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Research Article

Broad Antibacterial Spectrum and High Performance Liquid Chromatography Profiles of *Ocimum sanctum* Leaf Extract

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Abstract

Medicinal plants are the promising source of bioactive compounds which are well known for their several biological activities including antibacterial property. The current study assesses the antibacterial activity of two indigenous varieties (Dark and Bright) of *Ocimum sanctum* leaf extracts against gram-positive and gram-negative clinical bacteria, and determines the HPLC profiles of the extracts. Following disc diffusion method, the antibacterial activity of ethanolic leaf extracts (DOSE and BOSE) and aqueous leaf extracts (AqDOS and AqBOS) of both varieties *O. sanctum*, was determined and their phytochemical analysis was performed by HPLC. The HPLC chromatogram showed the presence of 11, in DOSE, and 9, in BOSE, major compounds with the RTs 1.77 - 5.29 min and 1.92 - 6.27 min, respectively, while both AqDOS and AqBOS had 4 major compounds, with the respective RTs of 1.66 - 6.17 min and 1.64 - 2.51 min. The ZDI values, due to the action of DOSE (6.25 mg), were recorded as 20 mm, for gram-positive bacteria, and 20 - 24 mm, for gram- negative bacteria, while BOSE (6.25 mg) had ZDIs 17 - 19 mm and 14 - 24 mm, respectively, for gram-positive and gram-negative bacteria. The DOSE and BOSE were more active compared to the AqDOS and AqBOS, in terms of the antibacterial activity by disc diffusion. Thus, the *O. sanctum* leaves might be utilized as the source of biotherapeutic agents to be administered against broad range of bacterial infections.

Keywords: *Ocimum sanctum*; Broad Spectrum Antibacterial Activity; Zone Diameter of Inhibition; Phytocomponents; High Performance Liquid Chromatography

Abbreviations

AqBOS: Aqueous Leaf Extract of *O. sanctum* Bright Variety; AqDOS: Aqueous Leaf Extract of *O. sanctum* Dark Variety; BOSE: Ethanolic Leaf Extract of *O. sanctum* Bright Variety; DOSE: Ethanolic Leaf Extract of *O. sanctum* Dark Variety; HPLC: High Performance Liquid Chromatography; MDR: Multi Drug Resistant; MTCC: Microbial Type Culture Collection; RT: Retention Time; SD: Standard Deviation; ZDI: Zone Diameter of Inhibition

Introduction

The bacterial pathogens, such as *Staphylococcus aureus* (*S. aureus*), *Acinetobacter baumannii* (*A. baumannii*), *Klebsiella pneumonia* (*K. pneumoniae*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) are the important cause of nosocomial infections [1]. The antibiotic resistances among such human pathogenic bacteria have been reported worldwide [2-5]. In developing countries, including India, the burden of the infectious diseases has been recorded high because of the emergence of MDR bacterial pathogens resulting from the poor health-care facilities, and over-the-counter use of antimicrobial agents [6]. This fact has directed the clinicians and the scientists around the world to search for novel affordable nontoxic antimicrobials, alternative to the synthetic antibiotics, with promising therapeutic potential to overcome the situation of MDR bacterial infections.

Various parts of different plants have been shown in several studies to be one of the most promising sources for obtaining antimicrobial compounds [7-9]. Among different medicinal plants, *Ocimum sanctum* L. (= *Ocimum tenuiflorum*) is a unique one, and is known as tulsi in Bengali: the holy basil, belonging to the family Lamiaceae [10]. It is an herbaceous aromatic plant that grows abundantly in the tropical and subtropical regions of the Indian subcontinents, and widespread as a cultivated plant throughout the world, due to its great therapeutic importance to cure various infectious and non-infectious diseases [11,12]. There are commonly two varieties of *O. sanctum* found in India, which include Dark variety or the 'Krishna tulsi' and 'Bright variety or the 'Rama tulsi' [12-14]. The earlier authors from different parts of the globe documented the presence of various bioactive compounds in *O. sanctum* leaf extracts by HPLC analysis [15-17], and validated their antibacterial efficacy against gram-negative as well as gram-positive pathogenic bacteria [18-21].

In our part of the globe, though both varieties (Dark and Bright) of *O. sanctum* are found enormously, the extensive scientific studies remain to be explored in terms of the antibacterial activity or phytochemical analysis of the plant. Therefore, the above mentioned background prompted us to explore the broad spectrum antibacterial activity of *O. sanctum* leaf extracts along with HPLC profiles of phytochemical contained in them, in order to validate the traditional usage of *O. sanctum* leaves against bacterial infection to humans.

Materials and Methods

Bacterial strains

The gram-positive, such as *S. aureus* (n = 2), and gram-negative, such as *A. baumannii, K. pneumonia* and *P. aeruginosa* (n = 2), clinical bacteria were utilized as the indicator strains in testing the antibacterial activity of *O. sanctum* leaf extracts.

Preparation of *O. sanctum* leaf extracts

The ethanolic and aqueous extracts of *O. sanctum* leaves were prepared for the present study. The collection, processing and preparation of ethanolic and aqueous *O. sanctum* leaf extracts have been described in the previous publication [11]. The concentrations of the ethanolic (DOSE and BOSE) and aqueous (AqDOS and AqBOS) leaf extracts were 125 µg/µl.

Bacterial growth inhibition property of *O. sanctum* leaf extracts

The bacterial growth inhibition property of the plant extracts were assessed by disk diffusion method [22], and the details of the method have been mentioned elsewhere [2]. Briefly, two different concentrations (3.75 and 6.25 mg) of each, of the prepared extracts, were dropped on sterile blank paper disc, placed on the surface of the already inoculated nutrient agar plates, with test bacterial fresh broth culture, by swabbing. The sensitivity of the test bacterial isolates, to the plant extracts after incubation for 24h at 37^0C, was considered with ZDIs ≥ 7 mm, obtained around the discs loaded with the extracts [23].

HPLC analysis

The HPLC analysis of the ethanolic (DOSE and BOSE) and aqueous (AqDOS and AqBOS) *O. sanctum* leaf extracts was done in the YL 9100 HPLC system associated with C_{18} column (5 µm; 100Å; 4.6 × 250 mm). The sample volume used for injection into the column was 10 µl, and flow rate was 1.0 ml/min. The mobile phase used in this system comprised of acetonitrile and water (2:3 ratio). The detection of the eluting compounds was done at 230 nm, at 35^0C.

Statistical analysis

The results were expressed as the mean ± SD, and evaluated by 't' test in comparing the data (ZDI values from the action of *O. sanctum* leaf extracts), using MS Excel 2010 software; a 'p' value of ≤ 0.05 was considered for statistical significance.

Results

The antibacterial activity of *O. sanctum l*eaf ethanolic (DOSE and BOSE) and aqueous (AqDOS and AqBOS) extracts, following disc diffusion technique, is depicted in Figure 1.

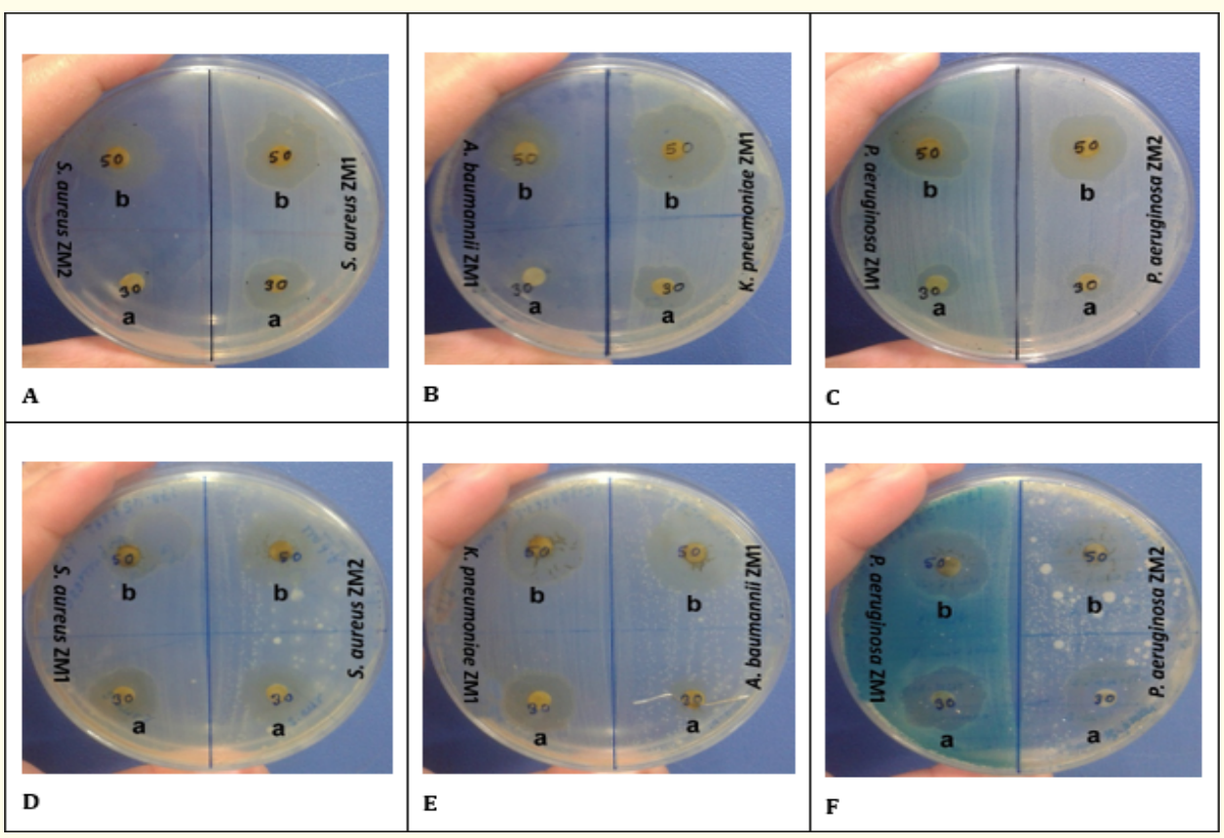


Figure 1: Antibacterial activity of *O. sanctum* leaf extracts; A-C: dark variety ethanolic extract; D-F: bright variety ethanolic extract. a: 3.75 mg; b: 6.25 mg. The clear halos around each disc on the plates are indicative of growth inhibitory action of the extracts.

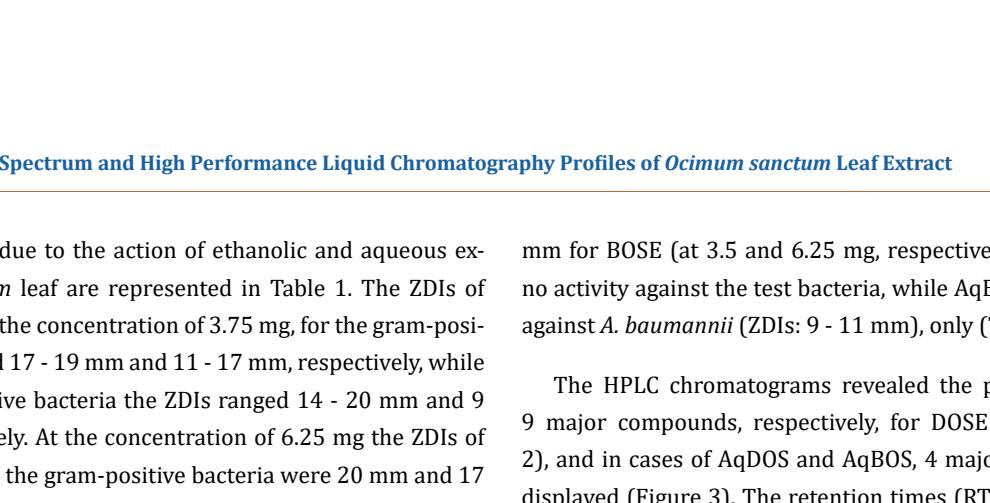
The ZDI values due to the action of ethanolic and aqueous extracts of *O. sanctum* leaf are represented in Table 1. The ZDIs of DOSE and BOSE, at the concentration of 3.75 mg, for the gram-positive bacteria ranged 17 - 19 mm and 11 - 17 mm, respectively, while for the gram-negative bacteria the ZDIs ranged 14 - 20 mm and 9 - 15 mm, respectively. At the concentration of 6.25 mg the ZDIs of DOSE and BOSE for the gram-positive bacteria were 20 mm and 17 - 19 mm, respectively, and for the gram-negative bacteria, the ZDIs ranged 20 - 24 mm and 14 - 24 mm, respectively. The overall ZDIs recorded were 17.5 ± 2.07 mm and 21 ± 1.67 mm for DOSE (at 3.5 and 6.25 mg, respectively), and 12.5 ± 2.94 mm and 18.67 ± 3.33 mm for BOSE (at 3.5 and 6.25 mg, respectively); the AqDOS had no activity against the test bacteria, while AqBOS showed activity against *A. baumannii* (ZDIs: 9 - 11 mm), only (Table 1).

The HPLC chromatograms revealed the presence of 11 and 9 major compounds, respectively, for DOSE and BOSE (Figure 2), and in cases of AqDOS and AqBOS, 4 major compounds were displayed (Figure 3). The retention times (RTs) within which the compounds were detected ranged 1.77 - 5.29 min and 1.92 - 6.27 min, respectively, for DOSE and BOSE, and 1.66 - 6.17 min and 1.64 - 2.51 min, respectively, for AqDOS and AqBOS.

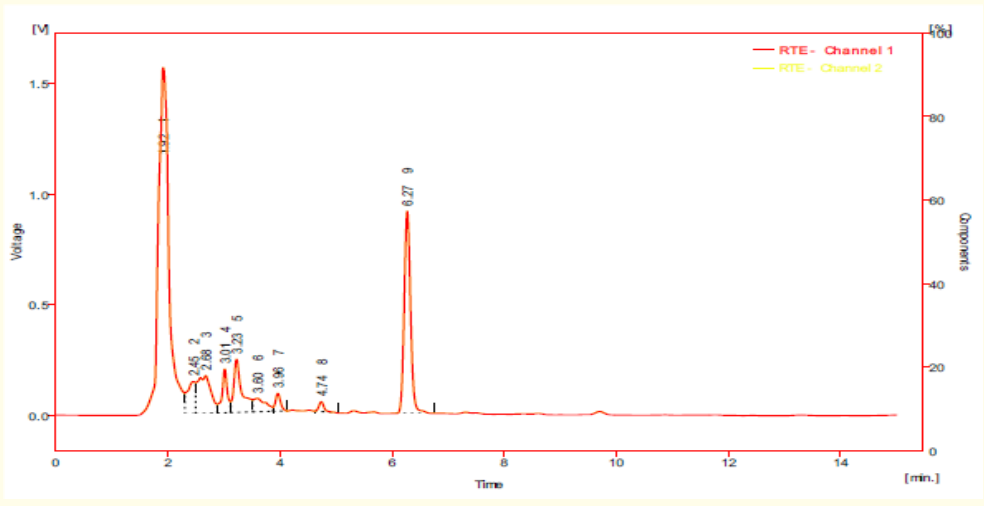
Bacterial strain	ZDI (mm) at different extract concentration (mg)							
	3.75		6.25		3.75		6.25	
	DOSE*	BOSE**	DOSE$^{\alpha}$	BOSE$^{\alpha\alpha}$	AqDOS*	AqBOS**	AqDOS$^{\alpha}$	AqBOS$^{\alpha\alpha}$
S. aureus ZM1	17	17	20	19	6	6	7	6
S. aureus ZM2	19	11	20	17	6	6	6	6
A. baumannii ZM1	14	9	24	14	6	9	6	11
K. pneumonia ZM1	17	15	20	24	6	6	6	6
P. aeruginosa ZM1	18	11	20	18	6	6	6	6
P. aeruginosa ZM2	20	12	22	20	6	6	6	6
Mean value	17.5	12.5	21	18.67	6	6.5	6.17	6.83
SD	±2.07	±2.94	±1.67	±3.33	0	±1.23	±0.41	±2.04
P value	0.003		0.083		0.181		0.232	

Table 1: The ZDI (Zone diameter of inhibition) values due to the action of *O. sanctum* leaf extracts at two different concentrations against pathogenic bacteria

DOSE: Ethanolic Leaf Extract of *O. sanctum*, Dark Variety; BOSE: Ethanolic Leaf Extract of *O. sanctum*, Bright Variety; AqDOS: Aqueous Leaf Extract of *O. sanctum*, Dark Variety; AqBOS: Aqueous Leaf Extract of *O. sanctum,* Bright Variety. *p value: <0.01; ** p value: < 0.01; α p value: 0.01; αα p value: < 0.01.

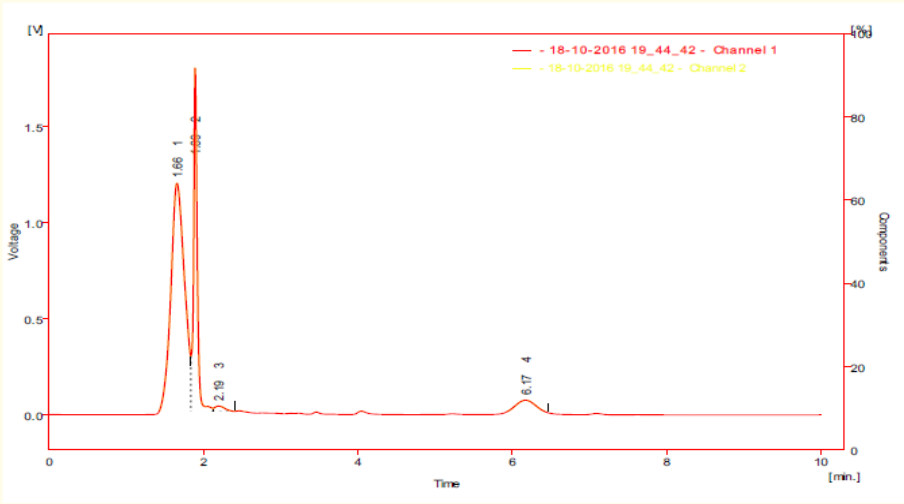


DOSE

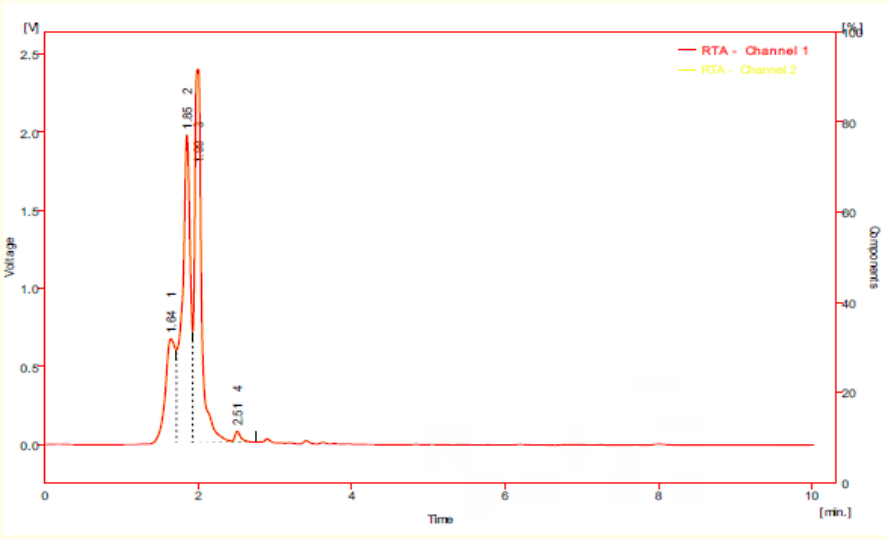


BOSE

Figure 2: The HPLC chromatograms of *O. sanctum* ethanolic extracts. DOSE: ethanolic leaf extract of *O. sanctum*, dark variety; BOSE: ethanolic leaf extract of *O. sanctum*, bright variety.



AqDOS



AqBOS

Figure 3: The HPLC chromatograms of *O. sanctum* aqueous extracts. AqDOS: aqueous leaf extract of *O. sanctum,* dark variety; AqBOS: aqueous leaf extract of *O. sanctum,* bright variety.

Discussion

The *O. sanctum* is regarded as the queen of herbs and has been used since the antiquity in traditional medicinal system to diverse range of therapeutic activities, for alleviating many diseases including bacterial infections [10,24]. The antibacterial activity of *O. sanctum* has been reported by earlier authors. Geeta *et al.* [25] reported the growth inhibitory activity of *O. sanctum* alcoholic leaf extract against *Vibrio cholerae*. Antibacterial efficacy of *O. sanctum* ethanolic leaf extract against gram-positive (*S. aureus* and *Bacillus cereus*) and gram-negative (*Escherichia coli, Proteus vulgaris, Salmonella typhi, K. pneumonia, P. aeruginosa* and *A. baumannii*) bacteria has been reported by Das *et al.* [11]. Auil *et al.* [26] reported the antibacterial activity of *O. sanctum* leaf extract against *S. aureus* having resistance to β-lactams. As per the report of Singh *et al.* [27], due to higher content of linolenic acid, the *O. sanctum* fixed oil was found to be active against *Bacillus pumilus, P. aeruginosa* and *S. aureus*. In the current study, ethanolic leaf extract of both varieties of *O. sanctum*, showed concentration dependant antibacterial activity against all the gram-positive and gram-negative bacteria tested, while most of the bacterial isolates had resistance (ZDI = 6 mm) to the aqueous extracts: AqDOS and AqBOS. A significant difference ($P < 0.01$) was observed in between the antibacterial activity of ethanolic and aqueous extracts of both varieties of *O. sanctum* leaf. At 3.75 mg extract concentration, the ZDIs of DOSE recorded were 17 - 19 mm and 14 - 20 mm, respectively, for the gram-positive and gram-negative bacteria tested; at the same concentration, BOSE displayed respective ZDIs of 11 - 17 mm and 9 - 15 mm, for the gram-positive and gram-negative test bacteria. The ZDI values, due to the action of DOSE (at 6.25 mg) were recorded as 20 mm, for gram-positive bacteria, and 20 - 24 mm, for gram-negative bacteria, while BOSE at the same concentration had ZDIs 17 - 19 mm and 14 - 24 mm, respectively, for the gram-positive and gram-negative test bacteria. Therefore, except at 3.75 mg concentration (p: 0.003), there was no significant differences in antibacterial action between DOSE and BOSE, and between AqDOS and AqBOS (p: 0.083 - 0.232). Similar to the current study, Sadul *et al.* [28], demonstrated the antimicrobial activity of *O. sanctum* leaf extract, and found ethanolic extract more efficacious than the aqueous *O. sanctum* leaf extracts.

The HPLC is an important tool that is utilized in the identification, quantification and purification of the individual components from the mixture of samples [29]. Several authors from different parts of the world using HPLC have identified medicinally important bioactive compounds from *O. sanctum* leaf extracts. Among the different biologically active components present in *O. sanctum* leaf, eugenol is a major one, which is most widely used as flavouring agents in foods and beverages; it has been detected, in HPLC system, at different RTs: 11.96 - 14.41 min, using methanol-water-acetonitrile mobile phases [16,30,31]. Tamilselvan *et al.* [32] identified three phenolic components (RT ranged: 3.33 - 12.57 min) in *O. gratissimum* chloroform extract using acetonitrile and water (60:40) as the mobile phase. In HPLC system, with the mobile phase of 10 mM hexane -1- sulphonic acid sodium salt containing 1% acetic acid and 0.13 % triethyl amine, and a flow rate of 1.0 ml/min, the presence of antibacterial components: ursolic acid and oleanolic acid, in *O. sanctum* leaf extracts at respective RT of 14.06 min and 14.43 min has been reported by Ali *et al.* [33]. Herein, the HPLC chromatograms demonstrated the presence of 11 and 9 major compounds, respectively in DOSE and BOSE, and 4 major compounds both in AqDOS and AqBOS. The RTs for each of the test extract ranged 1.77 - 5.29 min and 1.92 - 6.27 min, respectively for DOSE and BOSE, whereas for the AqDOS and AqBOS

the RTs ranged 1.66 - 6.17 min and 1.64 - 2.51 min, respectively, in detecting the compounds. Similar to the present study, Deo *et al.* [17] found the presence of 10 compounds (RTs: 1.78 - 11.55 min) in methanolic extract and 3 compounds (RTs: 1.72 - 3.45 min) in aqueous extract of *O. sanctum* leaf. Shanaida *et al.* [34] detected various amino acids: aspartic acid (RT: 1.92 min), asparagine (RT: 2.89 min), serine (RT: 3.12 min), arginine (RT: 3.28 min), alanine (RT: 3.62 min), proline (RT: 3.71 min) and isoleucine (RT: 4.81 min), in *O. americanum* seed; the RTs were closely similar to the compounds detected in DOSE and BOSE, in HPLC system. The HPLC analysis of *O. basilicum* methanolic leaf extract revealed the presence of seven phenolic components such as gallic acid (RT: 1.84 min), protocatechuic acid (RT: 2.43 min), caffeic acid (RT: 4.20 min), rutin (RT: 6.04 min), p-coumaric acid (RT: 6.66 min) and rosmarinic acid (RT: 10.53); among such components, antibacterial potentiality of caffeic acid and rosmarinic acid, against different gram-positive and gram-negative bacterial isolates, had been recorded [35]. Shafqatullah *et al.* [15] analysed the ethanolic *O. sanctum* leaf extract by HPLC, and detected 10 compounds at 210 nm, of which three compounds were identified as gallic acid (RT: 2.61 min), chloroganic acid (RT: 6.40 min) and vanillic acid (RT: 21.02 min). Depending on the earlier findings [15,34,35], the 7 plausible components detected in DOSE were: gallic acid (compound 1), aspartic acid (compound 2), asparagine (compound 6), serine (compound 7), arginine (compound 8), proline (compound 10) and caffeic acid (compound 11), while in BOSE, the possible detected components included aspartic acid (compound 1), protocatechuic acid (compound 2), gallic acid (compound 3), arginine (compound 5), alanine (compound 6), isoleucine (compound 8) and chloroganic acid (compound 9). In the aqueous *O. sanctum* leaf extracts gallic acid (compound 1), aspartic acid (compound 2) and chloroganic acid (compound 4) were detected (as in AqDOS), and gallic acid (compound 1), aspartic acid (compound 2) and protocatechuic acid (compound 3) (as in AqBOS). The phytochemicals, detected in the *O. sanctum* leaf extracts, might act alone or in combination in exerting growth inhibition activity against test pathogenic gram-positive and gram-negative bacteria.

Conclusion

The current study authenticated the presence of an array of phytochemicals in *O. sanctum* leaf ethanolic and aqueous extracts, attributing the excellence of the extracts in bacterial growth inhibition activity. The plant might be utilised in the preparation of non-antibiotic biotherapeutic agents in order to combat the life-threatening illnesses, caused due to the infection of different gram-positive and gram-negative pathogenic bacteria (at least in our part of the globe), thus having broad spectrum of antibacterial activity.

However, further studies are warranted in reverence to the dose determination, and safety and toxicity profiling of the *O. sanctum* plant (not studied before) available in the local niches.

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