Antimicrobial Activity and HPLC Fingerprinting of Crude *Ocimum* Extracts

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Abstract: The antimicrobial activity of crude methanolic and aqueous extracts of *Ocimum sanctum* and *Ocimum kilimandsacharicum* against gram positive, gram negative and antifungal activity was evaluated to find the zone of inhibition and to set a HPLC profile or fingerprint of these extracts. The crude methanolic extract of *Ocimum sanctum* showed strong antimicrobial activity against *S.aureus* and *C. albicans* and moderate activity against *E. coli* and *B. subtilis*. The crude methanolic extract of *Ocimum kilimandsacharicum* showed strong antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans* at higher concentration, same as that shown by the standard for *C. albicans*. It showed moderate activity against *B. subtilis*. The crude aqueous extracts of *Ocimum sanctum* showed strong antimicrobial activity against *S.aureus* and moderate against others. Whereas the crude aqueous extracts of *Ocimum kilimandsacharicum* showed moderate activity against the gram positive and gram negative organisms and strong activity against *C. albicans* at higher concentration, same as that shown by the standard for *C. albicans*.

Keywords: *Ocimum sanctum*, *Ocimum kilimandsacharicum*, Antimicrobial, HPLC fingerprint .

Introduction

Medicinal plants are widely used for curing various diseases since traditional times. Different plant parts like root, leaves, stems, seeds or even whole plants are known to have therapeutic potentials¹. Medicinal plants have been used as preservatives, in pharmaceuticals, natural therapies *etc.* There is a greater demand for medicinally important plants & cultivation of such plants is recommended. About 100 plant species are involved in 25% of all prescribed drugs in advanced countries². Among all families of plant kingdom members of Lamiaceae have been used for centuries in folk medicine. *Ocimum* genus and its various species, which is widely used in Ayurveda, have known to possess enormous medicinal potential.

The genus *Ocimum*, contains 200 species of herbs and shrubs³. *Ocimum* genus contains 50 to 150 species of herbs and shrubs from the tropical regions of Asia⁴. Plants have square stems, fragrant opposite leaves and whorled flower on spiked inflorescence. It is widespread over Asia, America, Africa *etc.* It is a annual herb having common name Tulsi [holy basil]. It is a sacred plant in Hindu culture. The roots are small and hairy and the color of the leaves vary from bright green to purple and even black.

Antimicrobial assay

Fatal infectious diseases are the world's leading cause of concern with drug & antibiotic resistant human pathogenic bacteria commonly reported from all over the world⁵⁻⁹. Multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease¹⁰. There is a constant need for new and effective therapeutic agents. There is a need to develop alternative newly synthesized antimicrobial substances or substance from other sources including plants¹¹. With increasing use of drugs, microorganisms are attaining resistance to commonly used antibiotics, which leads to downfall of effectiveness of conventional medicines and therefore, search for new antimicrobial agents has become necessary¹². Plants having antimicrobial activity have attracted attention in recent years¹³⁻¹⁵. The Lamiaceae family is one of the most employed medicinal plants & a source of extracts with strong antibacterial and antioxidant properties. *Ocimum* species are used as effective drugs for many applications in folk medicine especially in Africa and Asia¹⁶.

Adulteration is described as intentional substitution with another plant species or intentional addition of a foreign substance to increase the weight or potency of the product or to decrease the cost. Adulterations and substitutions are common in raw material trade of medicinal plants. In general, adulteration is considered as an intentional practice¹⁷. Unintentional adulterations also exist in herbal raw material trade due to various reasons and many of them are left unknown. The use of wrong plant species, adulteration with undeclared other medicines, contamination with undeclared toxic or hazardous material *etc.*, results in adverse effects¹⁸. Adulteration in market samples is one of the greatest drawbacks in promotion of herbal products¹⁹. Safety and quality assurance of the marketed herbal products has become an urge to overcome these problems and to ensure a good supply of medicinal plants. The growth of herbal medicinal market has on the part of pharma companies attracted much interest, which in turn stimulated the pre-clinical studies and well controlled clinical trials to prove their safety and efficacy²⁰.

The development of a routine quality control operation for assessment of herbal formulations, standardization of active principles or major components should be done with new tools and sophisticated instrumental technology^{21,22}. Chromatographic techniques to analyze crude extracts, such thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC) or gas chromatography (GC) hyphenated with detection techniques such as ultra-violet (UV), mass spectrometry (MS) and nuclear magnetic resonance have been successfully employed²³⁻²⁶. These techniques are useful for drug analysis because of small amount of samples required, high sensitivity, reproducibility and reliability as well as fast analysis.

In marketed formulations generally *Ocimum sanctum* is used. Names of some marketed formulations include Bresol (HK-07), Transina, Kofol cough syrup, Adulsa, Immue-21, Diabecon (D-400), CIM-Candy etc. Instead of *Ocimum sanctum* if other species is used or the formulation is adulterated with other species, the efficacy of the formulation may decline. *Ocimum sanctum* and *Ocimum kilimandsacharicum* can be confused on basis of physical appearance and phytochemical screening and therefore can be intentionally or unintentionally adulterated.

In the present work the antimicrobial activity of crude methanolic and aqueous extracts of *O. sanctum* and *O. kilimandsacharicum* against gram positive, gram negative and antifungal activity was evaluated to find the zone of inhibition. Bacitracin was used as a standard. With the view to set a profile or fingerprint of the extracts of *Ocimum sanctum* and *Ocimum kilimandsacharicum* prepared, as well as to differentiate the two species, all the extracts were subjected to profiling by chromatography with the use of sophisticated analytical techniques like HPLC.

Experimental

Test organisms taken for study were *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Candida albicans*.

Determination of zone of inhibition

Freshly prepared suspensions in sterile water (Optical Density: 0.6) of pure isolated cultures of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Candida albicans* were mixed with the sterilized nutrient agar and Sabouraud dextrose agar maintained at 42.0 ± 2.0 °C and poured in petri dish (6 inch) and allowed to solidify. Five wells of 6 mm diameter were bored in the medium with the help of sterile cork-borer having 6 mm diameter and were labeled properly and 100, 200 and 400 µg/mL of the working solution / vehicle and same volume of extraction solvent for control, as well as 25 µg/mL of the standard (Bacitracin) was filled in these wells with the help of micropipette. Similar sets were made for other extracts. Petri dishes containing nutrient agar for microbial and sabouraud dextrose agar for fungal growth were incubated at 37 ± 2.0 °C for 2 days and 24 ± 2.0 °C for 5 days respectively. Plates were observed for zone of inhibition.

High performance liquid chromatographic profiling of various extracts of Ocimum sanctum and Ocimum kilimandsacharicum

Chromatography is a method of separating a mixture into its various components. The application of high performance liquid chromatography (HPLC) to biochemical samples is now widespread. HPLC is the most popular technique among all the analytical techniques used in quality control of plant products²⁷⁻²⁹. HPLC systems has many advantages like high resolving power, Qualitative and quantitative measurements and isolation of compounds, fast analysis, small amount of samples required, high sensitivity etc^{30,31}. The mobile phase used in HPLC for the methanolic extracts was methanol of HPLC grade and for aqueous extracts was phosphate buffer of pH 7: acetonitrile (60:40).

The HPLC system binary gradient Shimadzu LC-10 VP with a UV detector was used for determination of fingerprints of methanolic and aqueous extracts of *Ocimum sanctum* and *Ocimum kilimandsacharicum*. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250x4.6 mm internal diameter, particle size 5 µm, Luna 5 µm C-18(2); phenomenex, at 26 °C. Running conditions included: injection volume, 5 µL; mobile phase, methanol (HPLC grade) or phosphate buffer of pH 7: acetonitrile (60:40); flow rate, 1 mL/min and the chromatogram monitored at 254.0 nm. Samples were filtered through an ultra membrane filter (pore size 0.45 µm; E-Merck, Darmstadt, Germany) and sonicated for 45 min before being used.

Results and Discussion

Diameter of the zone of inhibition (ZOI) was measured for the estimation of potency of the antimicrobial substance which is indicated in Table 1.

Table 1. Zone of inhibition (in mm) of different organisms and the different concentration of extracts used

S. No	Extracts	Concentration, µg/ mL	Solvent	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>C. albicans</i>
1.	Bacitracin (Standard)	25		18	16	20	16
2.	OSME	100	Methanol	8	0	10	10
		200	Methanol	10	8	12	12
		400	Methanol	14	8	10	14
3.	OKME	100	Methanol	10	0	12	10
		200	Methanol	12	8	16	10
		400	Methanol	14	10	16	16
4.	OSAE	100	Water	8	0	12	8
		200	Water	12	8	12	10
		400	Water	14	10	14	10
5.	OKAE	100	Water	10	0	10	10
		200	Water	10	0	10	12
		400	Water	12	10	14	16

Where OSME – *Ocimum sanctum* methanolic extract OKME – *Ocimum kilimandsacharicum* methanolic extract, OSAE - *Ocimum sanctum* aqueous extract, OKAE – *Ocimum kilimandsacharicum* aqueous extract

The methanolic extract of *Ocimum sanctum* showed strong antimicrobial activity against *S. aureus* and *C. albicans* at 400 µg/mL concentration and moderate activity against *E. coli* and *B. subtilis*. The methanolic extract of *Ocimum kilimandsacharicum* showed strong antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans* at higher concentration, same as that shown by the standard for *C. albicans*. It showed moderate activity against *B. subtilis*.

The aqueous extracts of *Ocimum sanctum* showed strong antimicrobial activity against *S. aureus* and moderate against others. Whereas the aqueous extracts of *Ocimum kilimandsacharicum* showed moderate activity against the gram positive and gram negative organisms and strong activity against *C. albicans* at higher concentration, same as that shown by the standard for *C. albicans*.

Figure 1. Antimicrobial activity of *Ocimum* extracts against *S. aureus*

Figure 2. Antimicrobial activity of *Ocimum* extracts against *B. subtilis*

Figure 3. Antimicrobial activity of *Ocimum* extracts against *E. coli*

Figure 4. Antifungal activity of *Ocimum* extracts against *C. albicans*

Plant essential oils and extracts have been used for long time, in food preservation, pharmaceuticals, alternative medicine and natural therapies. It is necessary to investigate such plants scientifically which have been used in traditional medicine to improve the quality of healthcare. Plant extracts are potential sources of novel antimicrobial compounds

especially against bacterial pathogens. *In vitro* studies in this work showed that the plant extracts inhibited bacterial growth but their effectiveness varied. The antimicrobial activity of these plant extracts has been previously reviewed and classified as strong, medium or weak³².

HPLC fingerprinting of *Ocimum* extracts

An HPLC chromatographic method was successfully developed using a reversed phase column with UV detection at 254 nm having methanol of HPLC grade as mobile phase that could resolve 10 & 11 peaks from methanolic extracts of *Ocimum sanctum* and *Ocimum kilimandscharicum* respectively. And a method using phosphate buffer of pH 7: acetonitrile (60:40) as a mobile phase with UV detection at 254 nm could resolve 3 & 2 peaks from aqueous extracts of *Ocimum sanctum* and *Ocimum kilimandscharicum* respectively. The fingerprint profile of the plant extracts obtained can be used for identification purposes and for differentiation of the two species (Figure 5).

Figure 5. HPLC fingerprint of (a) OSME; (b) OKME; (c) OSAE and (d) OKAE

The HPLC ‘fingerprints’ of the methanolic extracts of *Ocimum sanctum* and *Ocimum kilimandsacharicum* showed major peaks (more concentration of components) at the retention times (min.) of 2.63, 2.85, 3.03, 2.61, 7.37 respectively at wavelength of 254 nm, whereas the aqueous extracts of *Ocimum sanctum* and *Ocimum kilimandsacharicum* showed major peaks at the retention times (min.) of 1.72, 3.45, 1.72 respectively at wavelength of 254 nm.

Conclusion

In conclusion, the results of the present study support the usage of the studied plants and suggest that the plant extracts possess compounds with antimicrobial potentials that can be further explored for antimicrobial activity. The antimicrobial activity was increased in all testing systems with increasing concentrations of extract. Irrespective of similar findings in phytochemical screening, practically unable to differentiate the two species, methanolic extract of *Ocimum kilimandsacharicum* was found to be more potent than that of *Ocimum sanctum* against the organisms studied. The methanolic and aqueous extracts of *Ocimum kilimandsacharicum* at higher concentration were as potent as Bacitracin against *C. albicans*.

These facts justify the medicinal use of the plant for the treatment of various ailments but further work is necessary to ascertain the clinical safety of extracts from the plant and to determine appropriate concentration for therapy so as to safeguard the health.

The fingerprint profile of the *Ocimum* extracts showing significant antimicrobial activity, obtained by HPLC can be used for identification purposes and for differentiation of the two species.

The fingerprint profiles of these extracts in the present study may enable drug manufacturers to adjust the proportion of them and prepare a standardized product with consistent biological activity.

From the above study it can be concluded that, the HPLC method can be used as a tool in routine quality analysis of the raw materials of plants with medicinal value, to detect the presence of adulterants & common contaminants.

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