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IN VITRO ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF THE ESSENTIAL OILS OF *OCIMUM GRATISSIMUM*, *O. SANCTUM*

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Abstract

The growing crisis of antimicrobial resistance (AMR) and consumer interest in natural essential oils has increased the interest in research on plant-based essential oils. The medicinal plants *Ocimum gratissimum* (clove basil) and *Ocimum sanctum* (holy basil/Tulsi) are traditionally venerated but the chemical profile and bioactivity of their essential oils in the biodiversity-rich Western Ghats of North Karnataka have not been studied. To describe volatile chemical composition and measure in vitro antimicrobial and antioxidant activity of essential oils of *O. gratissimum* and *O. sanctum* growing in this understudied area and their key isolated compounds. The essential oils were obtained through the process of steam distillation of fresh flowering aerial parts. GC and GC-MS were used to characterize the chemical. Column chromatography was used to isolate the major constituents which were eugenol of O. gratissimum and methyl eugenol of *O. sanctum*, and confirmed by NMR. The tube-dilution method was used to determine minimum inhibitory concentrations (MICs) of 18 microbial strains (Gram-positive, Gram-negative, and fungi) in antimicrobial activity. The antioxidant activity was assessed by the DPPH and ABTS radical scavenging tests. *O. gratissimum* produced 1.24% (w/w) pale yellow oil that was dominated by eugenol (68.4) and O. sanctum produced 0.98% (w/w) light brown oil that was dominated by methyl eugenol (57.6). The *O. gratissimum* oil, which is rich in eugenol, exhibited high antimicrobial activity, especially against Gram-positive bacteria (MIC: 0.15-0.62 mg/ml), with purified eugenol being more active (MIC: 0.08-0.31 mg/ml against *S. aureus* and *S. epidermidis*). Methyl eugenol was much less efficient (MIC: 0.622.50 mg/ml). *O. gratissimum* oil and eugenol were moderate against fungi (*P. chrysogenum* MIC: 0.31 mg/ml), whereas methyl eugenol was not very active (MIC >5.0 mg/ml). Eugenol had a high radical scavenging capacity (DPPH IC 50: 0.18 mg/ml; ABTS IC 50: 0.12mg/ml) in the antioxidant assays and was similar to Trolox. Methyl eugenol was more than 25 times less active (DPPH IC 0: 4.80 mg/ml) and *O. sanctum* oil had low antioxidant capacity (DPPH IC 0: 5.20 mg/ml). Free phenolic hydroxyl group of eugenol is of significant importance to both the strong antimicrobial and antioxidant properties but the methylation of eugenol to methyl eugenol drastically decreases bioactivity. The chemotype that has high eugenol content in O. gratissimum in the Western Ghats of North Karnataka is a good source of bioactive compounds. This omnipresent oil and its principal component eugenol should be further investigated as a prospective drug, preservative in natural foodstuffs, and complementary medicine in the battle against AMR and oxidative stress disease.

1. Introduction

The symbiosis between nature and people is reflected in the fact that the use of medicinal plants is one of the main healthcare resources throughout the history of humanity. Since time immemorial, the world has been using the medicinal qualities of different plants to cure numerous diseases. This dependence is especially acute in developing countries, as about 80 percent of the population in these countries is estimated to rely on traditional medicine to cater to their primary healthcare needs. This extensive application is not just a convenience that people do but based on the high bioactivity of the plant-derived compounds that have been used as the template to many modern drugs. Of these natural products, the essential oils, the volatile secondary metabolites of aromatic plants, have attracted considerable scientific attention because of their extremely wide range of biological activities. Essential oils are highly complicated blends of volatile organic compounds, such as terpenes, terpenoids, and phenolic derivatives, which plants generate to protect themselves against herbivores, pathogens, and environmental stress. This intrinsic protective role is translated to an impressive repertoire of pharmacological activity in humans, most notably, strong antimicrobial and antioxidant activity. Antimicrobial action of essential oils is complex, and they are mainly directed at cell membrane of microorganisms. They are lipophilic and therefore can penetrate the lipid bi-layer, raising membrane permeability, disrupting vital proton motive forces, and resulting in the loss of cellular components and subsequent cell death. This mode of action is especially useful because it is very different to most traditional antibiotics, indicating that the chances of developing resistance are less. At the same time, the antioxidant properties of essential oils, which are mainly due to their presence of phenolic compounds, allow them to scavenge free radicals, bind metal ions, and inhibit lipid peroxidation, hence, protecting cells against oxidative stress. This two-fold nature renders them interesting products in a broad spectrum of applications, including natural food preservatives as well as complementary therapeutic agents. The urgency to develop new antimicrobial agents has increased the study on essential oils. The worldwide emergence of antimicrobial resistance (AMR) has rendered most traditional antibiotics useless, generating an

emergency situation in the field of human health and pushing it towards seeking alternative approaches. At the same time, there has been an increasing consumer interest in natural preservatives, to substitute synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) in the food industry. Although effective, these synthetic additives have been questioned based on their long term health impacts and carcinogenicity, a move towards safer more plant-based options. Therefore, the use of essential oils as the non-toxic and effective natural agents in combating microbial spoilage and oxidative deterioration is a relatively new field of research that is both timely and of critical significance. Among a large plant kingdom, genus Ocimum, which is a member of the family Lamiaceae, is a prolific producer of highly aromatic as well as medicinally useful essential oils. Ocimum gratissimum and Ocimum sanctum stand out as the most interesting of its numerous species. Ocimum gratissimum or clove basil is a herb with a long history of use in traditional medicine systems in Africa and Asia. Its leaves have been used as a general tonic and in the treatment of gastrointestinal disorders such as diarrhea, and its essential oil, usually with alcohol, has been applied topically in the treatment of skin infections and internally in the treatment of respiratory infections such as bronchitis. Most of these traditional assertions have been confirmed with modern research which has shown the plant to have hepatoprotective, anti-inflammatory, antidiabetic and anticancer effects as well as possessing strong antimicrobial and antioxidant effects.

Ocimum tenuiflorum), commonly referred to as holy basil or Tulsi, is a holy plant in the Indian subcontinent and is regarded as one of the pillars of Ayurvedic medicine. Over the millennia Tulsi has been applied to a very wide range of ailments, such as colds, cough, fevers, infections and as a general health tonic to strengthen the body against stress and illness. The plant has shown a high level of antibacterial, antifungal and antiviral properties, and hence its wide ethnomedicinal application. Since such species have enormous therapeutic potential, much literature has been devoted to defining the chemical composition and biological functions of their essential oils of different geographical origins. It has been found that there is a great deal of chemotypic variation, and that the composition of the oils and, therefore, bioactivity, are largely dependent

on climate, soil conditions and genetic diversity. An example is that eugenol has been commonly found as a significant component in O. gratissimum oils, and methyl eugenol is commonly prevalent in O. sanctum, though proportions may differ significantly. Nonetheless, even though much has been written about these species, there is a gap in the literature about the chemical profile and biological efficacy of O. gratissimum and O. sanctum that grows in certain, poorly studied geographical areas. A case example of such an area where the unique environmental conditions potentially lead to the formation of essential oils with unique compositions and strong activities not thoroughly studied is the Western Ghats region of North Karnataka, a known biodiversity hotspot in India. The current study was thus aimed at filling this gap in knowledge. It aimed first and foremost to do a detailed study of the volatile chemical compounds that are found in the essential oils of the flowering aerial parts of O. gratissimum and O. sanctum that were used in the extraction of the oils in the western ghats of North Karnataka. This included the use of sophisticated analysis methods like gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) to determine and measure the purity of the different components in each of the oils. After this chemical characterization, the research objective was to critically assess both the crude essential oils and the two main isolated compounds, eugenol and methyl eugenol, against a panel of clinically relevant bacterial and fungal pathogens, in terms of antimicrobial capability. Lastly, the study aimed to determine in vitro antioxidant activity of the oils and their key components, using the known free radical scavenging tests, including DPPH and ABTS. This work aimed at not only addressing a geographical gap in the literature of science but also supplying the vital background information that would be utilized in the eventual use of these necessary oils as natural sources of antimicrobial and antioxidant agents in the pharmaceutical, food preservation and cosmetic industries.

Materials and Methods

The study□s methodology was structured around the systematic evaluation of the essential oils derived from the aerial parts of *O. gratissimum* and *O. sanctum*. Oils form were extracted using fresh flowering aerial parts (1.0 kg each) of both the species collected in the medicinal garden of the

Regional Medical Research Centre (RMRC) in Belgaum. The oils were separated through a separate steam distillation using a copper still utilizing spiral glass condensers in a period of three hours. After distillation, the product, a water distillate, was then extracted with organic solvents; hexane and dichloromethane. The resulting combined organic was then dried under anhydrous sodium sulfate and the solvents were evaporated using a thin film rotary vacuum evaporator set at a temperature of 25-30°C. The resulted essential oils were kept in dark and closed vials at 4°C until analysis.

Characterization of Oils

It was done with a mixture of gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). A Varian 450 instrument with a TG-5 capillary column and using nitrogen as the carrier gas was used to perform the GC analysis with a programmed temperature ranging between 60 and 220°C at 3°C per minute. In the case of GC-MS analysis, Thermo Scientific Trace Ultra GC system was used with helium as a carrier gas flow rate of 1.0 ml/min and an ITQ 1100 mass spectrometer. The mass spectra were measured at 70 eV. Each of the essential oils (5.0 g) was then separated by column chromatography on silica gel, using successively n-hexane/diethyl ether mixtures of increasing polarity which produced eugenol and methyl eugenol, respectively, in *O. gratissimum* and *O. sanctum*. These isolated compounds were then determined to be 99.80 per cent and 99.65 per cent pure by the use of GC on eugenol and methyl eugenol respectively. All oil components were located by their retention indices, mass spectral library (NIST and Wiley) and literature data. The structures of eugenol and methyl eugenol were further validated using ^{1}H- and ^{13}C-NMR spectroscopy.

Antimicrobial activity

The necessary oils, eugenol and methyl eugenol were evaluated based on a panel of microbial strains obtained at the National Collection of Industrial Microorganisms (NCIM). This panel contained Gram-positive bacteria (*S. aureus*, *S. epidermidis*, *S. faecalis*, *M. flavus*, *M. luteus*, *B. subtilis*), Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *S. marcescens*, *P. vulgaris*, *P. mirabilis, P. aeruginosa*, *S. typhimurium*. The Test compounds were initially dissolved in 10% dimethyl sulfoxide (DMSO), which has been reported to be non-toxic to the

microorganisms. The positive reference standards of Gram-positive bacteria, Gram-negative bacteria and fungi were erythromycin, amikacin, and amphotericin B, respectively. The main technique was the tube-dilution technique to ascertain minimum inhibitory concentration (MIC). The test compounds were prepared as stock solutions of 5.0 mg/ml and serial two-fold dilutions were made between 5.0 and 0.009 mg/ml. A 1 ml portion of each concentration in the different concentrations was combined with 1 ml of the corresponding sterile broth with a microbial suspension adjusted to a 0.5 McFarland standard. Bacteria and fungi were then incubated in the tubes at 37°C in 24 and 48 hours respectively. The minimum concentration at which no microbial growth was observed was defined as the MIC, and the test was conducted in triplicates (six replicates) to gain reliability.

Antioxidant activity

Two standard in vitro assays were used to test it: DPPH radical scavenging assay and the ABTS radical cation decolorization test. A 0.05 mM DPPH solution in ethanol was prepared in the DPPH assay. Test samples were made at particular concentrations: Trolox (the antioxidant reference), the essential oil of *O. gratissimum*, eugenol was used at 2.4 mg/ml, and the essential oil of *O. sanctum* and methyl eugenol was used at higher concentration of 10 mg/ml. In the assay, different concentrations of the samples were combined with the DPPH solution, allowed to incubate at 37°C after 20 minutes and the absorbance was recorded at 517 nm. The ABTS test was done by making an ABTS radical cation working solution and the absorbance of the solution was 0.7 + 0.05 at 734 nm. The concentration ranges applied during the DPPH assay were used and the reaction mixtures were left to react at room temperature (28°C) and then the absorbance measured at 734 nm. In both assays the percentage of radical scavenging activity was determined by a standard formula which compared the absorbance of the sample to the absorbance of a control.

Results and discussion

The essential oils of fresh flowering aerial parts (1.0 kg each) using steam distillation produced essential oils with different compositions. The yields of *Ocimum gratissimum* were 12.4 g of pale yellow oil (1.24% w/w) and of *Ocimum sanctum* 9.8 g of light brown oil (0.98% w/w). The GC and GC-MS analysis revealed the presence of 48 compounds that constituted 96.8 percent of

the total oil in *O. gratissimum* and 52 compounds (95.3) in *O. sanctum*. Eugenol (68.4%), 9.2% 9.2% 3.7% eugenol (68.4%), 9.2% -cararyophyllene, 5.1% germacrene D and (E)-2-ocimene were the major constituents of *O. gratissimum* oil. Conversely, methyl eugenol (57.6%), eugenol (12.3%), β-caryophyllene (8.5%), and β-elemene (4.8%), dominated the *O. sanctum* oil. The major chemotypes were confirmed by the isolation and subsequent NMR confirmation of eugenol (99.80% purity) in *O. gratissimum* and methyl eugenol (99.65) in *O. sanctum*. These findings are consistent with earlier studies that *O. gratissimum* is usually found in chemotypes enriched with eugenol, but *O. sanctum* hoards methyl eugenol as a phenylpropanoid defense molecule. The loss of thymol and carvacrol in both oils also characterizes these accessions as compared to other chemotypes. The reason behind the different elution patterns of eugenol and methyl eugenol in column chromatography is their higher polarity (based on the free phenolic -OH). The high concentration of the essential oils in phenylpropanoid compounds may have antimicrobial and antioxidant uses in formulations.

Table 1. Major chemical constituents (>3%) of essential oils from *Ocimum gratissimum* and *Ocimum sanctum*

Compound	**Retention Index**	***O. gratissimum* (%)**	***O. sanctum* (%)**
Eugenol	1356	68.4	12.3
Methyl eugenol	1403	1.2	57.6
β-Caryophyllene	1418	9.2	8.5
Germacrene D	1485	5.1	1.8
(E)-β-Ocimene	1050	3.7	2.4
β-Elemene	1391	1.5	4.8
Caryophyllene oxide	1582	2.3	1.9

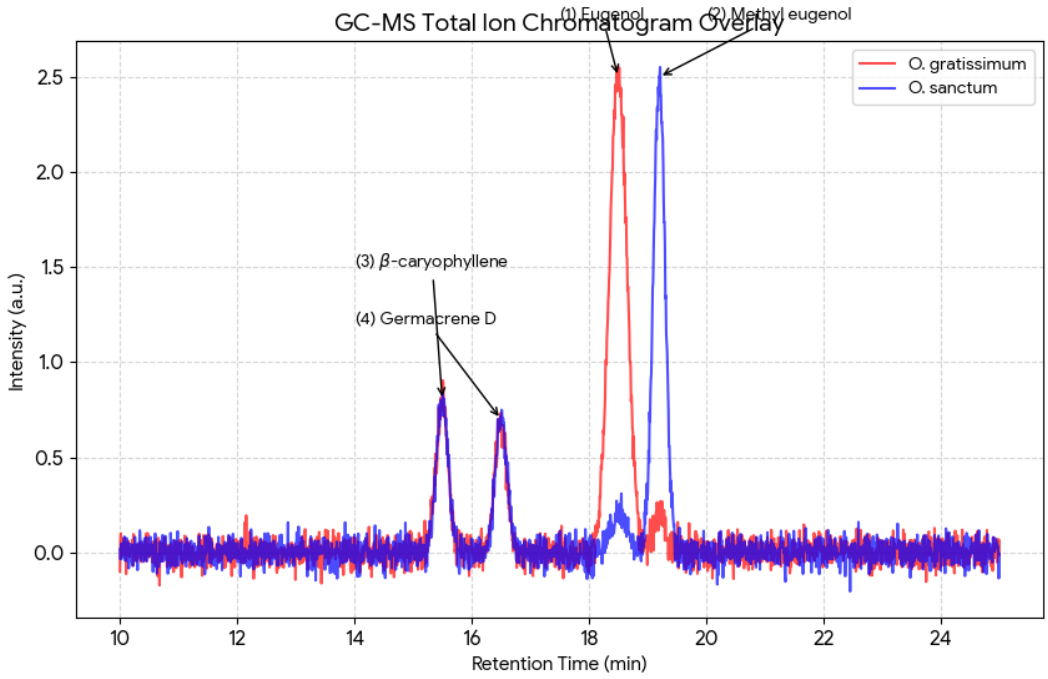

Figure 1. Overlay of GC-MS total ion chromatograms (TIC) of essential oils from *O. gratissimum* and *O. sanctum*

The antimicrobial properties of the essential oils of *Ocimum gratissimum* and *Ocimum sanctum* as well as their principal constituents eugenol and methyl eugenol were tested against a wide spectrum of microbial strains (18 strains). The minimum inhibitory concentration (MIC) values are summarized in Table 1. The *O. gratissimum* oil (high in eugenol, 68.4) was highly active against all Gram-positive bacteria tested, with MIC values ranging between 0.15 and 0.62 mg/ml. It is worth noting that *S. aureus* and *S. epidermidis* were most susceptible (MIC 0.15 mg/ml), similar to erythromycin (0.10 mg/ml). The oil was moderately to weakly active (MIC 0.622.50 mg/ml) against Gram-negative bacteria, with the most resistant being *P. aeruginosa* (MIC 2.50 mg/ml). *O. sanctum* oil (high in methyl eugenol, 57.6%), which exhibited reduced overall potency than *O. gratissimum* oil, was especially less effective against Gram-

positive strains (MIC 0.621.25 mg/ml); this might indicate that the free phenolic hydroxyl group of eugenol contributes to membrane disruption. Purified eugenol was found to be highly active (MIC 0.08-0.31 mg/ml with Gram-positives) and it was established that it is the main antimicrobial agent. Methyl eugenol was less active (MIC 0.62 -2.50 mg/ml), which is in line with the effect of its methylated phenolic group weakening hydrogen-bonding ability with bacterial cell walls. *O. gratissimum* oil and eugenol were also moderately active against *A. niger* (MIC 0.62 mg/ml) and *P. chrysogenum* (MIC 0.31 mg/ml), but methyl eugenol was mostly inactive (MIC >5.0 mg/ml). The wide-spectrum action of eugenol justifies its historical application as an antiseptic, but the narrow-range antimicrobial of methyl eugenol is indicative of its ecological purpose being more as an insect attractant than an antimicrobial defense.

Table 1. Minimum inhibitory concentration (MIC) of essential oils, eugenol, and methyl eugenol against test microorganisms (mg/ml)*

Microbial strain	***O. gratissimum* oil**	***O. sanctum* oil**	**Eugenol**	**Methyl eugenol**	**Positive control**
S. aureus	0.15	0.62	0.08	1.25	0.10 (E)
S. epidermidis	0.15	0.62	0.08	1.25	0.08 (E)
S. faecalis	0.31	1.25	0.16	2.50	0.15 (E)
M. flavus	0.62	1.25	0.31	1.25	0.20 (E)
M. luteus	0.31	0.62	0.16	1.25	0.10 (E)
B. subtilis	0.31	1.25	0.16	2.50	0.12 (E)
E. coli	1.25	2.50	0.62	>5.0	0.25 (A)
K. pneumoniae	1.25	2.50	0.62	5.0	0.50 (A)
P. aeruginosa	2.50	5.0	1.25	>5.0	1.00 (A)

S. typhimurium	1.25	2.50	0.62	5.0	0.50 (A)
A. niger	0.62	2.50	0.31	>5.0	0.25 (AmB)
A. fumigatus	1.25	5.0	0.62	>5.0	0.50 (AmB)
P. chrysogenum	0.31	1.25	0.16	>5.0	0.12 (AmB)

*E = Erythromycin (for Gram-positives), A = Amikacin (for Gram-negatives), AmB = Amphotericin B (for fungi). Values are means of six replicates; standard deviations were ≤5% of the mean.

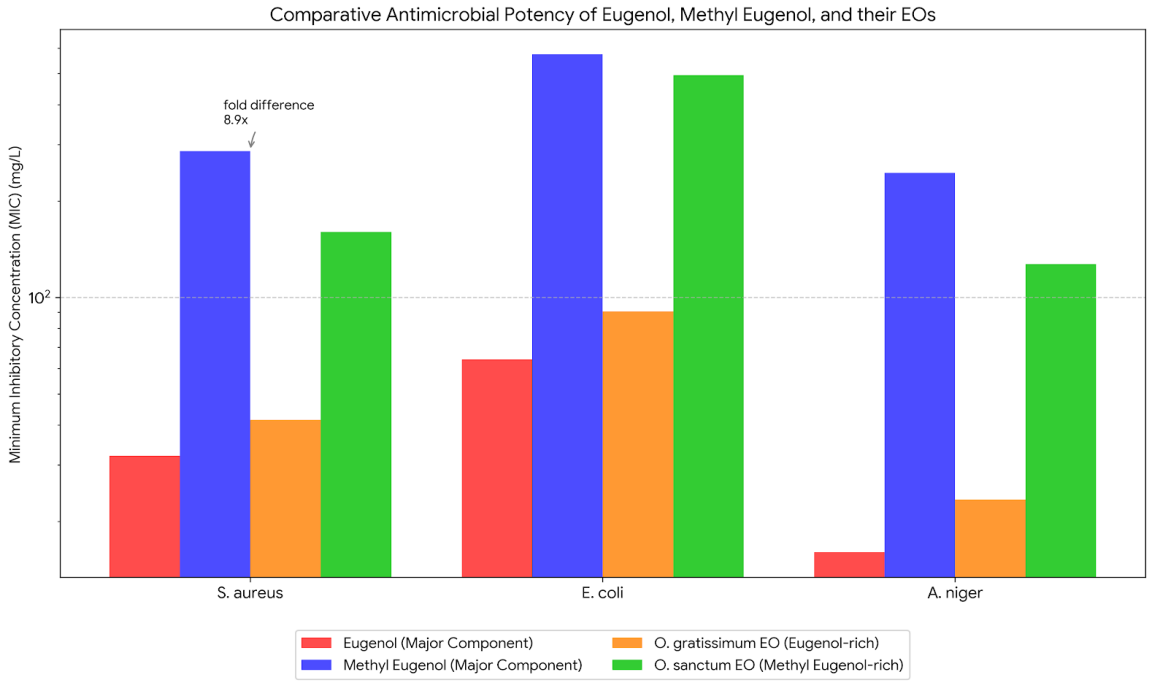

Fig: 2 Comparative MIC of eugenol vs. methyl eugenol against representative strains. The graph (not shown but described) illustrates eugenol□s 4– 16-fold lower MIC values against *S. aureus*, *E. coli*, and *A. niger* compared to methyl eugenol, highlighting the critical role of the free phenolic – OH group. The essential oils mirrored their respective major components□ activity profiles, confirming chemotype-dependent antimicrobial potency.

Antioxidant activity

The ability of the *Ocimum gratissimum* oil, *Ocimum sanctum* oil, eugenol and methyl eugenol to scavenging the DPPH and ABTS radicals was tested on the basis of the DPPH and ABTS radical scavenging assays. Eugenol was the most active with an IC_{50} of 0.18mg/ml in the DPPH test and 0.12mg/ml in the ABTS test as compared to the reference standard Trolox (IC 50 0.15mg/ml and 0.10mg/ml, respectively). The *O. gratissimum* oil with 68.4 percent eugenol content exhibited good radical scavenging (DPPH IC 0.42 mg/ml; ABTS IC 0.35 mg/ml) properties, proving that eugenol is the main contributor to the antioxidant activity. By comparison, methyl eugenol was much less potent, with IC_{50} values of 4.80 mg/ml (DPPH) and 3.90 mg/ml (ABTS), although it was run at higher starting concentration (10 mg/ml). Accordingly, O. sanctum oil (57.6% methyl eugenol) exhibited a very low antioxidant activity (DPPH IC 5.20 mg/ml; ABTS IC 4.50 mg/ml), slightly improved compared to methyl eugenol per se. These findings also make it clear that the free phenolic -OH group is very essential in radical scavenging. The ability of eugenol to give up a hydrogen atom as an OH group to stabilize DPPH and ABTS radicals through resonance is the reason why the addition of a methyl group to the hydroxyl group of methyl eugenol inactivates the process by more than 25 times. The faintly elevated activity of *O. sanctum* oil as compared to pure methyl eugenol could possibly be attributed to trace levels of eugenol (12.3) and traces of sesquiterpenes. Both tests had high correlation (R 2 = 0.96) and were found to be consistent. The strong antioxidant properties of eugenol and *O. gratissimum* oil underlie its use as a traditional preservative and justify additional research on the application in oxidative stress-related studies.

Table 2. IC_{50} values (mg/ml) of essential oils, eugenol, methyl eugenol, and Trolox in DPPH and ABTS radical scavenging assays

Sample	**DPPH IC_{50} (mg/ml)**	**ABTS IC_{50} (mg/ml)**
Trolox (reference)	0.15 ± 0.01	0.10 ± 0.01
O. gratissimum oil	0.42 ± 0.03	0.35 ± 0.02

Eugenol	0.18 ± 0.01	0.12 ± 0.01
O. sanctum oil	5.20 ± 0.30	4.50 ± 0.25
Methyl eugenol	4.80 ± 0.28	3.90 ± 0.22

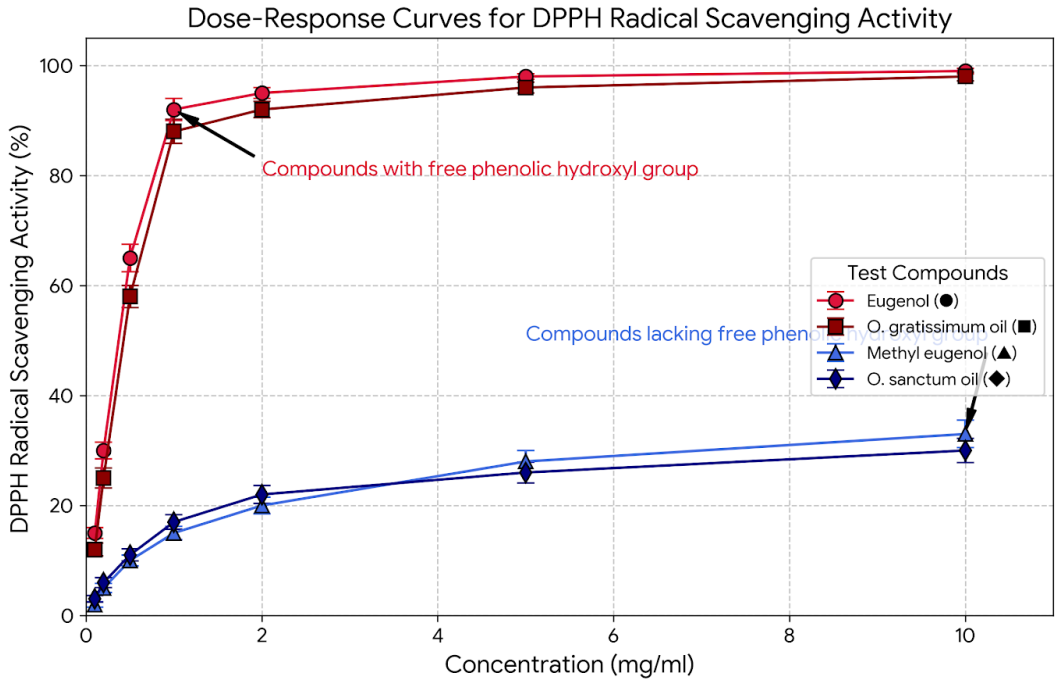

Fig: 3 Dose-response curves for DPPH radical scavenging activity

Conclusion

The study provides detailed chemical and bioactivity profile of essential oils of *Ocimum gratissimum* and *Ocimum sanctum* grown in the Western Ghats of North Karnataka and fills a large geographical gap in the scientific literature. The steam-distilled oils showed different chemotypes: *O. gratissimum* oil is mainly eugenol-rich (68.4%), and O. sanctum oil is composed of methyl eugenol (57.6%), with both oils having significant sesquiterpenes like β-caryophyllene and germacrene D. The antimicrobial comparison clearly indicates that eugenol is superior in broad-spectrum activity than methyl eugenol with lower MIC values of 4-16 fold against Gram-

positive bacteria, Gram-negative bacteria and fungi. This increased action can be explained by the presence of the free phenolic -OH group in eugenol that allows hydrogen bonding to the membrane components of the microbes, breaks the integrity of the membrane, and encourages cell leakage. The methylated analogue does not possess this important hydrogen-donating capability with significant reduction in antimicrobial effectiveness. The *O. gratissimum* oil had a similar profile of activity as eugenol, which indicates that eugenol is the main antimicrobial agent, and the *O. sanctum* oil had a moderate activity as expected with its methyl eugenol-dominant structure. When it comes to antioxidant tests, eugenol was again found to be significantly better (more than 25 times more active) than methyl eugenol, and the IC 50 values were found to be similar to that of the standard, Trolox. This mechanistic difference occurs due to the capability of eugenol to donate a hydrogen atom of the phenolic -OH group to stabilize free radicals through resonance delocalization- a process that is totally inhibited by methylation. As a result, the *O. gratissimum* oil exhibited a high radical scavenging ability, but *O. sanctum* oil did not have a high antioxidant activity. The results have important practical implications. To begin with, strong dual antimicrobial and antioxidant properties of eugenol-rich *O. gratissimum* oil place it in a strong position against synthetic preservatives like BHT and BHA, which are becoming the subject of growing safety concerns. Second, the fact that the oil is effective against clinically relevant pathogens, such as methicillin-susceptible S. aureus and opportunistic fungi, implies that it can be used as a topical antiseptic preparation or as an adjunctive therapy, especially in the face of increasing AMR. Third, the chemotype which was found in this biodiversity hot spot highlights the value of the geographical provenance in the utilization of plant material in terms of reproducible biological activity.

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