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Research Article

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS AND ANTI-METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ACTIVITY OF OLIVE FRUIT ETHANOLIC EXTRACT

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ABSTRACT

The current study explores the antibacterial activity and HPLC (high performance liquid chromatography) profiles of olive (*Elaeocarpus floribundus*) fruits. The ethanolic extract of olive fruit parts: seed (OSE) and mesocarp-epicarp (OME), were prepared, and tested for their antibacterial activity, by agar-well diffusion method using 1.875 – 6.25 mg/well, against the clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA; n=3). The HPLC profiles of the extracts were prepared. For the test MRSA isolates, the OSE, at concentrations 1.875, 3.125 and 6.25 mg/well, had ZDI (zone diameter of inhibition) values 8 ± 1.73 mm (range: 6 – 9 mm), 9 ± 1.73 mm (range: 7 – 10 mm) and 11.67 ± 1.53 mm (range: 10 – 13 mm), respectively, and the ZDIs for OME recorded were 12.33 ± 2.51 mm (range: 10 – 15 mm), 13.66 ± 2.08 mm (range: 12 – 16 mm) and 16.33 ± 1.53 mm (range: 15 – 18 mm), respectively (when the values expressed as mean ± standard deviation). The HPLC chromatograms for both OSE and OME displayed 9 major compounds with retention times 1.54 – 6.14 min and 1.79 – 9.47 min, respectively. Thus, the olive fruit extracts (OME and OSE) possessing various phytochemicals, showed anti-MRSA activity, suggesting the plausible usage of the extracts in the preparation of antibacterial leads in combating various life threatening diseases caused due to *S. aureus* infection, including MRSA.

Keywords: *Elaeocarpus floribundus*; anti-MRSA activity; zone diameter of inhibition; high performance liquid chromatography, phytochemicals

INTRODUCTION

The rapid emergence and dissemination of multidrug resistant pathogenic bacteria in various niches led treatment difficulties of several life-threatening infectious diseases, mainly in the developing countries including India, due to the lack of antibiotic(s) of choice[1]. Among the human pathogenic bacteria causing most fatal infection, *Staphylococcus aureus* is a very dangerous opportunistic pathogen possessing the capacity to cause skin and soft tissue infection to most sever diseases, such as endocarditis, osteomyelitis, and severe sepsis[2-4], and is potential to cause staphylococcual food poisoning[5]. Additionally, the emergence of methicillin resistant *S. aureus* (MRSA), in the hospital as well as in the community, causes the problem more serious[6-8]. Therefore, the scientists are still in search of new sources of antimicrobial lead molecules to be useful in the preparation of cost-effective bio-therapeutic agents, such as plant extracts, honey and probiotics[9-11], alternative to the conventional antibiotic therapy of *S. aureus* infection, including MRSA.

Among the plants, the Indian olive plant, *Elaeocarpus floribundus*, which is known as 'Jalpai' in Bengali belongs to the family Elaeocarpaceae, and, though famous for its fruits, is an excellent medicinal plant, possessing antioxidative, antifungal and antibacterial activities. Medina *et al.*[12] reported the growth inhibition potentiality of olive fruit oils against food-borne gram-positive bacteria, such as, *Listeria monocytogenes* and *S. aureus*. Zaman[13] confirmed the antibacterial capacity of various extracts of *E. floribundus* leaves against *S. aureus*. Lee and Lee[14] reported the antibacterial effect of phenolics mixture prepared from olive leaf extract against *Bacillus cereus*. Owen *et al.*[15] demonstrated the efficacy of olive leaves against *Escherichia coli*, *S. aureus* and *B. cereus*. Antimicrobial activity has also been reported in olive oil mill wastewaters[16,17] and olive leaf extracts [18,19] as well.

It has been reported that olive contains high amount of phenolic compounds (phenolic acids, phenolic alcohols, flavonoids and secoiridoids), and its flavonoids include flavonol glycosides, such as, luteolin-7-glucoside and rutin, found in the olive leaves, fruits and seeds [20,21], displaying various bioactivities, including bacterial growth inhibition capacity. The earlier authors from different parts of the world documented various olive phenolic compounds by HPLC analysis[22-24], and validated their antibacterial efficacy against gram-negative as well as gram-positive pathogenic bacteria, including *S. aureus*[25,26]. The oleuropein extracted from olive leaf had an excellent antibacterial agent against *S. aureus*, as has been reported by earlier authors[27,28]. However, no scientific data is available on antibacterial activity of olive, *E. floribundus*, from within and around Malda, West Bengal state, India.

The above scientific studies and reports prompted us to explore the anti-*S. aureus* activity of *E. floribundus* fruits parts ethanolic extracts and to determine the HPLC profiles of phytochemicals present in the extracts, in order to evaluate the traditional usage of *E. floribundus* against microbial infection from this part of the globe.

MATERIAL AND METHODS

Bacterial strains and media

The *Staphylococcus aureus* isolates (n=3) procured from three different clinical samples (throat swab, urine and pus) were utilized as the indicator strains in testing the antibacterial activity of *E. floribundus* fruit parts extracts. The *S. aureus* pure cultures were maintained in cystine tryptone agar (Hi-Media, India) stabs, and inocula were prepared in nutrient broth (Hi-Media, India). All the *S. aureus* isolates had resistance to cefoxitin (ZDI: 6 mm), and thus considered as MRSA.

Preparation of plant extracts

The wild mature olive, *E. floribundus*, fruits were collected from Hemtabad block of Uttar Dinajpur district, West Bengal state, India, and were washed with double distilled water. The seeds and mesocarp-epicarp parts of the fruits were separated, shed-dried, and thereafter (mesocarp-epicarp parts and seeds) were grinded. The ethanolic extracts of the grinded materials (25 g in 200 ml) were prepared as described earlier[29,30]. The olive seed ethanolic extract (OSE; 125 µg/µl)) as well as the olive mesocarp-epicarp ethanolic extract (OME; 125 µg/µl), after filtration, were stored at 4^0C, for further work.

Antibacterial activity

The olive fruit parts extracts (OME and OSE) were tested against three clinical isolates of MRSA, by agar-well diffusion method[31], as described elsewhere[11]. The concentrations used for each of the extracts, OME and OSE, were 15-, 25- and 50-µl/well, which, were equivalent, respectively, to 1.875, 3.125 and 6.25 mg/well (6 mm, diameter) prepared on the Nutrient agar (Hi-Media, India) plate, swabbed inoculated with the test bacterial isolate. Following 24 h incubation, at 35 °C, the ZDI (zone diameter of inhibition) values (nearest whole) produced around the wells due to the action of the test extracts at various concentrations were recorded, and interpretation of the results was done as per the criteria mentioned earlier[11]: the extract was considered highly active for ZDIs ≥15 mm, less active for ≤10 mm, or moderately active for 11 – 14 mm.

HPLC analysis

The chemical components of olive fruit extracts, OME and OSE, were analyzed with a HPLC unit (YL 9100 HPLC system). The samples, 10 µl, each of OME and OSE, were loaded by injection into the HPLC associated to a stationary phase, C18 column (5 µm; 100Å; 4.6×250 mm). The mobile phase used consisted of acetonitrile (solvent A) and water (solvent B), in 2:3 ratio, and the flow rate was 1.0 ml/min, with an elution performed at 35 °C. The chromatograms were recorded at 230 nm, and eluting compounds were identified following[23,24].

Statistical analysis

The data were expressed as the mean ± SD (standard deviation), and evaluated by one-way ANOVA (analysis of variance) tests using *MS Excel 2010* software, while comparison of the mean values was assessed using the Tukey's test, and statistical significance was estimated at $p < 0.05$.

RESULTS AND DISCUSSION

The current research was designed to explore the anti-MRSA activity and phytochemical profiles of ethanolic fruits parts extracts from olive, *E. floribundus*, an indigenous medicinal plant having the capacity to display an array of biological activities, including antibacterial property. The growth inhibition property of the olive fruits extracts, OME and OSE, was determined by agar-well diffusion method, and the phytochemical profiling was done by HPLC analysis.

The anti-MRSA activity of OSE and OME, following agar-well diffusion method, is represented in Figure 1. The *E. floribundus* extracts showed concentration dependant activity against the test MRSA isolates, and the ZDIs recorded were 8 ± 1.73 mm (range: 6 – 9 mm), 9 ± 1.73 mm (range: 7 – 10 mm) and 11.67 ± 1.53 mm (range: 10 – 13 mm) due to the action of OSE, at concentrations, 1.875, 3.125 and 6.25 mg/well, respectively, while for OME, the ZDIs were 12.33 ± 2.51 mm (range: 10 – 15 mm), 13.67 ± 2.08 mm (range: 12 – 16 mm) and 16.33 ± 1.53 mm (range: 15 – 18 mm), respectively (Table 1). Therefore, the growth inhibition activity of OME, compared to that of the OSE, at all the extract concentrations (1.875 – 6.25 mg/well) against the MRSA isolates, was higher, and a significance difference was observed between the anti-MRSA activities of OME and OSE (p value: <0.05). Similar to the current study with olive fruit antibacterial activity, the leaves extracts from two olive varieties, *Dathier* and *Chemlal*, had concentration dependant activity against *S. aureus*, with ZDIs 9 – 17 mm and 13 – 15 mm, at the extracts concentrations 25% - 100%, respectively[25]. It has been reported by Zaman[13] that the *E. floribundus* leaves extracts had antibacterial activity against several human pathogenic bacteria, including *S. aureus*, by disc diffusion method, with the ZDIs 10 – 22 mm.

Table 1: Zone diameter of inhibition (ZDI) values from the action of olive fruit parts ethanolic extracts against the clinical MRSA isolates by agar-well diffusion technique

Bacterial strain	ZDI (mm) values from the action of *E. floribundus* extract					
	1.875 mg/well		3.125 mg/well		6.25 mg/well	
	OSE$^{\alpha}$	OME$^{\alpha}$	OSE$^{\beta}$	OME$^{\beta}$	OSE$^{\gamma}$	OME$^{\gamma}$
MRSA1	9	10	10	12	13	15
MRSA2	9	12	10	13	12	16
MRSA3	6	15	7	16	10	18
Statistical value						
Mean value	8	12.33	9	13.67	11.67	16.33
SD	± 1.73	± 2.51	± 1.73	± 2.08	± 1.53	± 1.53
p value	$^{\alpha}$0.039		$^{\beta}$0.021		$^{\gamma}$0.010	

MRSA: methicillin resistant *S. aureus*; OME: olive mesocarp-epicarp ethanolic extract; OSE: olive seed ethanolic extract; SD: standard deviation.

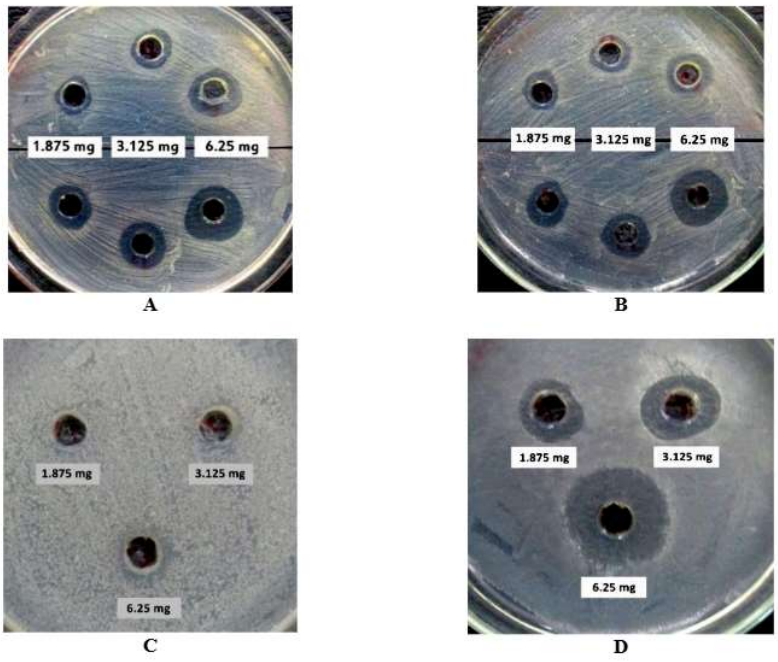


Figure 1: Agar-well diffusion technique representing the anti-MRSA activity of olive fruit parts extracts on Nutrient agar plate; A: OSE on upper half and OME on lower half against MRSA1; B: OSE on upper half and OME on lower half against MRSA2; C & D: OSE and OME, respectively, against MRSA3. MRSA: methicillin resistant *S. aureus*; OME: olive mesocarp-epicarp ethanolic extract; OSE: olive seed ethanolic extract.

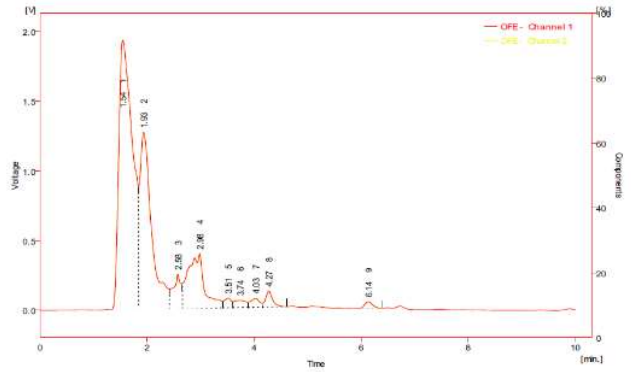


Figure 2: The HPLC chromatograms of olive, *Elaeocarpus floribundus*, fruit parts extracts. OME: olive mesocarp-epicarp ethanolic extract; OSE: olive seed ethanolic extract

In the current study, the OSE had ZDIs 10.66 ± 2.08 mm (range: 9 – 13 mm), 10.33 ± 1.53 mm (range: 9 – 12 mm) and 7.66 ± 2.08 mm (range: 6 – 10 mm), against MRSA1, MRSA2 and MRSA3, respectively, while the OME showed ZDIs 12.33 ± 2.52 mm (range: 10 – 15 mm), 13.66 ± 2.08 mm (range: 12 – 16 mm) and 16.33 ± 1.53 mm (15 – 18 mm), for the isolates, respectively (Table 1). Thus, the OME was highly active (≥ 15 mm) against MRSA3, at all the extracts concentrations (1.875 – 6.25 mg/well), and against all the test MRSA isolates at concentration 6.25 mg/well, while the extract (OME) was moderately active (ZDI: 12 – 13 mm) against MRSA2 (at 1.875 - 3.125 mg/well of OME) and against MRSA1 with 3.125 mg/well of OME; herein, the OSE had less to moderate activity against the MRSA isolates tested, based upon the previously mentioned criteria[11]. Gokmen *et al.*[32] demonstrated the growth inhibition property of olive leaf extract against *S. aureus* having ZDI value of 19 mm.

Djenane *et al.*[27] reported that oleuropein, from olive leaf extract, had an excellent antibacterial efficacy against *S. aureus*, having ZDI 30 mm. The HPLC chromatograms for both OSE and OME displayed 9 major compounds with retention times 1.54 – 6.14 min and 1.79 – 9.47 min, respectively (Figure 2). Earlier, Kuo *et al.*[22] reported the presence of phenolic compounds in olive fruit extracts by HPLC, demonstrating 10 major peaks and detecting 3 compounds, such as, gallic acid (RT: 5.75 min), ferulic acid (RT: 41.63 min) and rutin (RT: 45.95 min). The HPLC system in a mobile phase consisting of distilled water (92%), acidified with 0.10 M ortho-phosphoric acid, and acetonitrile (21%), with a flow rate of 1.0 ml/min, demonstrated oleuropein in olive leaf extract at RT of 8 min[25]. Olive mill wastewater HPLC chromatograms provided the separation of the major biophenols and detected the presence of hydroxytyrosol glucoside, hydroxytyrosol and tyrosol, at RTs 3.12 – 10.73 mm[17]. Kanakis *et al.*[23], with a flow rate of 0.4 mL/min, using the solvent system of water acidified with 0.1% acetic acid and acetonitrile, demonstrated HPLC for olive extracts displaying peaks for hydroxytyrosol (RT: 1.79), hydroxytyrosol hexoside (2 isomers; RT: 1.44 and 1.98), tyrosol hexoside (RT: 3.40), tyrosol (RT: 3.29), hydroxylated products of the decarboxyl elenolic acid (two isomers; RT: 3.77 and 4.39), hydroxylated form of elenolic acid (RT: 4.83), glucosyl-methyloleoside (RT: 6.08), and hydroxytyrosol acetate (RT: 9.51 min). Caffeic acid, which was detected in olive leaves extract at RT of 4.24 min, in the HPLC system described by Khattab *et al.*[24], was equivalent to the compound 6, in OSE, and compound 8, in OME, with RT of 4.27 min, in the instant study.

Considering the findings of Kanakis *et al.*[23], OME contained hydroxytyrosol hexoside (2 isomers: compound 1 and compound 2), tyrosol (compound 4), tyrosol hexoside (Compound 5), hydroxylated products of the decarboxyl elenolic acid (Compound 6) and glucosyl-methyloleoside (Compound 9), while in case of OSE, the compounds 1, 2, 3, 4, 8 and 9 were detected as hydroxytyrosol, hydroxytyrosol hexoside, tyrosol, tyrosol hexoside, hydroxylated form of elenolic acid and hydroxytyrosol acetate, respectively, along with 2 isomers of hydroxylated products of the decarboxyl elenolic acid (coumpounds 5 and 7) (Figure 2). Himour *et al.*[25] determined the presence of phenolic compounds in leaves extracts of *Dathier* and *Chemlel* olive varieties, by reversed phase HPLC, and reported the ZDIs of 17 mm and 16 mm, respectively, against *S. aureus.* A total of 7 olive leafs extracts phenolic compounds, which have been identified by Pereira *et al.*[26], include caffeic acid, verbascoside, oleuropein, luteolin 7-*O*-glucoside, rutin, apigenin 7-*O*-glucoside and luteolin 4'-*O*-glucoside, and had growth inhibition activity against gram-positive food-borne bacteria such as *Bacillus cereus*, *B. subtilis* and *S. aureus*. Thus, various phenolic compounds have been detected in olive extracts, as has also been recorded in OSE and OME in the instant investigation, and due to the presence of such active principles, the olive fruits parts ethanolic extracts displayed anti- MRSA activity (Figure 1).

CONCLUSION

The phytochemical and pharmacological study results represented herein might be helpful in expanding the accessible curative potentiality of the edible olive fruits from *Elaeocarpus floribundus* plant and in offering strong support to its (olive fruit) prospective therapeutic usefulness in 'modern medicine' against *S. aureus* as well as MRSA infections. However, further studies in determining therapeutic dosage of the extracts for optimum benefits are warranted.

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