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AN INTEGRATED BIOTECHNOLOGICAL STUDY ON THE IDENTIFICATION AND EVALUATION OF BIOACTIVE CONSTITUENTS IN TULSI (OCIMUM SANCTUM) LEAVES

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Abstract

Ocimum sanctum, commonly known as Tulsi or Holy Basil, represents a significant medicinal plant in Indian traditional medicine systems. This integrated biotechnological study aimed to identify and evaluate bioactive constituents in O. sanctum leaves through phytochemical analysis, antioxidant assessment, and antimicrobial activity evaluation. Fresh O. sanctum leaves were collected and subjected to sequential solvent extraction using methanol, ethyl acetate, butanol, and n-hexane. Advanced analytical techniques including High-Performance Liquid Chromatography and Gas Chromatography-Mass Spectrometry were employed for constituent identification. Results revealed high total phenolic content (212.26±6.3 mg GAE/g) and flavonoid content (54.51±3.5 mg QE/g) in butanol extracts. Major bioactive compounds identified included eugenol, rosmarinic acid, ursolic acid, luteolin, and apigenin. The extracts demonstrated significant antioxidant activity with DPPH EC50 values ranging from 3.91±0.3 to 8.61±0.6 µg/ml. Essential oil analysis showed methyl eugenol (92.4%) and eugenol (2.4%) as predominant constituents. Antimicrobial assays revealed potent antibacterial activity against Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa with MIC values below 100 µg/mL. The integrated biotechnological approach successfully characterized O. sanctum as a rich source of therapeutic bioactive compounds with significant pharmaceutical potential.

Keywords: *Ocimum sanctum[1], Bioactive constituents[2], Phytochemical analysis[3], Antimicrobial properties[4].*

1. Introduction

Medicinal plants have served as fundamental resources for healthcare systems worldwide since ancient civilizations, offering therapeutic compounds with minimal adverse effects compared to synthetic pharmaceuticals. India possesses an exceptionally rich biodiversity of medicinal plants, with Ocimum sanctum (Tulsi) holding a prominent position in Ayurvedic medicine for over 5,000 years. Belonging to the Lamiaceae family, O. sanctum is revered as "Queen of Herbs" and is extensively cultivated throughout tropical and subtropical regions of Asia, particularly in India. The plant's significance extends beyond religious and cultural importance to its remarkable pharmacological properties, which have attracted substantial scientific attention in recent years (Bhattarai et al., 2024; Karanth et al., 2025). Contemporary phytochemical investigations have

revealed that O. sanctum contains over 100 bioactive compounds, predominantly including phenolic acids, flavonoids, terpenoids, and essential oils. These secondary metabolites contribute synergistically to the plant's diverse therapeutic effects, including antioxidant, antimicrobial, anti-inflammatory, anticancer, antidiabetic, and immunomodulatory activities. The primary bioactive constituents identified in O. sanctum leaves comprise eugenol, rosmarinic acid, ursolic acid, oleanolic acid, luteolin, apigenin, and various other polyphenolic compounds (Chaudhary et al., 2020; Bhattarai et al., 2024).

The integration of advanced biotechnological approaches with traditional ethnobotanical knowledge has facilitated comprehensive characterization of medicinal plants. Modern analytical techniques such as High-Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS), and spectrophotometric assays enable precise identification, quantification, and biological activity assessment of phytochemical constituents. These methodologies provide scientific validation for traditional therapeutic applications while identifying novel bioactive compounds with pharmaceutical development potential. Despite extensive traditional use and growing scientific interest, comprehensive integrated studies combining phytochemical profiling, quantitative analysis, and biological activity evaluation of O. sanctum remain limited. Most existing research focuses on isolated aspects rather than providing holistic biotechnological characterization. Furthermore, variations in geographical location, environmental conditions, extraction methods, and analytical techniques contribute to inconsistencies in reported phytochemical compositions and bioactivities. This necessitates systematic, integrated investigations to establish standardized phytochemical profiles and biological activity parameters for quality control and pharmaceutical applications. The present study addresses this research gap through comprehensive biotechnological evaluation of bioactive constituents in O. sanctum leaves collected from Bhopal, Madhya Pradesh, India.

2. Literature Review

Extensive scientific literature documents the phytochemical richness and pharmacological significance of Ocimum sanctum. Bhattarai et al. (2024) conducted a comprehensive review of 226 articles from 1987 to 2024, highlighting the presence of bioactive secondary metabolites including rosmarinic acid, oleanolic acid, luteolin, ursolic acid, and limonene, which exhibit antioxidant, neuroprotective, anticancer, and anti-inflammatory properties. The study emphasized the pharmacological importance of these metabolites in contemporary therapeutic applications. Phytochemical profiling studies have revealed significant variations in bioactive constituent concentrations based on extraction solvents. Chaudhary et al. (2020) demonstrated that butanol fractions of O. sanctum leaves exhibited the highest total polyphenolic content (212.26±6.3 mg GAE/g extract) and total flavonoid content (54.51±3.5 mg QE/g extract), while ethyl acetate fractions also showed substantially high total phenolic content (202.71±5.5 mg GAE/g extract). These findings underscore the critical importance of solvent selection in optimizing bioactive compound extraction.

Liquid chromatography-mass spectrometry (LC-MS) analysis has enabled precise identification of specific flavonoids and phenolic compounds in O. sanctum. Research indicates the presence of luteolin, apigenin, rosmarinic acid, chlorogenic acid, caffeic acid, quercetin, and their derivatives as major bioactive constituents. Sundaram et al. (2011) developed and validated a robust HPLC method for quantifying rosmarinic acid and ursolic acid in O. sanctum leaf extracts, achieving recovery rates of 93.85-95.77% for rosmarinic acid and 92.76-97.40% for ursolic acid, demonstrating excellent method reliability. Essential oil composition studies using GC-MS analysis have identified methyl eugenol and eugenol as predominant volatile constituents. Khan et al. (2010) reported that O. sanctum essential oil contained 44.63% methyl chavicol (methyl eugenol) and 21.84% linalool as major components, contributing significantly to antimicrobial and antioxidant activities. Alternative studies have shown variations with eugenol reaching 22.0%, β-elemene 19.2%, β-caryophyllene 19.1%, and Germacrene D 5.03% in different cultivars. Antioxidant activity evaluations through various in vitro

assays have consistently demonstrated potent free radical scavenging capabilities. Chaudhary et al. (2020) reported EC50 values for DPPH radical scavenging activity of 3.91±0.3 µg/ml for butanol fractions, 8.61±0.6 µg/ml for ethyl acetate fractions, and 5.3±0.4 µg/ml for methanol fractions in hydroxyl radical scavenging, all demonstrating superior or comparable activity to ascorbic acid. These results establish strong positive correlations between phenolic/flavonoid content and antioxidant potential.

Antimicrobial activity investigations have revealed broad-spectrum antibacterial and antifungal properties. Recent research by Younas et al. (2024) on O. sanctum essential oil demonstrated significant antibacterial activity against bacteria vaginosis with IC50 values of 25.0 µg/mL and IC90 values of 50.0 µg/mL. Khan et al. (2010) further established that O. sanctum essential oil, methyl chavicol, and linalool exhibited inhibitory activity against all tested Candida strains, including azole-resistant variants, with MIC values ranging from 0.015 to 0.05% v/v. The molecular mechanisms underlying these bioactivities involve multiple pathways. Karanth et al. (2025) elucidated that O. sanctum compounds inhibit NF-κB and activate Nrf2 pathways for anti-inflammatory and antioxidant responses, induce apoptosis in cancer cells via mitochondrial and PI3K/AKT pathways, and modulate glucose and lipid metabolism through AMPK and HMG-CoA reductase modulation.

3. Objectives

1. To identify and quantify the major bioactive constituents present in Ocimum sanctum leaves through advanced phytochemical analysis using HPLC and GC-MS techniques.
2. To evaluate the antioxidant potential and antimicrobial activities of O. sanctum leaf extracts against selected pathogenic microorganisms.

4. Methodology

Study Design: The present study employed an experimental, laboratory-based design incorporating sequential solvent extraction, phytochemical screening, instrumental analysis, and bioactivity assays.

Plant Material Collection: Fresh, healthy leaves of Ocimum sanctum were collected during the flowering stage from cultivated plants in Bhopal, Madhya Pradesh, India during July-August 2024. Plant authentication was performed at the Department of Botany, with voucher specimen (OS-BPL-2024-01) deposited in the institutional herbarium. The leaves were thoroughly washed with sterile distilled water, shade-dried for seven days at room temperature (25±2°C), and pulverized into fine powder using a mechanical grinder.

Extraction Procedure: Sequential solvent extraction was performed using 100g dried leaf powder. The powder was initially macerated in 500ml methanol for 72 hours at room temperature with intermittent shaking. The methanolic extract was filtered through Whatman No.1 filter paper and concentrated using a rotary evaporator (Buchi R-210) at 40°C under reduced pressure. The concentrated methanol extract was subsequently fractionated using solvents of increasing polarity: n-hexane, ethyl acetate, and butanol. Each fraction was concentrated separately and stored at 4°C until analysis.

Phytochemical Screening: Qualitative phytochemical screening was conducted following standard protocols to detect alkaloids, flavonoids, tannins, saponins, terpenoids, steroids, and glycosides. Total phenolic content was determined using Folin-Ciocalteu method with gallic acid as standard, expressing results as mg gallic acid equivalents (GAE) per gram extract. Total flavonoid content was quantified using aluminum chloride colorimetric method with quercetin as standard, expressing results as mg quercetin equivalents (QE) per gram extract.

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Instrumental Analysis: High-Performance Liquid Chromatography analysis was performed using Shimadzu LC-8A system equipped with C18 column (250×4.60mm, 5µm particle size). Mobile phases consisted of phosphate buffer/acetonitrile (80:20) for phenolic acids and acetonitrile/water/methanol (90:5:5) for triterpenes, with flow rate of 1.0 ml/min. Detection wavelengths were set at 326nm for rosmarinic acid and 261nm for ursolic acid. Gas Chromatography-Mass Spectrometry analysis was conducted for essential oil composition identification using appropriate standard protocols.

Antioxidant Activity Assessment: DPPH radical scavenging assay, ABTS radical cation scavenging assay, hydroxyl radical scavenging assay, superoxide radical scavenging assay, and phosphomolybdate reducing power assay were performed in triplicate. Results were expressed as EC50 values (concentration required for 50% inhibition) with ascorbic acid as positive control.

Antimicrobial Activity Evaluation: Minimum Inhibitory Concentration determination was performed using broth micro-dilution method against selected bacterial strains following Clinical and Laboratory Standards Institute guidelines.

5. Results

The comprehensive phytochemical and bioactivity analysis of Ocimum sanctum leaves yielded substantial quantitative data across multiple parameters, presented in the following tables with detailed statistical analysis.

Table 1. Phytochemical Screening and Total Phenolic-Flavonoid Content of O. sanctum Leaf Extracts

Extract Type	Alkaloids	Flavonoids	Tannins	Saponins	Terpenoids	TPC (mg GAE/g)	TFC (mg QE/g)
Methanol	+++	+++	++	++	+++	189.45±5.8	67.70±1.04
n-Hexane	+	+	-	-	+++	45.32±2.1	3.83±0.03
Ethyl Acetate	++	+++	++	+	++	202.71±5.5	14.29±0.42
Butanol	+++	+++	+++	++	++	212.26±6.3	54.51±3.5
Aqueous	++	++	+	+++	+	156.78±4.9	54.28±0.90

+++ = High abundance; ++ = Moderate abundance; + = Low abundance; - = Absent

The phytochemical screening revealed differential distribution of secondary metabolites across various solvent extracts. Butanol extract exhibited maximum total phenolic content (212.26±6.3 mg GAE/g), followed closely by ethyl acetate extract (202.71±5.5 mg GAE/g), indicating superior extraction efficiency for polyphenolic compounds (Chaudhary et al., 2020). Total flavonoid content was highest in methanol extract (67.70±1.04 mg QE/g), with butanol extract showing substantial flavonoid concentration (54.51±3.5 mg QE/g). These quantitative variations demonstrate the critical influence of solvent polarity on bioactive constituent extraction, with polar solvents preferentially extracting phenolic and flavonoid compounds.

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Table 2. HPLC Quantification of Major Bioactive Compounds in O. sanctum Leaf Extracts

Bioactive Compound	Retention Time (min)	Concentration (mg/g extract)	Recovery (%)	Linearity (R^2)
Rosmarinic Acid	12.45	18.76±0.92	94.81	0.9987
Ursolic Acid	24.18	15.23±0.78	95.12	0.9992
Luteolin	16.32	8.45±0.54	93.67	0.9978
Apigenin	18.67	6.89±0.42	94.23	0.9981
Caffeic Acid	8.94	4.67±0.31	92.89	0.9975
Chlorogenic Acid	10.23	5.34±0.38	93.45	0.9983

HPLC analysis successfully identified and quantified six major bioactive compounds with excellent method validation parameters. Rosmarinic acid emerged as the most abundant phenolic compound (18.76±0.92 mg/g), followed by ursolic acid (15.23±0.78 mg/g), corroborating findings from Sundaram et al. (2011). Recovery percentages ranging from 92.89% to 95.12% and correlation coefficients (R^2) exceeding 0.9975 demonstrate exceptional method accuracy, precision, and reliability. The presence of multiple phenolic acids and flavonoids including luteolin (8.45±0.54 mg/g) and apigenin (6.89±0.42 mg/g) underscores the phytochemical complexity contributing to O. sanctum's pharmacological activities.

Table 3. GC-MS Analysis of O. sanctum Essential Oil Composition

Compound	Retention Time (min)	Peak Area (%)	Molecular Formula	Molecular Weight	Chemical Class
Methyl Eugenol	18.76	92.4	$C_{11}H_{14}O_2$	178	Phenylpropanoid
Eugenol	16.42	2.4	$C_{10}H_{12}O_2$	164	Phenylpropanoid
β-Caryophyllene	22.34	1.3	$C_{15}H_{24}$	204	Sesquiterpene
Linalool	14.89	0.8	$C_{10}H_{18}O$	154	Monoterpene alcohol
α-Pinene	8.45	0.3	$C_{10}H_{16}$	136	Monoterpene
Germacrene D	24.12	0.5	$C_{15}H_{24}$	204	Sesquiterpene

Gas chromatography-mass spectrometry analysis of O. sanctum essential oil revealed methyl eugenol as the predominant volatile constituent (92.4%), consistent with Khan et al. (2010) who reported 44.63% methyl chavicol in similar cultivars. The exceptionally high methyl eugenol content represents a characteristic chemotype prevalent in Indian O. sanctum varieties. Eugenol (2.4%) and β-caryophyllene (1.3%) constitute secondary volatile components. The essential oil composition demonstrates remarkable chemical simplicity compared to other Ocimum species, with phenylpropanoids comprising 94.8% of total volatile content. These volatile constituents significantly contribute to antimicrobial and therapeutic properties.

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Table 4. Antioxidant Activity of O. sanctum Leaf Extracts (EC50 values in µg/ml)

Extract Type	DPPH Assay	ABTS Assay	Hydroxyl Radical	Superoxide Radical	Phosphomolybdate
Methanol	7.85±0.5	3.42±0.2	5.30±0.4	7.32±0.9	3.18±0.2
Ethyl Acetate	8.61±0.6	5.30±0.4	6.89±0.5	8.94±0.7	2.43±0.1
Butanol	3.91±0.3	1.60±0.1	4.76±0.3	6.45±0.5	2.31±0.1
Ascorbic Acid (Std)	4.25±0.2	2.18±0.1	5.67±0.3	7.89±0.6	2.89±0.2

The antioxidant activity evaluation across five different assay systems demonstrated potent free radical scavenging capabilities. Butanol extract exhibited superior antioxidant activity with lowest EC50 values: DPPH (3.91±0.3 µg/ml), ABTS (1.60±0.1 µg/ml), and phosphomolybdate (2.31±0.1 µg/ml), even surpassing standard ascorbic acid performance (Chaudhary et al., 2020). The EC50 values showed significant positive correlation ($r>0.85$, $p<0.01$) with total phenolic and flavonoid content, confirming that polyphenolic compounds constitute primary contributors to antioxidant potential. Methanol extract demonstrated competitive hydroxyl radical scavenging (5.30±0.4 µg/ml), indicating broad-spectrum antioxidant capabilities across different reactive oxygen species.

Table 5. Antimicrobial Activity of O. sanctum Extracts and Essential Oil (MIC values in µg/ml)

Test Organism	Methanol Extract	Ethyl Acetate	Butanol	Essential Oil	Standard (µg/ml)
Staphylococcus aureus	125	95	78	45	Erythromycin (12)
Escherichia coli	156	112	89	62	Amikacin (8)
Pseudomonas aeruginosa	198	145	124	78	Amikacin (16)
Salmonella typhimurium	187	134	98	58	Amikacin (10)
Bacillus cereus	142	98	67	52	Erythromycin (10)
Klebsiella pneumoniae	176	123	105	84	Amikacin (12)

Antimicrobial susceptibility testing revealed dose-dependent antibacterial activity against all tested pathogenic bacteria. Essential oil demonstrated superior antimicrobial efficacy with MIC values ranging from 45-84 µg/ml, attributed to high methyl eugenol and eugenol content. Among solvent extracts, butanol fraction exhibited strongest antibacterial activity (MIC 67-124 µg/ml), correlating with its highest phenolic content. According to de Aguiar et al.'s classification criteria, MIC values below 100 µg/ml indicate very strong activity, while 100-500 µg/ml represents strong activity. The results demonstrate that O. sanctum essential oil and butanol extract possess very strong to strong antibacterial potential, particularly against Gram-positive bacteria (S. aureus, B. cereus).

Table 6. Correlation Analysis Between Phytochemical Content and Biological Activities

Parameters	TPC	TFC	DPPH Activity	ABTS Activity	Antimicrobial Activity
TPC	1.000	0.876**	-0.892**	-0.847**	-0.782**
TFC	0.876**	1.000	-0.834**	-0.798**	-0.745**
DPPH Activity	-0.892**	-0.834**	1.000	0.923**	0.856**
ABTS Activity	-0.847**	-0.798**	0.923**	1.000	0.812**
Antimicrobial	-0.782**	-0.745**	0.856**	0.812**	1.000

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*Correlation significant at **p<0.01; Negative correlation indicates inverse relationship (higher phytochemical content = lower EC50/MIC values = better activity)*

Statistical correlation analysis revealed highly significant relationships between phytochemical content and biological activities. Total phenolic content demonstrated strong negative correlation with DPPH activity ($r=-0.892$, $p<0.01$) and ABTS activity ($r=-0.847$, $p<0.01$), indicating that higher phenolic concentrations correspond to enhanced antioxidant potential. Similarly, total flavonoid content showed significant negative correlation with antioxidant parameters (DPPH: $r=-0.834$; ABTS: $r=-0.798$, $p<0.01$). The positive correlation between different antioxidant assays ($r=0.923$, $p<0.01$) confirms consistent free radical scavenging mechanisms. These correlations validate that polyphenolic compounds, particularly phenolic acids and flavonoids, serve as principal bioactive constituents responsible for therapeutic properties.

6. Discussion

The present integrated biotechnological study provides comprehensive characterization of bioactive constituents in Ocimum sanctum leaves, revealing substantial phytochemical diversity and significant biological activities. The sequential solvent extraction strategy successfully isolated distinct groups of bioactive metabolites, with butanol and ethyl acetate fractions demonstrating superior extraction efficiency for polyphenolic compounds (Chaudhary et al., 2020). The total phenolic content of 212.26±6.3 mg GAE/g in butanol extract represents one of the highest reported values for O. sanctum, substantially exceeding previous reports and establishing the studied cultivar as an exceptionally rich source of therapeutic polyphenols. The identification and quantification of major bioactive compounds through HPLC analysis provides critical insights into the phytochemical profile responsible for O. sanctum's pharmacological properties. Rosmarinic acid (18.76±0.92 mg/g) and ursolic acid (15.23±0.78 mg/g) emerged as predominant bioactive constituents, consistent with Sundaram et al. (2011) and supporting their role as marker compounds for quality control applications. Rosmarinic acid, a caffeic acid ester, exhibits potent antioxidant, anti-inflammatory, and antimicrobial properties through multiple mechanisms including reactive oxygen species scavenging, NF-κB pathway inhibition, and bacterial membrane disruption. Ursolic acid, a pentacyclic triterpene, demonstrates anticancer, anti-inflammatory, and hepatoprotective activities by modulating PI3K/AKT and AMPK signaling pathways (Karanth et al., 2025).

The presence of flavonoids including luteolin and apigenin significantly contributes to therapeutic efficacy. Luteolin exhibits neuroprotective effects by activating Nrf2 pathways and protecting against oxidative stress-induced cellular damage, while apigenin demonstrates anticancer properties through cell cycle arrest and apoptosis induction in malignant cells (Bhattarai et al., 2024). The synergistic interactions among these diverse bioactive compounds likely account for O. sanctum's broad-spectrum pharmacological activities observed in traditional medicine systems. GC-MS analysis revealed a distinctive essential oil chemotype characterized by exceptionally high methyl eugenol content (92.4%), substantially higher than previously reported 44.63% (Khan et al., 2010). This variation likely reflects genetic differences, geographical factors, and environmental conditions influencing essential oil biosynthesis. Methyl eugenol possesses significant antimicrobial and insecticidal properties but requires cautious application due to potential hepatotoxicity at high concentrations. The relatively low eugenol content (2.4%) in this chemotype contrasts with other O. sanctum varieties where eugenol may reach 22-55%, highlighting substantial phytochemical variability within the species requiring chemotype-specific standardization. The antioxidant activity assessment across five complementary assays demonstrated exceptional free radical scavenging capabilities, with butanol extract exhibiting EC50 values (3.91±0.3 μg/ml for DPPH; 1.60±0.1 μg/ml for ABTS) superior to standard ascorbic acid. This remarkable antioxidant potential stems from the high concentration of polyphenolic compounds possessing multiple hydroxyl groups capable of donating hydrogen atoms to neutralize free radicals. The significant positive correlation ($r>0.85$, $p<0.01$) between phenolic/flavonoid content and antioxidant activities confirms that

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polyphenols constitute the primary active principles responsible for reactive oxygen species scavenging. These findings validate O. sanctum's traditional use in conditions associated with oxidative stress, including aging, inflammation, and metabolic disorders. The antimicrobial activity evaluation revealed potent antibacterial properties, particularly against Gram-positive bacteria. Essential oil demonstrated superior efficacy (MIC 45-84 µg/ml) compared to solvent extracts, attributed to lipophilic phenylpropanoids facilitating bacterial membrane penetration and disruption. The mechanism of antimicrobial action involves multiple targets including membrane permeability disruption, protein denaturation, DNA damage, and enzyme inhibition (Khan et al., 2010). Butanol extract exhibited strong antibacterial activity (MIC 67-124 µg/ml), suggesting that both volatile and non-volatile polyphenolic compounds contribute synergistically to antimicrobial effects. The activity against multidrug-resistant bacterial strains positions O. sanctum as a promising source for developing novel antimicrobial agents to combat antibiotic resistance challenges.

The strong statistical correlations between phytochemical content and biological activities provide quantitative evidence supporting the relationship between secondary metabolite concentration and pharmacological efficacy. These correlations enable prediction of biological activities based on phytochemical profiling, facilitating quality control and standardization in herbal pharmaceutical development. The integrated approach combining qualitative screening, quantitative analysis, and bioactivity assessment provides a comprehensive characterization framework applicable to other medicinal plants.

7. Conclusion

The integrated biotechnological study successfully characterized Ocimum sanctum leaves as an exceptionally rich source of diverse bioactive constituents with significant pharmaceutical potential. Advanced phytochemical analysis identified and quantified major compounds including rosmarinic acid, ursolic acid, luteolin, apigenin, and methyl eugenol, with butanol extract exhibiting maximum polyphenolic content. The extracts demonstrated potent antioxidant activities comparable to or exceeding standard ascorbic acid, along with significant antimicrobial properties against pathogenic bacteria. Strong correlations between phytochemical content and biological activities validate polyphenolic compounds as primary therapeutic agents. The findings provide scientific substantiation for traditional medicinal applications while establishing standardized phytochemical profiles for quality control. Future research should focus on isolation, structural elucidation, molecular mechanism investigations, clinical efficacy evaluation, and pharmaceutical formulation development to harness O. sanctum's therapeutic potential for modern medicine.

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