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Original Research Article

In-vitro antimicrobial activity of *Oscimum sanctum* leaf extract against pathogens isolated from otitis media infection

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Abstract

Introduction: Otitis media (OM) is the primary contributor to hearing loss in children younger than 5 years, a vital time for the development of speech, language, and cognition. Medications obtained from plants are favoured over synthetic treatments because of their potent pharmacological effects, low toxicity, and cost-effectiveness. *O. sanctum* has traditionally been used to treat various infections.

Aims and Objective: This study highlights the need for molecular identification of microorganisms linked to OM infection and examining the antimicrobial effects of extracted leaves from *O. sanctum* on OM infection pathogens.

Materials and Methods: The OM swab samples were processed for microbial identification and antimicrobial susceptibility through the Vitek 2 system, and molecular identification was carried out to identify the pathogens. The five non-polar to polar solvents were used to evaluate the zone of inhibition of all solvents against *S. aureus*, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, and *C. albicans*. The highest ZOI was reported with methanolic extract against *S. aureus*, and the best MIC of 0.162 mg/ml was found against *C. albicans*.

Conclusion: In this study, the *O. sanctum* methanolic leaves extract has shown good inhibitory activity against OM drug-resistant pathogens.

Keywords: Otitis media, Multidrug resistance, *O. sanctum*, Antimicrobial activity

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1. Introduction

Otitis media (OM) is the foremost cause of hearing loss in children underneath 5 years old, a critical period for speech, language, and cognition development. Childhood OM is linked to adult sensorineural hearing loss and tinnitus and can lead to permanent hearing loss at any age.[1] In the US, over 9 million children under the age of 5 require medical attention each year owing to OM. OM is a severe health and economic burden, particularly in underdeveloped nations where access to specialized medical care is limited. The associated lifelong morbidity and childhood death are unacceptable.[2,3]

In high-income nations, acute otitis media (AOM) along with otitis media with effusion (OME) are the most widespread children illnesses, leading to antibiotic prescriptions and consultations to general practitioners.[4] Chronic suppurative otitis media (CSOM), a severe form of condition, is predominantly found in low- to middle-income countries and high-risk populations, including indigenous communities in high-income countries.[5]

The diagnosis and microbiology of these disease entities are critical for treating these diseases as the associated microorganisms possess resistance mechanisms to recent

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antibiotics and antifungals. Global studies have reported significant variations in the prevalence of bacterial ear infections. For instance, it was reported that 78.8% of bacterial isolates were pathogenic, with gram-negative bacteria (57.1%) being more common than gram-positive strains (42.9%). The most common pathogenic microorganisms linked with these illnesses were *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella spp*., and *Hemophilus influenzae*. The growth of multidrug-resistant bacterial infections creates serious concerns within the healthcare Sector, restricting therapeutic options.[6]

Different predisposing factors for fungal ear infections have been identified, including a compromised immune system, injury, use of steroids, swimming, ear cleaning and treatment, the use of helmets and oils, childhood malnutrition, and the presence of fungal diseases in other areas of the body.[7] Recently, a range of antifungal medications, including azoles (like fluconazole), echinocandins, and polyenes (such as nystatin), have been suggested for treating fungal infections. However, several studies have recently uncovered multidrug-resistant (MDR) fungal strains that show resistance to a broad spectrum of antifungals. Moreover, in many instances, infections are mainly attributed to these antifungal-resistant organisms, especially in patients with weakened immune systems.[8] Because of these concerns, there is an increasing demand for molecular detection of related microorganisms and innovative methods such as natural antimicrobial treatments to effectively address antimicrobial resistance in otitis media (OM).

Plant-derived medications are widely preferred over synthetic treatments due to their powerful pharmacological effects, low toxicity, and economic feasibility. According to a World Health Organization (WHO) assessment, traditional medicines are still used by over 80% of the global population to treat various diseases.[9]

The *Ocimum sanctum* (Tulsi), is known as the 'Queen of Herbs' and the 'Mother of Nature' due to its numerous health advantages. It is one of the most revered holistic medicinal plants in traditional India. For centuries, its herbal compounds have been utilized as a home treatment for many diseases. This plant thrives throughout India and certain Southeast Asian countries.[10]

O. sanctum has long been valued in traditional medicine for treating various ailments, such as diarrhoea, malaria, fever, bronchitis, dysentery, skin disorders, arthritis, asthma, and insect bites.[11] Beyond its therapeutic uses, it exhibits a wide range of biological activities.[12] This medicinal herb is rich in bioactive compounds like terpenoids, flavonoids, phenolic acids, glycosides, and propenyl phenols.[13] Additionally, it provides essential nutrients, including vitamins A and C, along with minerals such as calcium, iron, zinc, and phosphorus. Nutritional analysis reveals that per 100 g, *O. sanctum* contains 4.2 g of protein, 0.5 g of fat, 25 mg of carbohydrates, 287 mg of phosphorus, 25 mg of calcium, and 15.1 mg of iron.[14]

The present study emphasizes the molecular identification of microorganisms associated with OM infection and exploration of antimicrobial activity of *O. sanctum* leaves extract against the pathogens identified from OM infection.

2. Materials and Methods

2.1. Sample collection

Ear swab samples with otitis media (OM) infection were collected from Otorhinolaryngology Department of IMS and SUM Hospital Bhubaneswar, Odisha, India, as part of routine care in between 2023 to 2025. Ethical clearance was not required for the study, as it did not involve the direct participation of human subjects.

2.2. Isolation and identification and antimicrobial susceptibility pattern analysis

The swab samples were processed for microbial identification through Vitek 2 identification following the standard procedures.[15] The antimicrobial susceptibility patterns of both bacterial and candidal isolates were analysed in the Vitek 2 analysis following the CLSI guidelines.[16]

2.3. Molecular identification of bacterial and candidal isolates

2.3.1. Extraction of genomic DNA from bacteria cells

Genomic DNA was isolated from all resistant bacterial strains using the QIAamp DNA Micro Kit, and their resistance profiles were assessed via the Vitek 2 system. To prepare bacterial liquid cultures, individual colonies were picked from nutrient agar plates and inoculated into sterile 2 mL nutrient broth tubes. These cultures were then incubated overnight at 37°C with continuous shaking at 180 rpm. The resulting broth was subsequently processed for DNA extraction according to the manufacturer's protocol. Following isolation, DNA purity and concentration were examined by 0.8% agarose gel electrophoresis. The purified DNA samples were stored at -20°C for further downstream analyses.

2.3.2. Genomic DNA isolation of Candidal isolates

Genomic DNA was extracted from all resistant Candida spp. isolates, which had been previously identified and assessed for resistance patterns via the Vitek 2 system, using the MP Biomedical Kit. To prepare candidal liquid cultures, a single colony from *Candidal* Differential Agar (CDA) plates was transferred into a sterile 2 mL yeast extract peptone dextrose (YPD) broth tube. The cultures were incubated overnight at 37°C with continuous shaking at 180 rpm. Subsequently, freshly grown broth cultures were utilized for DNA isolation in accordance with DNA isolation kit guidelines. Following

extraction, the purity and concentration of the DNA were assessed using 0.8% contain agarose in gel electrophoresis unit. The resulting purified DNA samples were preserved at -20°C for subsequent analysis.

2.3.3. PCR amplification of bacterial genomic DNA using 16S rRNA gene

Genomic DNA from all bacterial isolates that showed resistance was amplified using universal primers 16S rRNA gene, are: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), targeting partial 16S rRNA sequences.[17] The PCR was executed in a 25 µl reaction mix. The thermal cycling conditions were set up with protocol included an initial denaturation step at 94°C for 4 minutes, followed by 34 cycles consisting of denaturation (94°C for 30 seconds), annealing (50°C for 1 minute), and extension (72°C for 2 minutes), culminating in a final extension at 72°C for 7 minutes. The PCR products obtained were examined on a 1% agarose gel, where the prominent bands were chosen for sequencing. Purification was carried out with a commercial PCR cleanup kit, and Sanger sequencing was performed using an ABI Prism 310 genetic analyzer with the same universal primers.

2.3.4. PCR amplification of candidal genomic DNA using 16S rRNA gene

Similarly, the candidal genomic DNA of the resistant candidal isolates were amplified using 18S rRNA gene. PCR was performed in a 25 µL volume with 10 pmol of forward primer ITS1: TCCGTAGGTGAACCTGCGG and reverse primer ITS4: 5'TCCTCCGCTTA TTGATATGC-3'.[18] Sequences were compared to the GenBank nucleotide database using Blastx and Blastn to confirm the consensus *Candida* sp. Sequence submitted in the NCBI database data (**Table 1**).

Table 1: GenBank accession numbers of organism

Organism Name	GenBank Accession No.
Escherichia coli	PV571925
Mammaliicoccus sciuri	PV571903
Enterobacter mori	PV571900
Staphylococcus cohnii	PV571897
Staphylococcus aureus	PV571892
Enterobacter cloacae	PV571833
Pseudomonas aeruginosa	PV571831

2.4. Plant collection and extraction

Fresh, healthy leaves of *O. sanctum* were obtained from BPMPKC (Biju Patnaik Medicinal Plant Knowledge Centre) in Bhubaneswar, India. The plant specimen was authenticated by Dr. Pratap Chandra Panda (Voucher specimen No. provided) and preserved at the Centre for Biotechnology, Siksha 'O' Anusandhan (Deemed to be University), Bhubaneswar, India. The leaves were thoroughly rinsed 2 to 3 times with normal water, followed by a final wash with sterile distilled water. Afterward, washed leaves were shade-dried away from direct sunlight and finely ground into powder.

For extraction, approximately 5 grams of the powdered leaves were mixed with 50 mL (1:10 ratio) of organic solvent in a Soxhlet apparatus. A sequential extraction process was performed using five solvents of varying polarity: ethyl acetate, n-hexane, methanol, water and chloroform. The extraction was conducted according to the boiling points of the respective solvents. The resulting extract was collected in flask and concentrated the samples using evaporator technique to obtain a semisolid residue. The remaining plant material in the Soxhlet apparatus was dried at 40°C in an oven and subsequent extractions with the next solvent.

2.5. Determination of antimicrobial activities of extract

2.5.1. Determination of Zone of inhibitions

The antimicrobial properties of different solvent extracts were assessed employing the agar well diffusion technique to estimate zones of inhibition (ZOI). One strain from each microbial species that was resistant to the most antibiotics/antifungals was selected to assess the antibacterial properties of various solvent extracts. To create microbial broth cultures, nutrient broths (NB for bacteria and YPDB for Candida) were used. Then, 100 µL of each inoculum was placed on agar plates (MHA for bacteria and SDA for *Candida*) and dried. The media plates were made with 6mm thick wells and incubated with 5 mg/ml solvent extracts and standard antimicrobials as positive controls based on strains. The ZOI was calculated by measuring the diameter of the inhibitory zones after an overnight incubation at 37ºC. For a better understanding of the activities of all extracts against all strains of microorganisms, a control antibiotic/antifungal was used in the antimicrobial activity assay.

2.5.2 Assessment of minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), and Minimum fungicidal concentration (MFC)

The solvent extract that has good inhibitory action in ZOI was used to perform MIC and MBC. To calculate the MIC and MBC/MFC, the plant extract was diluted from its original stock solution in the appropriate solvent and inoculated in 96 well plates. The plant extract had an initial stock concentration of 5000 µg/ml. The control antimicrobials employed were Colistin (10 µg/ml for gram-negative bacteria), Amikacin (30 µg/ml for gram-positive bacteria), and Amphotericin B (20 µg/ml). This was followed by the micro broth dilution method, and the MBC/MFC value was calculated by sub-culturing the microbial inoculum from each well of the microplate on their corresponding agar plates.

3. Results

3.1. Identification of microorganisms

A total of 93 OM infected swab samples were collected, of which 85 (91.39 %) samples were reported positive growth and the rest were reported with no growth results after processing of samples. Both bacterial and candidal isolates were identified from OM patients, with four strains of bacteria and one strain of *Candida* sp. Among all, *Pseudomonas aeruginosa* was reported with the highest frequency of 28 (30.11 %), followed by *Klebsiella pneumoniae* (24.73 %), *Staphylococcus aureus* (20.43 %), *Acinetobacter baumannii* (11.83 %), and *Candida albicans* (4.30 %) (**Table 2**).

3.2. Antimicrobial susceptibility patterns of microorganisms

The antimicrobial activity patterns of microorganisms by the Vitek 2 system reported the resistance pattern of both bacterial and candidal strains. The pattern reported 15 strains having multi-drug resistance drug, two strains of methicillin-resistant *S. aureus* and multidrug-resistant strains of *K. pneumoniae*, *P. aeruginosa*, *A.baumannii* (**Table 3**). The Vitek 2 system analysis also reported the antifungal activity of *C. albicans* and found that the azole groups of antifungals were more resistant than others.

Table 2: Frequency and percentages of microorganisms isolated from OM

S.No	Microorganisms	Frequency	Percentage
1	*P. aeruginosa*	28	30.11
2	*K. pneumoniae*	23	24.73
3	*S. aureus*	19	20.43
4	*A. baumannii*	11	11.83
5	*C. albicans*	4	4.30
6	No growth	8	8.60
	Total	93	

Table 3: Antimicrobial resistance pattern of microorganisms reported by Vitek 2

Methicillin-Resistant *Staphylococcus aureus* (MRSA)											
Isolates	**CX**	**β-Lactams**	**Aminoglyc-osides**	**Fluoroquin-olones**	**Macrolides**	**Others**					
		BEN	OX	GEN	CIP	LE	E	CLN	LNZ	DAP	TE
MRSA Isolate 1	POS	0.6/R	4/R	0.4/S	7/R	8/R	8/R	4/R	2/S	4	1/S
MRSA Isolate 2	POS	0.5/R	4/R	16/R	8/R	8/R	8/R	4/R	2/S	1/S	16/R
Klebsiella pneumoniae											
Isolates	**Penici-llins**	**Cephalosp-orins**	**Carbapenems**	**Aminoglyc-osides**	**Fluoroquin-olones**	**Tetracy-clines**	**Folate Inhibitors**				
	TI	PI	CAZ	CPZ	CPM	AT	DOR	IMP	MRP	AK	GEN
KP 1	128/R	128/R	62/R	64/R	64/R	1/S	8/R	14/R	16/R	64/R	14/R
KP 2	128/R	128/R	64/R	64/R	64/R	64/R	8/R	2/I	16/R	64/R	2/S
KP 3	128/R	128/R	64/R	64/R	64/R	64/R	8/R	2/I	16/R	64/R	16/R
Acinetobacter baumannii											
Isolates	**β-Lactams**	**Cephalosp-orins**	**Carbapenems**	**Aminoglyc-osides**	**Fluoroquin-olones**	**Tetracy-clines**	**Folate Inhibitors**				
	TI	PI	CAZ	CPZ	CPM	AT	DOR	IMP	MRP	AK	GEN
AB 1	128/R	128/R	64/R	32/I	64/R	64/R	8/R	16/R	16/R	8/S	16/R
AB 2	128/R	128/R	64/R	64/R	64/R	64/R	8/R	16/R	16/R	64/R	16/R
AB 3	128/R	128/R	64/R	64/R	64/R	1/S	8/R	16/R	16/R	64/R	16/R
AB 4	128/R	128/R	64/R	32/I	64/R	64/R	8/R	16/R	16/R	8/S	16/R
Pseudomonas aeruginosa											
Isolates	**β-Lactams**	**Cephalosp-orins**	**Carbapenems**	**Aminoglyc-osides**	**Fluoroquin-olones**	**Others**					
	TI	PI	CAZ	CPZ	CPM	AT	DOR	IMP	MRP	AK	GEN
PA 1	128/R	128/R	128/R	64/R	64/R	64/R	8/R	16/R	16/R	64/R	4/S
PA 2	NA	128/R	NA	64/R	64/R	NA	NA	8/R	16/R	16/R	16/R
PA 3	128/R	128/R	64/R	64/R	64/R	64/R	8/R	16/R	16/R	64/R	16/R
PA 4	128/R	128/R	64/R	64/R	16/I	64/R	8/R	16/R	16/R	64/R	16/R

Candida spp. (Antifungal Susceptibility)											
Species	**Azoles**	**Echinoc-andins**	**Polyenes**	**Pyrimidine Analogs**							
	FLC	MICO	VORI	CAS	MICA	AMB	5-FC				
C. albicans 1	1.1/R	1.05/R	1.1/R	0.8/R	0.6/S	0.9/S	0.8/S				
C. albicans 2	1.1/R	1.05/R	1.1/R	0.8/R	0.6/S	0.9/S	0.8/S				

Table 4: Determination of the Zones of Inhibition of different solvent extracts of *O. sanctum* leaves

S.No.	Name of microorganisms	N-Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous	Control
1	*P. aeruginosa*	18	15	17	16	16	15
2	*K. pneumoniae*	17	18	18	17	16	16
3	*S. aureus*	17	15	19	19	18	15
4	*A. baumannii*	16	14	15	18	14	14
5	*C. albicans*	15	16	14	16	17	14

Table 5: MIC and MBC analysis of *O. sanctum* leaves in methanol extract

S.No.	Microorganisms	MIC in concentration mg/ml	MBC in concentration mg/ml
1	*S.aureus*	0.325	2.5
2	*K. pneumoniae*	0.75	1.25
3	*P. aeruginosa*	0.325	2.5
4	*A. baumannii*	1.25	1.25
5	*C. albicans*	0.162	0.325

3.3. Molecular identification of microorganisms

The bacterial strains were identified using the molecular method of 16S r RNA gene sequencing. Similarly, the candidal strain was identified using the molecular method of 18S rRNA gene sequencing.

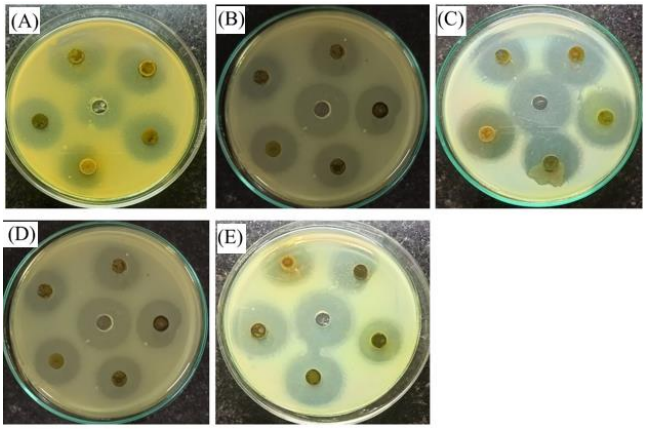

Figure 1: Zone of inhibition determined by well diffusion method: **A:** *P. aeruginosa;* **B:** *K. pneumonia;* **C:** *S. aureus;* **D:** *A. baumannii;* **E:** *C. albicans*

3.4. Antimicrobial screening of different solvent extracts of O. sanctum leaves

The antimicrobial screening of *O. sanctum* leaves extract against both bacterial and candidal isolates demonstrated good inhibitory actions following ZOI, MIC, and MBC. The inhibitory action of all extracts showed effective zones compared to the control antibiotic/antifungal drugs. The highest 19 mm of ZOI was reported with methanol extract against *S. aureus*, followed by 18 mm against *A. baumannii*, and 18 mm of ZOI with aqueous extract against *S. aureus* itself. The detailed ZOIs of all extracts against all microorganisms were mentioned in **Table 4** and **Figure 1.**

The antimicrobial activity was analysed through MIC and MBC and is mentioned in **Table 5**. The MIC of 0.162 mg/ml was reported against *C. albicans*, followed by 0.325 mg/ml reported against *P. aeruginosa* and *S. aureus*. Subsequently, the MBC was reported by culturing and did not yield any colonies.

4. Discussions

OM is a predominant cause of hearing loss in children, especially in poor countries. OM begins with middle ear mucosal irritation and inflammation. Mucosal edema is generated by an inflammatory response. Continuous inflammation finally causes mucosal ulcers and epithelial lining breakdown. Topical quinolones are effective and easy to administer, but still expensive. *P. aeruginosa* and *S. aureus* are the most prevalent pathogens responsible for OMwere reported earlier, whereas the current study reported some more microorganisms like *K. pneumoniae*, *A. baumannii,* and *C. albicans* along with the same.[19]

Determining the causal agent is essential for creating effective treatment strategies. Various methods, including biochemical and molecular techniques, are commonly used

to investigate pathogens. Each method presents its own set of pros and cons. While the biochemical method is straightforward, it often requires considerable time, and finding the right culture medium for different bacterial species can be difficult.[20] In such instances, molecular detection has emerged as the preferred method for identifying the bacterial community.

As antibiotic resistance continues to rise and the risks assessment associated with surgery increase, thereby there is a need for new therapeutic approaches to treat OM. In this research, the majority of strains were found to be multidrug-resistant, with most antibiotics and antifungals showing resistance activity. Among the advanced techniques, the Vitek 2 system identification system allows for prompt access to precise patterns of antibiotic susceptibility.[21,22] The Vitek 2 system has the benefit for evaluation and providing susceptibility results within 3.5 hours to 16 hours by matching its results against a big database.[23] Bacterial resistance, particularly MDR, is a natural part of microbial evolution. New products are needed to address bacterial resistance to present antimicrobials.[24]

O. sanctum, commonly referred to as Holy Basil, is a fragrant plant indigenous to the tropical regions of Asia and Africa and holds considerable medicinal significance within the Lamiaceae family. The Lamiaceae family is among the most utilized families of medicinal plants globally, serving not only as a source of healing herbs but also for its essential oils that are employed as spices and flavorings in a variety of food products.[25] The antimicrobial activities reported in this study against both bacterial and candidal isolates were significant, as most of the studies have the same opinion that *O. sanctum* is a good inhibitor of microorganisms.[26,27]

In this study, five solvents were used to analyse the antimicrobial activity of *O. sanctum* leaves extract, where the methanol extract showed good inhibitory action among all. However, in a previous report, it was stated that the aqueous extract of *O. sanctum* inhibited *K. pneumoniae, E. coli, Proteus spp., S. aureus*, and *C. albicans* more effectively than the alcoholic extract using the agar disk diffusion method.[28] The reported highest inhibitory zone of this study was 19 mm against *S. aureus*, followed by 18 mm against *A. baumannii*, which was a significant inhibition activity when compared to the control drugs. At a concentration of 5000 µg/ml the best MIC was reported with 0.162 mg/ml against *C. albicans.* The selected antimicrobial investigations employed, and anticandidal characteristics were assessed using the zone of inhibition (ZOI). All investigations indicated that *O. sanctum* has potent anticandidal properties, suggesting it could serve as an effective and cost-efficient "supplement" to conventional treatments for both systemic as well as candidal infections. The literature also points out that eugenol and linalool are likely the main compounds in *O. sanctum* contributing to its anticandidal effects.[29] However, the precise mechanism through which these components operate is still unclear.

5. Conclusion

The finding of study represents the first assessments of the antimicrobial effects of *O. sanctum* leaf extract on pathogens linked to otitis media (OM), especially those that lead to more severe infections. Due to the differences in microbial strains studied in earlier research, making direct comparisons with past studies on the antibacterial properties of *O. sanctum* is difficult. With the existing literature being sparse on its effectiveness against pathogens responsible for OM, this study highlights findings that suggest *O. sanctum* is a potential antibacterial treatment for OM infections. Additionally, further evidence could help tackle the challenges posed by multidrug-resistant (MDR) infections in treating OM, providing complementary support to current standard care practices.

6. Source of Funding

None.

7. Conflict of Interest

None.

8. Authors' Contributions

All authors contributed to the conception, design, and writing of the manuscript. All authors read and approved the final manuscript.

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