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Exploring the Antimicrobial Properties of *Pippali* (*Piper longum*) and *Haritaki* (*Terminalia chebula*) Against Human Microflora

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Abstract

This study investigates the potential antimicrobial properties of *Pippali* (*Piper longum*) and *Haritaki* (*Terminalia chebula*) against human microflora. Microbial infections pose a significant threat to human health, and the search for alternative antimicrobial agents has gained prominence. *Pippali* and *Haritaki,* two traditional medicinal plants in Ayurveda, have been historically recognized for their diverse pharmacological properties. The aim of this research is to assess the antimicrobial efficacy of *Pippali* and *Haritaki* extracts against a range of human-associated microorganisms. The study employs various microbiological techniques, including agar disc diffusion assays, minimum inhibitory concentration (MIC) and Minimum lethal concentration(MLC) determination, to evaluate the inhibitory effects of *Pippali* and *Haritaki* extracts. Additionally, the potential synergistic or additive effects of combining these herbal extracts with conventional antimicrobial agents are explored. The investigation also delves into the phytochemical composition of *Pippali* and *Haritaki* extracts, aiming to identify specific bioactive compounds responsible for their antimicrobial activity. Preliminary findings suggest that *Pippali* and Haritaki extracts exhibit notable antimicrobial activity against a spectrum of human microflora, including both Gram-positive and Gram-negative bacteria. The results of this study contribute valuable insights into the therapeutic potential of these traditional medicinal plants as natural antimicrobial agents, paving the way for further research and potential development of alternative strategies to combat microbial infections[1,2].

Keywords: Antimicrobial; Human microflora; Agar disc diffusion assays; Minimum inhibitory concentration; Synergistic effects; Additive effects; Phytochemical composition; Bioactive compounds

Introduction

Otitis externa, a prevalent ailment affecting the external auditory canal and auricle, has become a noteworthy global health concern, impacting 5% to 20% of patients seeking care at ear, nose, and throat (ENT) clinics [3]. The rise of drug-resistant strains, exacerbated by the indiscriminate use of commercial antimicrobial drugs, has spurred a worldwide exploration for alternative sources of antimicrobials. In this pursuit, India, celebrated for its profound traditional herbal knowledge, emerges as a promising repository. Among the diverse array of potential candidates, *Terminalia chebula* [4], commonly known as Black Myrobalan, stands out for its extensively documented medicinal uses, making it a focal point in the quest for substitutes to synthetic agents.

The investigation into plant-derived compounds has played a pivotal role in reshaping drug discovery paradigms, especially in addressing challenges posed by bacterial diversity and antibiotic resistance. This is particularly relevant for strains such as Pseudomonas, where conventional antibiotics are witnessing a decline in effectiveness[5,6]. Medicinal plants, typified by Piper longum, have demonstrated efficacy against bacterial diseases, presenting a potential avenue for the development of potent antibacterial agents. The chemical analysis of Piper longum has unveiled constituents like *piperine,* contributing to both bioavailability and therapeutic diversity[7].This study endeavors to contribute meaningfully to the ongoing search for novel antibacterial agents[8,9] by evaluating the antimicrobial potential of *T. chebula* fruits and isolating key constituents from Piper longum fruits. Aligned with the historical use of plant-derived compounds in traditional medicine[10], these efforts hold promise for the development of safer and more effective therapeutic agents against microbial infections.

Moreover, the unique cultivation conditions of Piper longum, thriving in limestone soil in the Cherrapunji region with heavy rains and high humidity, add an intriguing dimension to this

exploration. The detailed cultivation practices and harvesting methods underscore the significance of environmental factors[11] in obtaining potent medicinal compounds from plants like *Piper longum*.As a native plant in India, *Terminalia chebula*, commonly known as *Karakkaya* in Telugu (Harad in Hindi), has been traditionally employed for its medicinal properties[11], particularly as a cough reliever. The study also emphasizes the extensive use of *Terminalia chebula* in Ayurvedic formulations for infectious diseases, chronic ulcers, fungal infections of the skin, and its role in promoting longevity, immunity, and overall body resistance against diseases. The "king of medicines"[12] holds immense potential for contemporary medicine in combating microbial infections and contributing to global health[13,14]. *Haritaki* (*Terminalia chebula*) and *Pippali* (*Piper longum*) are traditional herbs in Ayurveda known for their potential benefits in promoting oral health. These herbs are believed to possess anti-inflammatory properties, contributing to the maintenance of healthy gums[15]. They can be incorporated into various oral care formulations to support gingival well-being. *Haritaki* is traditionally recognized for its analgesic properties and is occasionally applied topically to alleviate toothaches. The combination of *Haritaki* and *Pippali* may be integrated into natural toothpaste or mouthwash formulations[16].

Haritaki's astringent properties, along with *Pippali's* believed antimicrobial effects, can collectively contribute to oral hygiene by inhibiting the growth of harmful microorganisms [8] in the mouth. Moreover, the antimicrobial characteristics [17]of *Pippali* and the astringent nature of *Haritaki* may help address issues related to bad breath. Overall, the synergistic properties of these herbs make them potential candidates for holistic oral care [18,19]. *Pippali* fruit, a staple in traditional medicine, is utilized for various ailments such as cough, bronchitis, asthma, respiratory infections, constipation, gonorrhea, diarrhea, cholera, malaria, hepatitis, stomach-ache, spleen diseases, and tumors. Its primary strength lies in treating respiratory conditions like colds, coughs, and bronchitis, acting as a counterirritant to reduce inflammation. Particularly effective against asthma, it not only fights infections but also thins phlegm and alleviates congestion, reducing the intensity and frequency of asthma attacks [20].

Process of Preparing Plant Extractions

Plant Collection

The fruits of Piper longum was obtained from the local markets of Madugula and *Terminalia chebula* from the local markets of Visakhapatnam.

Processing and Extraction of Pippali and Haritaki Fruits

a) We washed the *Pippali* and *Haritaki* fruits thoroughly under running tap water and then rinsed them with sterile distilled water. After that, we dried them completely in a hot air oven at 50°C. Once dried, we ground them into a fine powder using a sterilized mixer grinder and stored the powder in sealed containers.

b) Next, we took 0.98 grams of the dried fruit powder from both *Pippali* and *Haritaki* and mixed it with 9.8 milliliters of ethanol in a conical flask. We sealed the flask with cotton wool and placed it on a rotary shaker set at 120 rotations per minute for 5 days to make sure all the active compounds were fully extracted.

c) Similarly, we prepared n-hexane extracts of *Pippali* and *Haritaki* using the same method.

d) After extraction, we filtered the extracts using Whatman filter paper. Then, we further purified the filtrate by centrifuging it at 4000 times the force of gravity for 5 minutes.

e) Finally, we stored the crude extracts in sealed containers at 4°C to preserve their potency.

Preparation of Sterile Discs

The Sterile filter paper discs were prepared from Whatman's No.1 filter paper. Discs of 6mm size were prepared on Petri plate and sterilized in an autoclave at 121°C for 15 minutes. Paper discs were soaked and allowed to stand for one hour to ensure complete saturation and air dried.

Antimicrobial activity

Bacterial strains

The fruit extracts were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL and evaluated for antibacterial activity using the agar disc diffusion assay. The bacterial culture in Muller Hinton broth was adjusted to a final inoculum density of $1x10^7$colony forming units per milliliter (CFU/mL) using the 0.5 McFarland standard and plated on molten Muller Hinton agar (MHA) plates. Streptomycin [21] served as the positive control in this assay. After 24 hours of incubation at 37 °C, the antibacterial activity was determined by measuring the diameter of inhibition zones around each disc containing either the plant extracts or the antibiotic. Each test was performed in triplicate to ensure the reliability of the results.

Determination of minimum inhibitory concentration (MIC)

The MIC of the fruit extracts against the tested bacteria was determined by broth micro-dilution procedure to find the lowest concentration of the extract at which no growth was visible. Stock solutions (10.24 mg/mL) of above extracts (*Pippali* and *Haritaki*) were prepared in DMSO, and serially diluted in Muller Hinton broth at concentrations of 5.12, 2.56, 1.28, 0.64, 0.32, 0.16, 0.08 and 0.04 mg/mL in a 96-well microtitre plate. The broth culture containing 0.5 McFarland ($1x10^8$ CFU/mL) inoculum density was then introduced to each of the microtitre wells at 1:10 ratio to maintain final inoculum density of $1x10^7$ CFU/mL. Microtitre plates were incubated for 18 h at 37°C, and the presence of visible growth in each well was inferred by measuring OD at 630 nm using ELISA reader.

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Determination of the Minimum Lethal Concentration (MLC)

The MLC (Minimum Lethal Concentration) for the antimicrobials was determined using the dilution in broth method. After 48 hours of incubation at 37°C, 0.1 mL of the solution was extracted from wells in the microtiter plates (Thermo Scientific) where no growth was observed. This sample was then plated onto Trypticase soy agar plates and further incubated for 48 hours at 37°C. The MLC was defined as the lowest concentration of the antimicrobial at which no colonies were observed on the agar plates.

Given that the detection limit of this method is 10 cfu/mL, the absence of growth on the Trypticase soy agar plates indicated that the concentration of bacteria was below this limit. Starting from an initial concentration of 105 cfu/mL, the MLC effectively reduced the bacterial count to below 10 cfu/mL. Therefore, the MLC represented the minimum concentration of the antimicrobial required to deactivate more than 99.99% of the bacteria present. Each strain and antimicrobial compound were tested in triplicate to ensure consistency and reliability of the results.

Discussion

The study uses extracts from *Terminalia chebula* and *Piper longum* fruits, employing various solvents like n-Hexane and ethanol along with antibiotics like streptomycin. The results indicate significant antibacterial properties against various bacterial strains [22,23]. A comparison study on *Terminalia chebula* fruits revealed antibacterial activity against tested strains, with the ethanolic extract being the most effective. The variations in results can be attributed to different plant materials, solvents, antibiotics, and bacterial strains used in each study [24-26].

Study 1: Utilizes various solvents like n-Hexane, ethanol, and antibiotics like streptomycin.

Diameter of Zone of Inhibition (mm) (Tables 1-4)

Table 1: Antibacterial properties of extracts of *Piper longum* fruits.

Bacteria	Ethanolic Extract	n- Hexane Extract	Standard (Streptomycin)
Staphylococcus epidermidis	17	10	25
Streptococcus mutans	10	12	18
Staphylococcus aureus	15	11	20
Escherichia coli	23	21	27
Streptococcus pneumoniae	21	20	21
Micrococcus luteus	13	10	26
Pseudomonas aeruginosa	12	11	18
Propionibacterium acnes	11	9	22

Table 2: Antibacterial properties of extracts of *Terminalia chebula* fruits.

Bacteria	Ethanolic Extract	n- Hexane Extract	Standard (Streptomycin)
Staphylococcus epidermidis	21	20	22
Streptococcus mutans	17	18	17
Staphylococcus aureus	22	15	25
Escherichia coli	15	17	24
Streptococcus pneumoniae	18	17	20
Micrococcus luteus	16	14	18
Pseudomonas aeruginosa	17	16	19
Propionibacterium acnes	24	19	28

Table 3: Antibacterial properties of extracts of *Terminalia chebula* fruits.

Bacteria	mg/ml	Ethanolic extract	n- hexane extract
Staphylococcus epidermidis	MIC	3.12	4.25
	MLC	6.25	6.12
Streptococcus mutans	MIC	12.5	10.8
	MLC	20.5	22.2

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Staphylococcus aureus	MIC MLC	4.25 15.5	6.2 13.33
Escherichia coli	MIC MLC	25.5 30.8	28.6 32.6
Streptococcus pneumoniae	MIC MLC	20.5 45.5	23.2 55.6
Micrococcus luteus	MIC MLC	8.54 48.42	12.25 50
Pseudomonas aeruginosa	MIC MLC	39.46 58.6	57.5 65
Propionibacterium acnes	MIC MLC	0.78 1.56	0.98 2.56

Table 4: Antibacterial properties of extracts of *Pippali* fruits.

Bacteria	mg/ml	Ethanolic extract	n- hexane extract
Staphylococcus epidermidis	MIC MLC	3.12 6.25	4.25 6.12
Streptococcus mutans	MIC MLC	12.5 20.5	10.8 22.2
Staphylococcus aureus	MIC MLC	4.25 15.5	6.2 13.33
Escherichia coli	MIC MLC	25.5 30.8	28.6 32.6
Streptococcus pneumoniae	MIC MLC	20.5 45.5	23.2 55.6
Micrococcus luteus	MIC MLC	8.54 48.42	12.25 50
Pseudomonas aeruginosa	MIC MLC	39.46 58.6	57.5 65
Propionibacterium acnes	MIC MLC	0.78 1.56	0.98 2.56

The ethanolic extract of *Pippali* exhibited a larger zone of inhibition compared to the n-hexane extract. Specifically, the zone of inhibition for the ethanolic extract against *E. coli* was 23 mm, while the smallest inhibition zone was observed against *Streptococcus mutans*, measuring 10 mm. On the other hand, the n-hexane extract of *Pippali* displayed a greater zone of inhibition against *E. coli*, measuring 21 mm. The smallest inhibition zone for the n-hexane extract was observed against Propionibacterium acnes, measuring 09 mm. Streptomycin acts as a control in this experiment.

The ethanolic extract of *Haritaki* exhibited a significant zone of inhibition against Propionibacterium acnes, measuring 24 mm, while displaying the smallest inhibition zone against *E. coli* at 15 mm. Similarly, the n-hexane extracts of *Haritaki* demonstrated notable inhibitory activity against Staphylococcus epidermidis, with a zone of inhibition measuring 20 mm, while exhibiting the lowest inhibition zone against *Micrococcus luteus* at 14 mm. The n-hexane extracts of *Haritaki* exhibited a larger inhibition zone of 18mm against *Streptococcus mutans* compared to the ethanolic extracts of *Haritaki.* This trend was also observed in the case of *E.coli*. Streptomycin acts as a control in this experiment.

Conclusion

In conclusion, the studies on isolated constituents from *Piper longum* and *Terminalia chebula* fruits offer valuable insights into the antibacterial properties of these fruit extracts. The findings suggest that both *Pippali* and *Haritaki* extracts possess antimicrobial properties against the tested bacterial strains. The choice of solvent (ethanol vs. n-hexane) influences the potency of the extracts against specific bacterial species. Additionally, *Haritaki* extracts tend to exhibit stronger inhibition against certain bacteria compared to *Pippali* extracts. Further research could explore the mechanisms underlying these differences and their potential applications in antimicrobial therapies (Figure 1).

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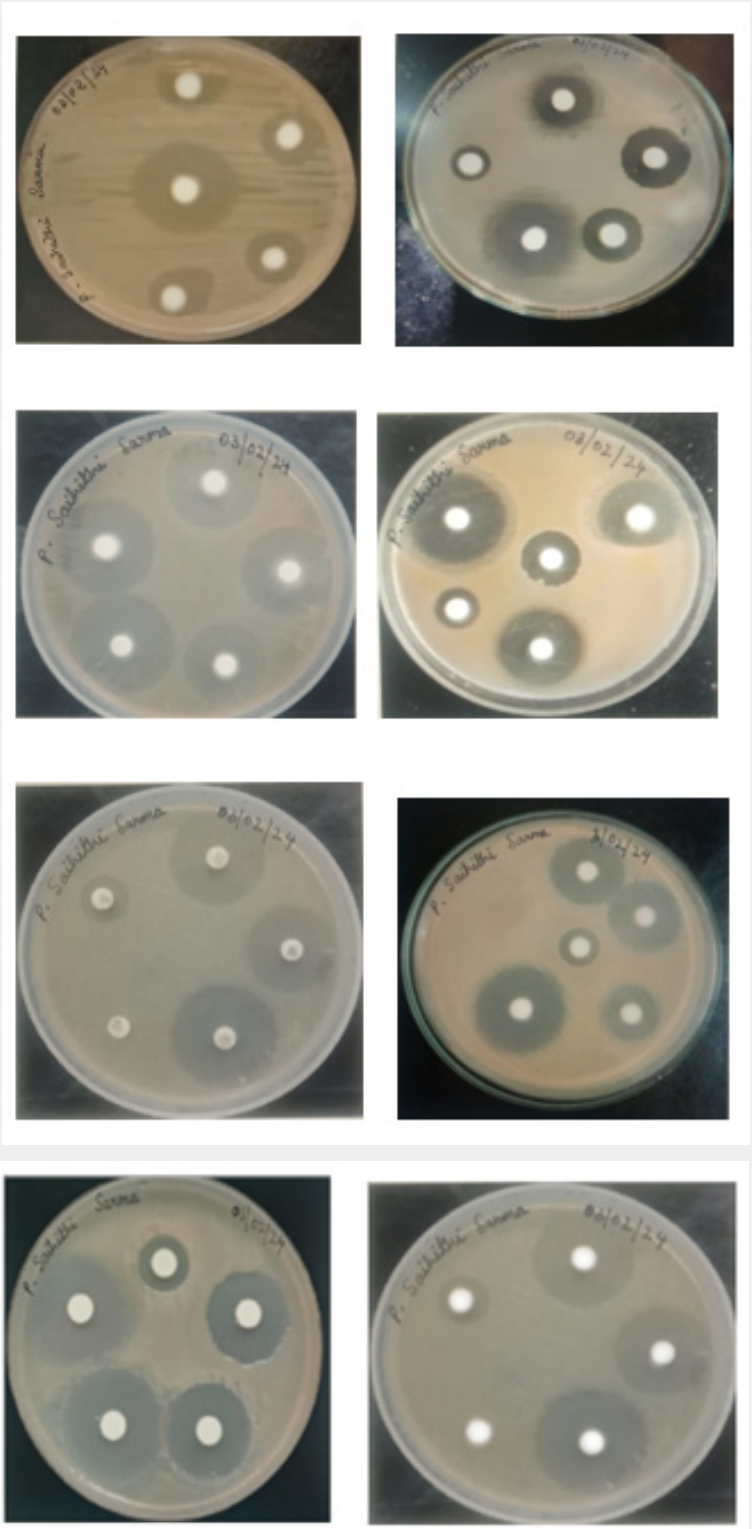

Figure: Zone of Inhibition of different microorganisms

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