sterile discs on the agar.

Add each extract to the wells or discs.

Incubate plates at the required temperature for 24-48 hours. Measure the zones of inhibition in millimetres.
Compare antimicrobial activity of maceration and Soxhlet extracts.

RESULT AND DISCUSSIN

Phytochemical test
Phytochemical tests are qualitative chemical test used to detect the presence or absence of various bioactive chemical constituents in plants, such as alkaloids, flavonoids, tannins, saponins, and glycosides.

Evaluation of phytochemical test (table1.2)

Phytochemical test	Test	Soxhlet extraction	Maceration extraction	Interpretation
Carbohydrates	Molish test	Purple appear rings	Purple appear rings	**Both extracts contain carbohydrates**
	Fehling test	Brick red ppt	Brick red ppt	
alkaloids	Wagner test	Reddish brown ppt	Reddish ppt brown	**Both extracts contain alkaloids**
	Hager test	Yellow ppt	Yellow ppt	
Amino acid	Ninhydrin test	Blue develops colour	Violet develops colour	**Both extracts contain Amino acid**
Proteins	Biuret test	Purple develops colour	Violet/purple colour develops	**Both extracts contain proteins.**
	Millon test	White ppt	White ppt	
Saponin	Foam test	Foam stays for above 10-15 minutes	Foam stays for above 10-15 minutes	**Both extracts contain saponin**
glycosides	Borntrager test	Red develops colour	Red develops colour	**Both extracts contain glycosides**
Phenol	Phenolic test	Violet colour	Violet colour	**Both extracts contain phenol**
tannins	Lead acetate	Cream ppt	Cream ppt	**Both extracts contain tannins**
	Ferric chloride test	Green develops colour	Green develops colour	

PHYSICOCHEMICAL ANALYSIS

MOISTURE CONTENT AND TOTAL SOLUBLE SOLID

Calculate formula

- Weight of empty petri dish = 57.68gm
- Weight of drug sample =5gm
- Weight before drying (petri dish +sample) =62.18gm
- Weight after drying = 4.5gm
- Loss on drying (%) =10%

DETERMINATION OF PH

pH value of sample of Haritaki (**Soxhlet-extraction)**:3.9

pH value of sample of Haritaki (**Maceration-extraction)**:3.6

DETERMINATION OF ASH VALUES

Total ash

- Weight of empty crucible = 40g
- Weight of the sample =5.00g
- Weight of crucible with ash=41g
- Total ash (%) =20%

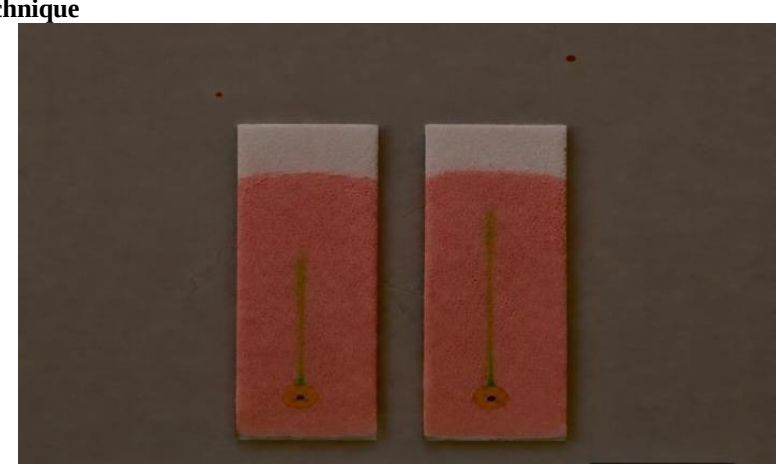
ACID- INSOLUBLE ASH:

- Empty Gooch crucible = 40gm
- Weight of the sample =5gm
- Acid-insoluble ash = 0.12gm
- Crucible with insoluble ash = 40.12gm
- Acid-insoluble ash (%) = 2.4%

WATER SOLUBLE ASH

- Empty crucible = 31gm
- Weight of the sample =5gm
- Total ash=0.72gm
- Acid-insoluble ash = 0.31gm
- Crucible with insoluble ash = 31.31gm
- Water soluble ash (%) = 8.2%

Chromatographic technique



It is a chromatographic technique used to separate, identify, and analyse the different chemical constituents present in a mixture. The TLC profiles of the Soxhlet and maceration extracts were compared with the reference standard (**Ursolic acid)** (fig1.5) to assess the presence of terpenoid constituents.

Calculate for thin layer chromatography

(table1.3)

FOR Soxhlet- extraction	**FOR Maceration- extraction**
Travelling of mobile phase –8 cm Travelling of solute –6.4 cm	Travelling of mobile phase –8 cm Travelling of solute –5.5 cm
RF=distance travelled by solute/distance travelled by solvent =6.4/8 =0.80	RF=distance travelled by solute/distance travelled by solvent =5.5/8 =0.6875≈0.69

INTERPRETATION

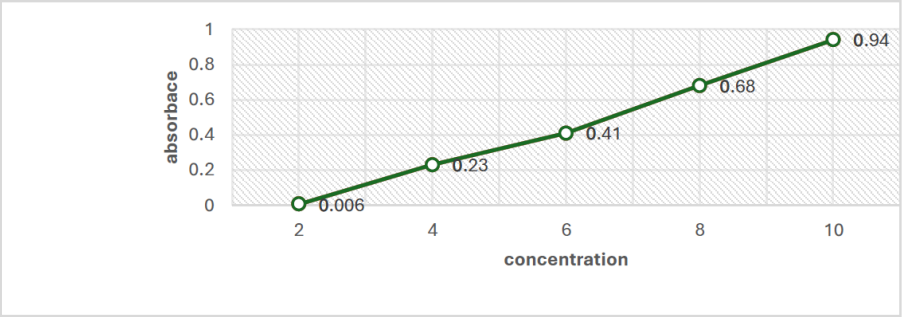
Terpenoid is a fruit derived from fruit that can produce a compound that produces Défense mechanism and improves the medicinal value such as anti-inflammatory, anti-microbial activities due to enhancing the metabolic activity and inducing biosynthesis of secondary metabolites. The extracts showed different TLC profiles with Rf values of 0.80 and 0.68.

TOTAL PHENOLIC CONTENT

The total phenolic content (TPC) is the total amount of phenolic compounds in a fruit extract or sample, measured using the folin-ciocalteu method which forms a complex with the sample.

Total phenolic content
(Table 1.4)

FOR Soxhlet extraction	FOR Maceration extraction
TPC=C×V/M =2.99×10/0.1 =299µg/ml (or) 0.299mg GAE/ml	TPC=C×V/M =2.97×10/0.1 =297µg/ml (or) 0.297mg GAE/ml

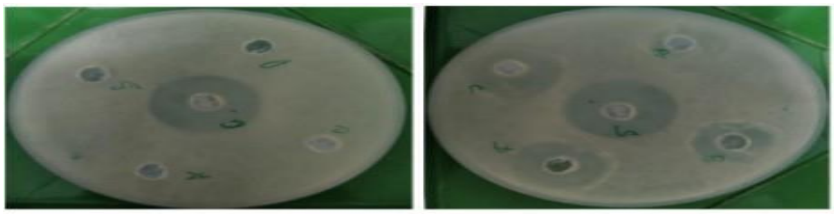


(fig 1.6)

Interpretation
Soxhlet extracted have more phenolic content than Maceration extracted Haritaki is a secondary metabolite that protect from fruit from insects as well as the light exposure induce the phenylpropanoid pathway.

ANTIMICROBIAL ACTIVITY
Antimicrobial activity is the ability of a fruit extract to inhibit or kill the growth of microorganisms, such as bacteria and fungi.

Soxhlet extraction



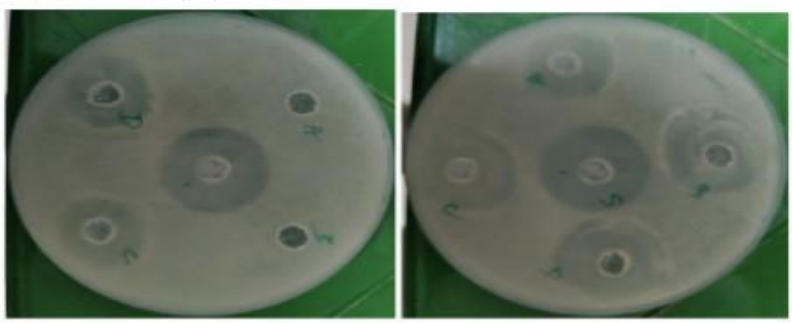
(fig1.7)

Zone of inhibition Soxhlet extraction
(table1.5)

Microbes	5µl	10µl	15µl	20µl	standard
Staphylococcus Aureus	9mm	14mm	17mm	19mm	21mm
E. coli	8mm	10mm	13mm	16mm	22mm

Mean for zone of inhibition
Staphylococcus Aureus =9+14+17+19/4 =14.75mm
E. coli = 8+10+13+16/4 =11.75mm

Maceration extraction



(fig1.8)

Zone of inhibition Maceration extraction
(table1.6)

Microbes	5µl	10µl	15µl	20µl	standard
Staphylococcus Aureus	7mm	11mm	14mm	16mm	21mm
E. coli	6mm	8mm	11mm	14mm	22mm

Mean for zone of inhibition.
Staphylococcus Aureus =7+11+14+16/4 =12mm
E. coli = 6+8+11+14/4 =9.75mm

DISCUSSION

Phytochemical screening:

s.no	Evaluation	Soxhlet extraction	Maceration extraction
1.	**Test for Carbohydrates**	++	++
	Test for alkaloids	++	+
	Test for Amino acid	+++	++
	Test for Proteins	+++	+++
	Test for saponin	+++	+++
	Test for glycosides	++	+
	Test for Phenol	+++	++
	Test for tannins	++	+
2.	**Total phenolic content**	+++	++
3.	**Terpenoids content**	+++	++
4.	**Antimicrobial activity**	+++	++

Soxhlet extraction vs Maceration extraction
(table1.7)
+++ =indicating the presence of constituents in higher level
++ =indicating the presence of constituents in moderated level
+ =indicating the presence of constituents in low level

Soxhlet extraction
Evaluated positive for Proteins, Carbohydrates, Amino acid, Saponin, glycosides, Phenol and, tannin.

Maceration extraction
indicated the presence of Proteins, Carbohydrates, Amino acid, Saponin, glycosides, Phenol and, tannin.

Physicochemical analysis
The sample is found to be stable and has a normal moisture content. The total Ash value was found to be within standard limits, which confirms the authenticity and purity of the sample. Furthermore, extractive values within the stipulated range indicate the non-existence of adulteration or depleted plant material.

TLC Analysis
Thin layer chromatography of the Soxhlet extract and Maceration extract revealed distinct bands at Rf values of 0.8 and 0.68.

TOTAL PHENOLIC CONTENT analysis
The extract of Haritaki present a phenolic content in separated from Soxhlet extraction and Maceration extraction at 0.99 and 0.97.

Antimicrobial activity
Soxhlet extraction has more anti-microbial activity than Maceration extraction due to the variation in the composition of phytochemical constituents, which has ability to damaging the cell membrane of microorganism.

CONCLUSION

The present comparative study showed that the extraction method effects the phytochemical profile, phenolic content, TLC fingerprint and antimicrobial activity of terminalia chebula Retz. The total phenolic content of the Soxhlet extract was slightly higher (299µg/ml=0.299 mg GAE/ml) than that of maceration extract (297 µg/ml=0.297GAE/ml). TLC Fingerprinting of the extracts, using Ursolic acid as a reference standard, showed distinct chromatographic profiles with Rf values of 0.80 for Soxhlet and 0.69 for maceration extracts. The anti-microbial evaluation revealed that the Soxhlet extract showed higher activity with inhibition zone of 14.75 mm against staphylococcus aureus and 11.75mm against Escherichia coli, whereas the maceration extract showed inhibition zones of 12mm and 9.75mm, respectively. These results suggest that Soxhlet extraction under the studied candied conditions gave relatively higher phenolic content, unique TLC characteristics and antimicrobial activity. The extraction technique is, thus, an important parameter affecting the recovery and biological properties of phytoconstituents from T. chebula fruit.

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