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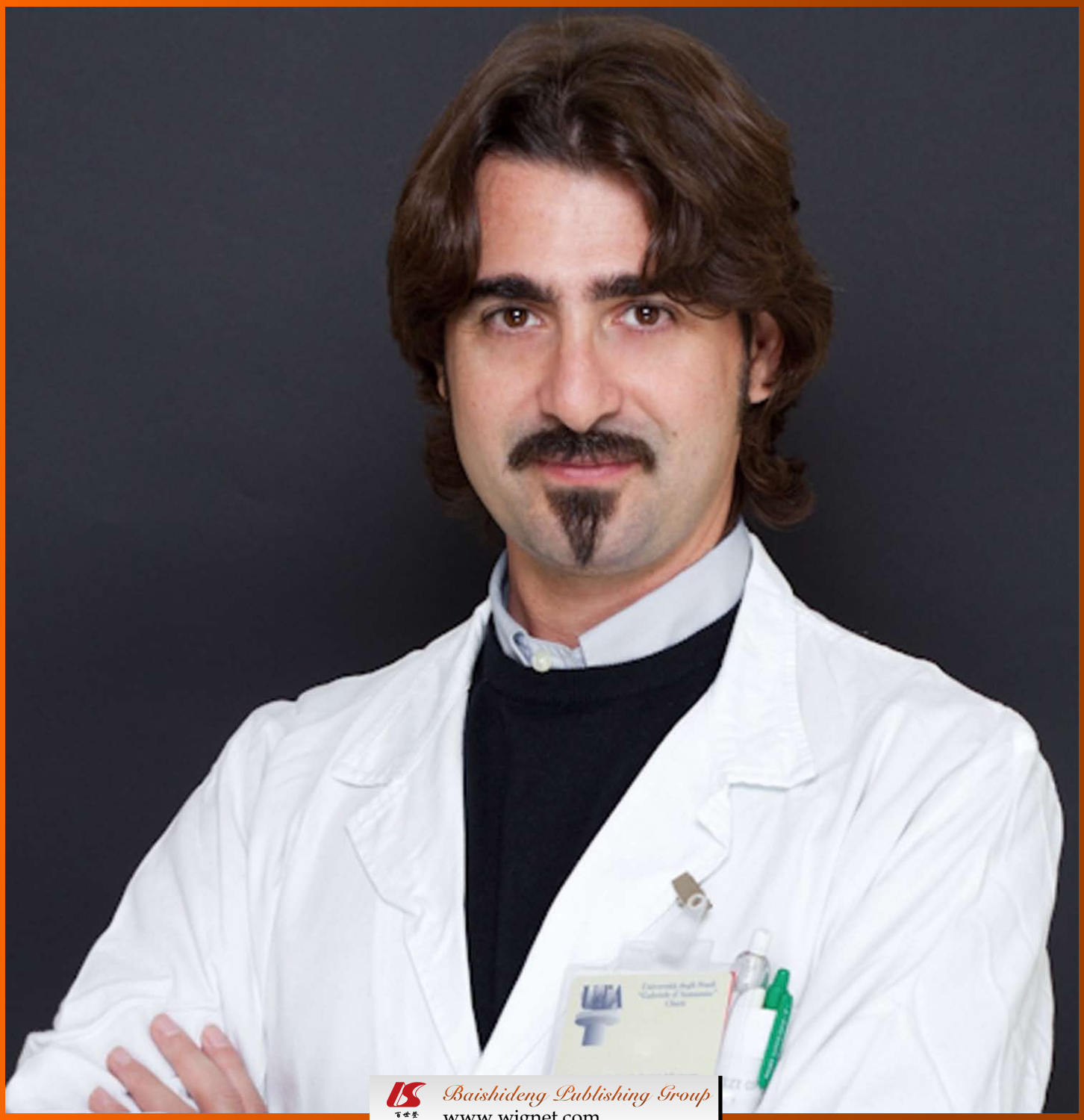

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BRIEF ARTICLE

Antimicrobial potential of *Terminalia chebula Retz*. fruit extracts against ear pathogens

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Abstract

AIM: To evaluate the antimicrobial potential of *Terminalia chebula* (*T. chebula*) extracts against pathogens causing otitis externa and compare it with ear drops.

METHODS: Four different extracts, methanol, ethanol, acetone and aqueous (hot and cold) extracts, from the *T. chebula* were tested for their antimicrobial activity through the agar well diffusion method and minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC) values were determined through the macrodilution broth method against six different microorganism, five bacterial (one gram positive and four gram negative) and one yeast.

RESULTS: Organic and aqueous fruit extracts displayed activity against all five tested bacterial ear pathogens with a maximum zone of inhibition of 31.6 mm against *Staphylococcus aureus*, followed by *Acinetobacter sp*. (24.6 mm), *Pseudomonas aeruginosa* (23.6 mm), *Proteus mirabilis* (21 mm) and *Escherichia coli* (19.3 mm). Of the four solvents evaluated, acetonic fruit extract of *T. chebula* was found to be best. The MIC values ranged between 0.78 mg/mL and 50 mg/mL for the different bacterial ear pathogens and MBC values ranged between 1.56 mg/mL and 50 mg/mL. The acetonic fruit extract showed larger inhibition zones compared to the herbal ear drops, Kan pip with lowest MIC of 0.78 mg/mL and MBC of 1.56 mg/mL.

CONCLUSION: Acetonic extract of *T. chebula* fruit may be used to treat otitis externa. However, more detailed studies, such as *in vivo* testing and pharmacokinetics properties, are needed to determine its therapeutic potential.

Key words: Otitis externa; *Terminalia chebula*; Antimicrobial activity; Minimum inhibitory concentration; Minimum bactericidal concentration; Organic and aqueous extracts

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INTRODUCTION

Otitis externa refers to a spectrum of infections of the external auditory canal and auricle, usually associated with microbial infection of macerated skin and subcutaneous cellular tissue. It affects between 5% and 20%

of patients attending ear, nose and throat (ENT) clinics. Manifestations of otitis externa include pain, pruritus and erythema but as the disease progresses, edema, otorrhea and conductive hearing loss may also develop[1-3]. The main causative agents of the diseases are *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Acinetobacter calcoaceticus*, *Proteus mirabilis* (*P. mirabilis*), *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus luchuensis*, *Aspergillus terreus*, *Penicillium sp.* and *Candida albicans* (*C. albicans*)[4-6].

Due to indiscriminate use of commercial antimicrobial drugs and development of multiple drug resistant strains of bacteria and fungi, there is a worldwide emphasis to search for new antimicrobials from natural sources. Plant metabolites and plant based drugs appear to be one of the better alternatives as they are known to have minimal toxicity and are cost-effective in contrast to synthetic agents[7].

India is sitting on a gold mine of well-recorded and traditional well practised knowledge of herbal medicine[8]. *Terminalia chebula* (*T. chebula*) *Retz.,* commonly known as Black Myrobalan and Harad, is a member of the family *Combretaceae*. It is native to the Indian subcontinent and the adjacent areas such as Pakistan, Nepal, south-west China and Sri Lanka. In Tibet, it is called the "king of medicine". It is a medium to large deciduous tree attaining a height of up to 30 m, with widely spreading branches and a broad roundish crown. The leaves are elliptical, oblong with an acute tip, cordate at the base, entire margins, glabrous above with a yellowish pubescence below. The flowers are monoecious, dull white to yellow, with a strong unpleasant odor, borne in terminal spikes or short panicles. The fruits are glabrous, ellipsoid to ovoid drupes, yellow to orange brown in color, containing a single angle stone[9-11].

The dried ripe fruit has traditionally been used in the treatment of asthma, sore throat, vomiting, hiccough, diarrhea, bleeding piles, gout and heart and bladder disease. In addition, the plant is commonly used for acidity, chronic lung disease, skin diseases and eye disorders[12-14]. It has been reported to exhibit a variety of biological activities, such as anticancer, antioxidant, antidiabetic, antibacterial, antifungal, antiviral, antianaphylactic, antiulcerogenic and antispasmodic. It also possesses cardioprotective, hepatoprotective, radioprotective and wound healing activities[9,15,16].

This plant is known to be an important source of secondary metabolites, of which, 33% of the total phytoconstituents are hydrolysable tannins (which may vary from 20%-50%) and are responsible for pharmacological activity. The chief constituents of tannin are chebulic acid, chebulagic acid, corilagin and gallic acid[17-19]. Tannin of *T. chebula* is of the pyrogallol (hydrolyzable) type. Hydrolyzable tannins (gallic acid, chebulic acid, punicalagin, chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulogic acid, chebulinic acid, 1,2,3,4,6- penta-O-galloyl-H-D-glucose, 1,6,-di-O-galloyl-D-glucose, casuarinin, 3,4,6-tri-O-galloyl-D- glucose and terchebulin) have been reported from *T. chebula* fruits[20-23].

In view of these reported medicinal values, the present study was carried out to examine the antimicrobial potential of *T. chebula* fruits against the locally isolated microbes, bacteria and yeasts, from otitis externa patients and to compare its efficacy with locally available ear drops.

MATERIALS AND METHODS

Plant collection

The fruits of *T. chebula* were obtained from the local market in Kurukshetra, Haryana. The taxonomic identity of the plant was confirmed by Dr. Vashishta BD, plant taxonomist, Chairman of Botany Department, Kurukshetra University, Kurukshetra.

Extraction of plant material

The samples were carefully washed under running tap water followed by sterile distilled water and air dried at room temperature (35-40 ℃) for 4-5 d, homogenized to a fine powder using a sterilized mixer grinder and stored in air tight bottles. Four different solvents, namely ethanol, methanol, acetone and aqueous (hot and cold), were used for extraction. Homogenized fruits, 10 g each, were separately soaked in conical flasks each containing 100 mL of acetone, ethanol, methanol (95%) and sterile distilled water. Also, an equal amount (i.e., 10 g) of homogenized fruits was immersed separately in 100 mL of hot sterile distilled water in conical flasks and allowed to stand for 30 min in a water bath (at 100 ℃) with occasional shaking, followed by keeping all the flasks on rotary shaker at 200 rpm for 24 h. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40 ℃ using a rota evaporator. The dried extract thus obtained was sterilized by overnight ultra violet-irradiation, checked for sterility on nutrient agar plates and stored at 4 ℃ in labelled sterile bottles until further use[24].

Test microorganisms

Five bacteria, namely *S. aureus* (HM626197, nucleotide sequence of all the five bacteria have been submitted to GenBank database which provided the GenBank accession number, HM626197-HM626201), *Acinetobacter sp.* (HM626198), *Proteus mirabilis* (HM626199), *E. coli* (HM626200), *P. aeruginosa* (HM626201) and one yeast, *C. albicans*, were isolated from patients with an ear infection from the local ENT clinics in Kurukshetra[4]. Bacterial strains were identified on the basis of gram staining, biochemical and molecular characteristics (16S rRNA sequencing)[25] and on the basis of staining, morphological and cultural characteristics for the yeast[26,27]. The bacterial isolates were subcultured on nutrient agar and *C. albicans* on malt yeast agar and incubated aerobically at 37 ℃. The media were procured from Hi Media Laboratory Pvt. Ltd., Bombay, India.

Ear drops

Three commonly prescribed ear drops by otolaryngologists, two allopathic ciplox (antibacterial), candid (antifungal) and a herbal ear drop Kan pip (antimicrobial), were procured from the local market in Kurukshetra.

Screening for antimicrobial activity

The acetone, methanol, ethanol and hot and cold aqueous *T. chebula* fruits extracts were used for evaluation of antimicrobial activity by the agar well diffusion method. In this method, a pure isolate of each microbe was grown on agar plates at 37 ℃ for 24 h. One plate of each microorganism was taken and a minimum of four colonies were transferred into normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted to be equal to that of 10^6 cfu/mL (standardized by 0.5McFarland standard) and used as the inoculum for performing an agar well diffusion assay. One hundred microliter (100 μL) of the inoculum of each test organism was spread onto the agar plates so as to achieve a confluent growth. The agar plates were allowed to dry and 8 mm wells were made with a sterile borer in the inoculated agar plates. The lower portion of each well was sealed with molten agar medium. The dried extracts were reconstituted to 20% in dimethylsulphoxide (DMSO) for the bioassay analysis. A 100 μL volume of each extract was propelled directly into the wells (in triplicate) of the inoculated agar plates for each test organism. The plates were allowed to stand for 1 h at room temperature (40 ℃) for diffusion of the extract into agar and incubated at 37 ℃ for 24 h. Sterile DMSO (20%) served as the negative control and ciplox (for bacteria), candid (for fungi) and Kan pip (antimicrobial) ear drops served as the positive controls. The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the extract, was recorded if the zone was greater than 8 mm. The experiments were performed in triplicate and the mean values of the diameter of inhibition zones ± standard deviations were calculated[24].

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) for each test organism was determined by the macrodilution broth method. A twofold serial dilution of each extract was prepared by first reconstituting the dried extract (100 mg/mL) in 20% DMSO, followed by dilution in Mueller-Hinton broth (bacteria) and Malt yeast broth (yeast) to achieve a decreasing concentration range of 50 mg/mL to 0.39 mg/mL. Each dilution was seeded with 100 μL of the standardized microbial inoculum (1.5×10^6 cfu/mL). The inoculated culture tubes were incubated at 37 ℃ for 24 h. A set of tubes containing only broth was kept as control. Afterwards, incubation tubes were examined for changes in turbidity as an indicator of growth. The lowest concentration that did not permit any visible growth was considered as MIC[28,29].

Determination of minimum bactericidal concentration

Minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial agent that will not allow the growth of an organism after subculturing on antibiotic free media. MBC was determined by subculturing the preparations that did not show any bacterial growth in the MIC determination. A 100 μL aliquot from the selected tube (showing MIC) was spread over the MHA plate and incubated at 37 ℃ for 24 h and examined for bacterial growth. The MBC, the lowest concentration of the plant extract giving 99.9% reduction of the bacterial growth of various plants parts against the bacterial pathogens, was recorded[29].

Determination of minimum fungicidal concentration

A loopful of culture from each set of tubes that did not show any visible growth of the yeast in MIC determination was subcultured on to fresh plates of MEA and incubated at 37 ℃ for 24-48 h. Minimum fungicidal concentration for each plant extracts against the tested yeast was recorded as the lowest concentration that did not yield any fungal growth on the solid medium[30,31].

Statistical analysis

The experimental results were repeated thrice in triplicate each time and expressed as mean ± SD and results were statistically evaluated using Dennett's *T*-test. *P* value less than 0.01 was considered significant.

RESULTS

The antibacterial activity of *T. chebula* fruits extracts on the agar plates varied in different organic (methanol, ethanol and acetone) and aqueous (hot and cold) extracts. Positive controls produced significantly sized inhibition zones against the tested bacteria (ranging between 16.3 mm and 56.3 mm) and the yeast (with zone of inhibition 21.6 mm), and the negative control produced no observable inhibitory effect against any of the test organism (Tables 1 and 2). A perusal of the data in Table 1 reveals that all the tested solvent fruit extracts possessed antibacterial activity against all five tested bacterial ear pathogens. However, the bioactivity against *C. albicans* was absent in all five extracts.

The acetonic fruit extract was found most effective against *S. aureus* (31.6 mm), followed by *Acinetobacter sp.* (24.6 mm), *P. aeruginosa* (23.6 mm), *P. mirabilis* (21 mm) and *E. coli* (19.3 mm). The ethanolic and methanolic fruit extracts showed moderate activity with inhibition zones of 28.6 mm and 27.3 mm against *S. aureus,* followed by *Acinetobacter sp.* (22.3 mm and 21.6 mm), *P. aeruginosa* (22.3 mm and 20.6 mm), *P. mirabilis* (20.6 mm and 19.3 mm) and *E. coli* (16.3 mm and 15.6 mm). Of the two tested aqueous extracts, hot aqueous extract exhibited more activity than the cold aqueous extract with zone of inhibition of 26.3 mm against *S. aureus,* followed by *Acinetobacter sp.* (18.3 mm), *P. mirabilis* (17.3 mm), *P. aeru-*

Table 1 Antibacterial and antiyeast activity of *Terminalia chebula* fruits extract on ear pathogens

Solvent extracts (mg/mL)	Diameter of growth of inhibition zones (mm)					
	Sa	Pm	Pa	Ec	As	Ca
Acetone	31.6[a,1] ± 1.52[2]	21.0 ± 1.0	23.6 ± 1.15	19.3 ± 1.52	24.6 ± 0.57	-
Ethanol	27.3 ± 1.15	19.3 ± 1.15	20.6 ± 1.15	15.6 ± 0.57	21.6 ± 1.15	-
Methanol	28.6 ± 0.57	20.6 ± 0.57	22.3 ± 1.52	16.3 ± 1.52	22.3 ± 1.15	-
Hot aqueous	26.3 ± 1.15	17.3 ± 0.57	16.3 ± 1.15	14.3 ± 1.15	18.3 ± 0.57	-
Cold aqueous	25.6 ± 0.57	15.6 ± 1.52	14.6 ± 1.52	13.6 ± 0.57	17.6 ± 1.52	-
Ciplox ear drop	56.3 ± 0.57	46.3 ± 1.52	34.0 ± 1.0	36.3 ± 0.57	32.6 ± 0.57	NT
Kan pip ear drop	26.3 ± 1.52	20.3 ± 0.57	18.3 ± 1.52	23.6 ± 1.15	21.6 ± 1.52	16.3 ± 1.15
Candid ear drop	NT	NT	NT	NT	NT	21.6 ± 0.57

[1]Values, including diameter of the well (8 mm), are means of three replicates, [2]± SD. The data were analyzed by one way analysis of variance followed by Dunnett's test. [a]P <0.01 *vs* positive control. Sa: *Staphylococcus aureus*; Pm: *Proteus mirabilis*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; As: *Acinetobacter sp.*; Ca: *Candida albicans*; NT: Not tested.

Table 2 Minimum inhibitory concentration and minimum bactericidal concentration of *Terminalia chebula* fruits extracts against bacterial ear pathogens

Solvent extract	mg/mL	Sa	Pm	Pa	Ec	As
Acetone	MIC	0.78	12.5	6.25	25	6.25
	MBC	1.56	6.25	6.25	25	6.25
Ethanol	MIC	3.12	25	12.5	50	12.5
	MBC	6.25	25	12.5	> 50	12.5
Methanol	MIC	3.12	12.5	12.5	50	12.5
	MBC	3.12	12.5	25	50	25
Hot aqueous	MIC	3.12	25	25	50	25
	MBC	6.12	50	50	50	25
Cold aqueous	MIC	3.12	25	25	50	25
	MBC	6.12	50	50	> 50	50

Sa: *Staphylococcus aureus*; Pm: *Proteus mirabilis*; Pa: *Pseudomonas aeruginosa*; Ec: *Escherichia coli*; As: *Acinetobacter sp.* MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration.

ginosa (16.3 mm) and *E. coli* (14.3 mm). The antibacterial activity of the acetonic extract was found better than the standard herbal ear drop (Kan pip) against all the tested bacterial ear pathogens (Table 1).

The MIC values ranged between 0.78 mg/mL and 50 mg/mL for the different bacterial ear pathogens and MBC values ranged between 1.56 mg/mL and 50 mg/mL. The results revealed that the MBC values were either equal or twofold higher than the MIC values against the corresponding pathogens. Of all the fruit extracts in different solvents tested, the acetonic extract was the best solvent where the lowest MIC of 0.78 mg/mL and MBC of 1.56 mg/mL was found against *S. aureus* that increased to MIC value of 6.25 mg/mL against *P. aeruginosa, P. mirabilis* and *Acinetobacter sp.*, and MIC was 25 mg/mL for *E. coli* (Table 2).

Kan pip ear drop showed the inhibition of all the tested pathogens with a zone of inhibition ranging between 26.3 mm and 16.3 mm but the allopathic antibacterial ear drop ciplox, containing ciprofloxacin and antifungal ear drop candid containing clotrimazole, produced a zone of inhibition ranging between 56.3 mm and 21.6 mm. The antibacterial activity of *T. chebula* acetonic extract was found to be better than the standard herbal ear drop (Kan pip) against all the tested bacterial ear pathogens. All the obtained results were statistically significant as they showed ($P < 0.01$) compared with control (Table 1).

DISCUSSION

Medicinal plants have been considered a boon to human society to cure a number of ailments[32]. Several works have documented the pharmacological screening of plant extracts which have been exploited as the source of innumerable therapeutic agents[33-35]. *T. chebula* is an important medicinal plant in Indian traditional medicine and it is the most frequently used herb in Ayurveda[36]. Therefore, in the present investigation, different organic (ethanol, methanol, acetone) and aqueous (hot and cold) fruit extracts of this plant were evaluated for their antibacterial and antifungal potential for the first time against the pathogens causing ear infection.

In our study, the organic fruit extracts of *T. chebula* were found to be the most active in inhibiting the growth of all the five tested bacterial ear pathogens compared to aqueous extracts. They showed a broad spectrum of antibacterial activity showing inhibition of gram-positive and gram-negative bacteria. Our work is supported by earlier studies on an alcoholic extract that exhibited greater activity than the aqueous extracts against bacteria[37,38]. There are several other reports about the antibacterial activity of *T. chebula* fruit extracts against uropathogenic *E. coli*, *Helicobacter pylori* and *S. aureus*, *Salmonella typhi*, *S. epidermidis*, *Bacillus subtilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*[7,10,36].

Among the tested bacterial ear pathogens, gram-positive bacterial strains have been found to be more susceptible than gram-negative bacterial strains. This may be attributed to the fact that the cell wall in gram-positive bacteria consists of a single layer, whereas, the gram-negative cell wall is a multilayered structure bounded by an outer cell membrane[39]. A majority of the described antimicrobial effects of *T. chebula* extracts have been attributed to their secondary metabolites, notably tannins. The antibacterial activities of tannins are well documented and are known to inhibit the growth of many fungi, yeasts, bacteria and viruses[10].

Of the three organic extracts of this plant screened, the acetonic extract has been found to be more active and have a better antibacterial activity than the corresponding ethanolic and methanolic extracts (Table 1). Our results confirm the finding of Nair *et al*[40], Cowan[41] and Eloff[42], who rated acetone as the best solvent. Interestingly, *T. chebula* extracts have been found to be more potent against the tested ear pathogens compared to the standard herbal ear drop (Kan pip), showing a great potential to be developed as a herbal ear drop to control microbial ear infections.

CONCLUSION

The present investigation revealed that the acetonic extracts of *T. chebula* showed promising antibacterial activity against all the tested bacterial ear pathogens, which explains its use in traditional system of medicines. *T. chebula* can be employed as a source of natural antimicrobials that can serve as an alternative to conventional medicines. However, further experiments, including phytochemical analysis, are needed to identify the active constituents responsible for the observed antibacterial activity and *in vivo* studies on this plant to determine its toxicity and their pharmacokinetics properties, for therapeutic utility in treating otitis externa infections.

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COMMENTS

Background

The incidence of otitis externa is mainly found in tropical areas of the world and in most of the patients visiting the ear, nose and throat clinics.

Research frontiers

Presently, most of us are consuming antibiotics for every ailment but misuse and overuse of these antibiotics leads to the development of antimicrobial resistance. So, to combat the antimicrobial resistance, the authors in this study evaluated the antimicrobial potential of a plant compared to the ear drops.

Innovations and breakthroughs

This recent study has highlighted the significance of a herbal plant [*Terminalia chebula* (*T. chebula*) fruit] extract for treating a bacterial ear infection. Fruit extract of *T. chebula* displayed a broad spectrum activity against all the tested bacterial pathogens. This is the first study showing the use of *T. chebula* fruit extracts against the pathogens causing otitis infection.

Applications

This study suggested that the fruit extract of *T. chebula* can be used for treating bacterial otitis infection. This fruit extract can be developed as a herbal ear drop or be used in any other formulation for human beings, after testing its toxicity and pharmacokinetics properties.

Terminology

Otitis externa: refers to a spectrum of infections of the external auditory canal and auricle; Otorrhea: any flowing/drainage/discharge from the ear; *Pruritus*: itching/irritation of the skin; Erythema: redness of the skin.

Peer review

This is a good descriptive study in which the authors evaluated the antibacterial potential of herbal plants and compare it with locally available ear drops. The results are interesting, with acetonic fruit extracts of *T. chebula* displaying more antibacterial activity compared to Ayurvedic ear drops, Kan pip. Hence, this extract can be used as a therapeutic substance that could be used in the pharmaceutical industry for treating otitis and other infections.

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Multidisciplinary Head and Neck Cancer Symposium
Phoenix, AZ, United States

January 27-28, 2012
Advanced Otology Update workshop
Surat, India

February 1, 2012
2nd Vestibular Assessment and Rehabilitation Therapy Course, University of Cape Town, South Africa

March 11-13, 2012
5th International Congress on Ear Reconstruction
Sydney, Australia

April 18-22, 2012
Combined Otolaryngology Spring Meeting
San Diego, CA, United States

April 26-May 28, 2012
10th International Otorhinolaryngology Head and Neck Surgery Congress
Ankara, Turkey

May 3-5, 2012
12th International Conference on Cochlear Implants and other Implantable Auditor
Baltimore, MD, United States

May 13-18, 2012
Acoustics 2012
Hong Kong, China

May 20-23, 2012
11th International Congress of the European Society of Pediatric Otorhinolaryngology
Amsterdam, The Netherlands

May 23-24, 2012
7th Global Otology-Neurotology Live Surgical Broadcast, Europe
Utrecht, The Netherlands

June 3-7, 2012
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Frontiers in Otorhinolaryngology Conference
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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No volume or issue

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Patent (list all authors)

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Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as

χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.
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