

# Gas Chromatography–Mass Spectrometry (GC-MS/MS) Analysis, Ultrasonic Assisted Extraction, Antibacterial and Antifungal Activity of *Embelica officinalis* Fruit Extract

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## History

- Submission Date: 24-10-2018;
- Review completed: 27-11-2018;
- Accepted Date: 30-11-2018.

DOI : 10.5530/pj.2019.11.47

## Article Available online

<http://www.phcogj.com/v11/i2>

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## ABSTRACT

**Introduction:** *Embelica officinalis* is one of the common plants being used traditionally in different ways to search for cures and relief from various diseases. Among these diseases, *Embelica officinalis* had many of medicinal uses in treating a wide variety of bacterial and fungal infections. In this study, Ultrasonic assisted extraction (UAE) was efficiently used for the preparation of aqueous and methanolic extracts of *Embelica officinalis* fruits (EOFE). The phytoconstituents was detected through GC-MS/MS analysis to confirm antimicrobial effect of EOFE. **Materials and Methods:** The extraction was carried out at 20°C, for 20 min using a solid-to-solvent ratio of 1: 60 w/v. The extracts were subjected to GC-MS/MS analysis. The *in-vitro* effect of extracts against twenty one microbial strains was investigated by an agar well diffusion method in different concentrations (25 µg/mL-1000 µg/mL). **Results:** The percentage yield was found to be 41.33% w/v and 23.0 % w/v with water and methanol, respectively. Phenol,3,5-bis(1,1 dimethylethyl, 2,4-di-tert-butylphenol and heptasiloxane1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl were confirmed by GC-MS/MS analysis. Effectiveness of extracts against Gram negative bacteria; *Providencia alcalifaciens* and Gram positive bacteria; *Bacillus pumilis*, *Bacillus polymyxa* and fungal strains; *Neurospora crassa*, *Aspergillus brasiliensis* and *Cladosporium oxysporum* are reported for the first time. Aqueous extract revealed excellent antibacterial activity at 50 µg/mL and antifungal activity at 100 µg/mL whereas methanolic extract showed antifungal activity at 250 µg/mL. **Conclusion:** Results highlight the considerable inhibitory effect of EOFE against various microbial species was mainly due to the presence of phenolic compounds and other phytocompounds.

**Key words:** GC-MS/MS, *Embelica officinalis*, Ultrasonic Assisted Extraction, Antibacterial, Antifungal.

## INTRODUCTION

The plant *Embelica officinalis* has the potential for wide therapeutic applications (Family Euphorbiaceae), commonly known as amla. It is reported *E. officinalis* fruit is widely producing bioactive molecules which are found to be useful for the treatment of various diseases including antibacterial, antifungal, antiviral, immunomodulatory, analgesic, antipyretic, lowering cholesterol level, etc.<sup>1</sup> *E. officinalis* seems to be a rich source of vitamin-C, amino acids and minerals like chromium, zinc and copper. Phenolic compounds generally distributed in *E. officinalis*, so it is consider to be a promising source of hydrolysable tannins, Emblicanin A and B, gallic acid and ellagic acid.<sup>2</sup> According to World Health Organization (WHO), today most of the drugs are obtained from medicinal plants. Bacteria and fungi are widely scattered as environmental contaminants and nearly 10 % of people worldwide have fungal allergy.<sup>3</sup> The secondary metabolism of the plant such as *E. officinalis* and *Coriandrum sativum*,<sup>4</sup> *Aloe vera* and *Vitex negundo*,<sup>5</sup> produce compounds

which give plant antibacterial and antifungal character. Various methods are claimed to carry out the extraction of active constituents from plant material however several factors are considered for extraction such as effectiveness, operating cost, simplicity of method and waste production. Many conventional extraction techniques such as Soxhlet, heated reflux and cold pressing, were used for the extraction from medicinal plants. However, ultrasonic assisted extraction (UAE) is a novel technique used to extract bioactive compounds from medicinal plants. This is an efficient method that, requires a very short time, less solvent, reduces the discharge of hazardous pollutants through reducing organic and synthetic solvent overexposure, eco-friendly and is comparatively simple to operate. It reduces processing time, minimal risk of oxidation, disintegration and decomposition of chemical constituents of plant.<sup>6</sup> Because of these excellent advantages, the UAE techniques have become one of the best techniques

**Cite this article:** Al-Samman AMMA, Kakhshan, Siddique NA. Gas Chromatography–Mass Spectrometry (GC-MS/MS) Analysis, Ultrasonic Assisted Extraction, Antibacterial and Antifungal Activity of *Embelica officinalis* Fruit Extract. Pharmacogn J. 2019;11(2):315-23.

for the extraction of bioactive constituents from medicinal plants.<sup>7-8</sup> UAE may be explored to extract the vital bioactive constituents of *E. officinalis*. GC-MS/MS is an analytical technique used for identifying the bioactive compounds from plant extract. Pyrogallol, 5-hydroxymethylfurfural, 5-methyl-2-furylmethyl ketone, Gallic-acid as major constituents in leaves of *E. officinalis* identified by Gas chromatography-mass spectrometry (GC-MS).<sup>9</sup> The aim of this current study is to analyse the components of *E. officinalis* fruit by Ultrasonic Assisted Extraction (UAE) in different solvents by using GC-MS/MS and investigate the *in-vitro* effects of it against bacterial and fungal strain. Although antimicrobial activity of *E. officinalis* fruit extract reported to date, for the best of our knowledge there are still bacterial and fungal strains for which antimicrobial activity are leaked in the previous studies. Hence our study increasingly turning its contribution to folk medicines, looking for new leads to develop better drugs against microbial infections. Further, UAE explore to extract the vital bioactive constituents whilst structure of HSTDM was elucidated first time in EOFE by GC-MS/MS analysis.

## MATERIALS AND METHODS

### Equipment and chemicals

The Extraction was performed on an ultrasonic bath (GT Sonic-1990Q Ts, China, Ultrasonic Power 200 W and Frequency 33/40 kHz). The Analysis was performed on Agilent 7000D GC-MS/MS (Triple Quad) system. Methanol was supplied by Merck (Darmstadt, Germany). All other inorganic chemicals and organic solvents were of reagent grade.

### Plant material

Fresh sample of *E. officinalis* were collected from Panchsheel Park-South Delhi, 110017, India, Plant was authenticated by National Herbarium of Cultivated Plants (NHCP), Pusa campus, New Delhi-110012 and specimen (NHCP/NBPGR/2017-1) was submitted to Bioactive Natural Compound Laboratory (BNCL), Department of Pharmacognosy and Phytochemistry, Glocal school of Pharmacy, Glocal University. The plant material initially washed thoroughly to remove impurities and was chopped in to small pieces with a blender and then placed in to shade to dry at room temperature. The shade dried plant materials were finely grained into powder using an electric grinder and store in airtight bottles for further investigations.

### Preparation of extracts

#### Ultrasonic Assisted Extraction (UAE)

The dried plant material (10 gm) was submitted to extraction with water and methanol (60 mL) using UAE apparatus (GT Sonic- 1990Q Ts, China) equipped with ultrasonic bath, digital meter and a temperature regulator. The device was operated at a frequency of 33/40 kHz, an ultrasonic input power of 200 W and the procedure was repeated for three times. Ultrasonication generates massive heat; to avoid this bath was run at room temperature (25°C). The extract was filtered and concentrated using rotary evaporator (Buchi, Switzerland) to remove the solvent completely.

### Chromatographic Analysis

#### Identification of component in EOFE using Gas chromatography-mass spectrometry (GC-MS/MS)

#### Preparation of samples

EOFE was submitted to GC-MS/MS evaluation and the sample was prepared by taken 10 mg of extract in volumetric flask and adjusted the volume up to 10 mL with water and methanol followed by centrifugation at 5000 rpm for 5 min. Supernatant was filtered through 0.22 µm syringe and 1 mL of this solution was taken for GCMS/MS analysis.

### GC-MS/MS conditions

The constituents in aqueous and methanolic extract of EOFE were examined on Agilent 7000D GC-MS/MS (Triple Quad – S. No. US1710UM1) system equipped with HP-5 MS capillary column with a 5% phenyl polysiloxane stationary phase (30.0 m × 0.25 mm, film thickness 0.25 µm). Different temperatures of oven were considered to achieve good separation in standard as well as in samples. The instrument was set to an initial temperature of 70°C and (maintained for 2 min) then in second stage temperature was allowed to raise by 3°C/min up to 150°C, then finally attended to 200°C by expanding the heating rate of 8°C/min. The flow rate of Helium carrier gas was kept 1.0 mL/min, injection port temperature was kept at 280°C and pressure was maintained 60 Kilo Pascal while the ionization voltage was 70eV. Mass spectra were examined in scanning mode at 25-500 m/z. Interpretation of GC-MS/MS spectra were, moreover, recognized and verified by using the NIST 14-MS database library stored in Standard Analytical Laboratory (SAL). The name, Molecular Weight (MW), Retention Time (RT), peak area and structure of the active constituents of EOFE were ascertained.

### Selection of microorganisms

#### Bacterial strains

Seven Gram negative bacteria *Pseudomonas aeruginosa* (MTCC 7837), *Escherichia coli* (MTCC 723), *Vibrio cholera* (MTCC 3906), *Salmonella typhi* (MTCC 3216), *Shigella dysenteriae* (MTCC 1842), *Proteus mirabilis* (MTCC 743), *Providencia alcalifaciens* (MTCC 4430), seven Gram positive *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 736), *Bacillus megaterium* (MTCC 4911), *Bacillus pumilis* (MTCC 1607), *Bacillus cereus* (MTCC 430), *Staphylococcus pyogenus* (MTCC 1927) and *Bacillus polymyxa* (MTCC 122) were used in the present study. Subsequent specification of Gram negative bacteria; *Providencia alcalifaciens* and Gram positive bacteria; *Bacillus pumilis* and *Bacillus polymyxa* were reported first time in our study.

#### Fungal strain

Seven pathogenic fungi *Aspergillus niger* (MTCC 1881), *Neurospora crassa* (MTCC 1855), *Penicillium chrysogenum* (MTCC 6891), *Trichoderma viridae* (MTCC 4329), *Aspergillus brasileinsis* (MTCC 1344), *Candida albicans* (MTCC 227) and *Cladosporium oxysporum* (MTCC 1777) were used in the present study. In order to antifungal study three new fungal strains *Neurospora crassa*, *Aspergillus brasileinsis* and *Cladosporium oxysporum* were reported first time in our study.

#### Antibacterial activity

Antibacterial activity was evaluated by agar-well diffusion assay technique. Initially, the potential of stock cultures of bacteria were checked by inoculating in broth media (Peptone-10 gm, NaCl-10 gm and Yeast extract- 5 gm, Agar- 20 gm in 1000 mL of distilled water) and grown at 37°C for 18 h. The wells were prepared on Agar plates which was subsequently inoculated with 18 h old cultures (100 µL, 10<sup>4</sup> CFU) and swabbed uniformly. Latter on 20 min, the wells were filled with extract at different concentration. Ciprofloxacin was inoculated as positive control with different concentrations (25, 50, 100, 250, 500 and 1000 µg/mL) while DMSO (Dimethyl sulfoxide) 2% solution was used as negative control. After addition of media, the plates were incubated at 37°C for 24 h and MIC (concentration at which no optically clear growth showed) were determined the zone of inhibition. Tests for *in-vitro* antibacterial activity were performed in triplicate and calculated mean values.

#### Antifungal activity

Czapek-Dox agar media were used for evaluating antifungal activity. Initially, the stock cultures of fungi were checked by inoculating in broth

media; sucrose (30.0 gm/mL), Sodium nitrate (2.0 gm/mL),  $K_2HPO_4$  (1.0 gm/mL),  $MgSO_4 \cdot 7H_2O$  (0.5 gm/mL), KCl (0.5 gm/mL),  $FeSO_4$  (0.01 gm/mL) and Agar (20 gm/mL) and grown at 37°C for 48 h. The wells were prepared on Agar plates which was subsequently inoculated with 48 h old cultures (100  $\mu$ l, 104CFU) and swabbed uniformly. Then after 20 min, the wells were filled with different concentrations of samples. Amphotericin was inoculated as positive control with different concentrations (25, 50, 100, 250, 500 and 1000  $\mu$ g/mL) to compare the activities of EOFE at the same concentrations while DMSO 2% solution was used as negative control. After addition of media, all plates were incubated at 37°C for 96 h and the diameter zones of inhibition were noted. Tests for *in-vitro* antifungal activity were performed in triplicate and calculated mean values.

## RESULTS

### Selection of Ultrasonic Assisted Extraction (UAE)

The conventional methods normally performed at high temperature for several hours and a large amount of solvents are the main disadvantage of conventional technique which was found not economically suitable for the extraction purpose,<sup>10-11</sup> in which food is subjected to the elevated pressure which is mostly between 100 to 800 MPa. HHPP is seen not only in food engineering, but also have other application areas, such as extraction of active ingredients from natural biomaterials. In this study, several extraction conditions such as two different solvents [methanol and solvent cocktail (dH 2O:ethanol:methanol:acetone:CH<sub>2</sub>Cl<sub>2</sub> - 1:2.5:2.5:2:2) in this study using UAE method high percentage yield of EOFE was

found in water (41.33 w/v) and methanol (23.00 w/v) at low temperature (25°C). Moreover, a few minutes of extraction time (20 min) and small amount of extraction solvent (60 mL) were required.

### Optimization of chromatographic conditions for GC-MS/ MS

Different temperature ramping were tried to achieve good separation of constituents in EOFE. The oven temperature was initially 70°C for 2 min. The temperature was increased to 150°C at rate of 3°C/min and then gradually increased to 200°C at a rate of 8°C/min. Good separation was found when inlet temperature (70°C), helium flow rate (1.0 mL/min), total running time (41.87 min) while the ionization voltage was 70 eV and MS were operated in SCAN mode.

### Peak identification

Molecular characterization and elucidation by GC-MS/MS spectra was conducted using the database of Standard Analytical Laboratory (SAL). The compounds in EOFE were recognized by their Retention Time (RT), Molecular Weight (MW), molecular structure and peak area in percentage which revealed good separation and structure of compounds.

### Determination of different compound by GC-MS/ MS

GC-MS/MS chromatogram of methanolic and aqueous extract of *E. officinalis* were given a well separated peaks of PBDME at Rt (8.541-8.614 min), DTBP at Rt (8.541-8.607 min) and HSTDm at Rt (36.199-36.00 min), as shown in [Figures 1, 2 and 3] respectively. The column

**Table 1: Description and relative composition of active constituents of *E. officinalis* fruit extract by GC- MS/MS.**

<i>E. officinalis</i> fruit extract	Name of compound	Formula	Mol. Wt. (gm/mol)	Peaks	RT (min)
Methanolic extract	Phenol, 3,5-bis (1,1-dimethylethyl)	C <sub>14</sub> H <sub>22</sub> O	206	24	8.5
Aqueous extract	2,4-Di-tert-butylphenol	C <sub>14</sub> H <sub>22</sub> O	206	15	8.6
	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl	C <sub>14</sub> H <sub>44</sub> O <sub>6</sub> Si <sub>7</sub>	503	29	36.2

RT: Retention Time; Mol. Wt.: (Molecular weight)

**Table 2: Antibacterial activity of methanolic fruit extract of *E. officinalis* on Gram negative and Gram positive bacteria.**

Types and name of organisms	Concentration of extract ( $\mu$ g/mL) and Zone of inhibition (mm) <sup>a</sup> $\pm$ SD							
	25 $\mu$ g	50 $\mu$ g	100 $\mu$ g	250 $\mu$ g	500 $\mu$ g	1000 $\mu$ g	MIC $\mu$ g	
Gram negative	<i>E. coli</i>	NS	NS	NS	NS	4.1 $\pm$ 0.9	15.9 $\pm$ 0.8	500
	<i>P. aeruginosa</i>	NS	NS	NS	10.2 $\pm$ 0.6	15.2 $\pm$ 0.6	18.3 $\pm$ 0.4	250
	<i>V. cholerae</i>	NS	NS	NS	NS	12.5 $\pm$ 0.4	13.2 $\pm$ 0.7	500
	<i>S. typhi</i>	NS	NS	NS	10.5 $\pm$ 0.8	13.9 $\pm$ 0.8	16.3 $\pm$ 0.7	250
	<i>S. dysenteriae</i>	NS	NS	NS	NS	5.7 $\pm$ 0.7	9.8 $\pm$ 0.7	500
	<i>P. mirabilis</i>	NS	6.2 $\pm$ 0.8	8.7 $\pm$ 0.6	11.1 $\pm$ 0.8	12.4 $\pm$ 0.4	12.9 $\pm$ 0.8	50
	<i>P. alcalifaciens</i>	NS	NS	NS	13.0 $\pm$ 0.8	14.0 $\pm$ 0.6	17.1 $\pm$ 0.7	100
Gram positive	<i>S. aureus</i>	NS	NS	7.3 $\pm$ 0.4	8.1 $\pm$ 0.6	14.2 $\pm$ 0.5	15.2 $\pm$ 0.7	100
	<i>B. subtilis</i>	NS	NS	NS	8.4 $\pm$ 0.4	10.2 $\pm$ 0.5	17.2 $\pm$ 0.5	250
	<i>B. megaterium</i>	NS	NS	NS	10.0 $\pm$ 0.8	10.5 $\pm$ 0.4	17.6 $\pm$ 0.5	100
	<i>B. pumilis</i>	NS	3.2 $\pm$ 0.6	6.5 $\pm$ 0.8	12.4 $\pm$ 0.4	13.5 $\pm$ 0.4	14.2 $\pm$ 0.5	50
	<i>B. cereus</i>	NS	NS	NS	4.4 $\pm$ 0.4	7.7 $\pm$ 0.7	10.4 $\pm$ 0.4	250
	<i>S. pyogenus</i>	NS	NS	NS	NS	7.1 $\pm$ 0.6	9.1 $\pm$ 0.6	500
	<i>B. polymyxa</i>	NS	NS	3.2 $\pm$ 0.8	6.4 $\pm$ 0.6	11.7 $\pm$ 0.7	13.2 $\pm$ 0.5	100
DMSO	Negative control	-	-	-	-	-	-	-

MIC  $\mu$ g: Minimum inhibitory concentration in microgram; NS: Not susceptible (did not exhibit any zone of inhibition); SD: Standard deviation, DMSO: Dimethyl sulfoxide; Values are expressed as mean  $\pm$  SD from three experiments (\*n=3).



**Table 3: Antibacterial activity of aqueous fruit extract of *E. officinalis* on Gram negative and Gram positive bacteria.**

Types and name of organisms		Concentration of extract (µg/mL) and Zone of inhibition (mm) <sup>±</sup> SD						MIC µg
		25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	
Gram negative	<i>E. coli</i>	NS	NS	NS	NS	5.0±0.8	19.1±0.7	500
	<i>P. aeruginosa</i>	NS	NS	NS	12.0±0.8	16.1±0.7	19.2±0.7	250
	<i>V. cholerae</i>	NS	NS	NS	NS	14.0±0.8	15.2±0.8	500
	<i>S. typhi</i>	NS	4.3±0.4	7.2±0.5	11.2±0.5	14.0±0.8	17.6±0.4	50
	<i>S. dysenteriae</i>	NS	NS	NS	3.2±0.5	8.1±0.8	12.2±0.71	250
	<i>P. mirabilis</i>	NS	8.2±0.5	11.2±0.5	12.1±0.4	14.0±0.4	14.3±0.5	50
	<i>P. alcalifaciens</i>	NS	NS	5.7±0.5	14.0±0.7	16.3±0.2	17.9±0.3	100
Gram positive	<i>S. aureus</i>	NS	5.5±0.2	10.8±0.6	10.9±0.8	14.7±0.6	17.8±0.6	50
	<i>B. subtilis</i>	NS	NS	NS	13.0±0.4	14.0±0.7	18.4±0.4	250
	<i>B. megaterium</i>	NS	NS	10.1±0.6	12.9±0.7	14.2±0.5	19.1±0.6	100
	<i>B. pumilis</i>	NS	4.1±0.3	7.0±0.4	13.1±0.3	14.5±0.4	15.8±0.5	50
	<i>B. cereus</i>	NS	NS	3.4±0.4	5.8±0.3	9.4±0.5	13.0±0.3	100
	<i>S. pyogenus</i>	NS	NS	NS	5.4±0.4	8.87±0.3	12.0±0.7	250
	<i>B. polymyxa</i>	NS	4.7±0.3	6.2±0.5	14.0±0.6	16.6±0.2	17.6±0.3	50
DMSO	Negative control	-	-	-	-	-	-	-

MIC µg: Minimum inhibitory concentration in microgram; NS: Not susceptible (did not exhibit any zone of inhibition); SD: Standard deviation, DMSO: Dimethyl sulfoxide; Values are expressed as mean ± SD from three experiments (\*n=3).

employed in this study for the separation of components was elite PBDME, DTBP and HSTDMD [Table 1]. HSTDMD was reported first time in EOFE.

### Antibacterial and antifungal activity

EOFE (methanolic and aqueous) have exhibited different degrees of antibacterial activity against seven Gram negative bacteria and seven Gram positive bacteria [Tables 2 and 3]. Similarly, the inhibition zones formed by standard antibiotics (CPF) are shown in [Table 4]. CPF was susceptible at 25 µg/mL against all Gram negative and Gram positive bacteria and exhibit maximum zone of inhibition (30.2±0.5mm) with *V. cholerae*. Maximum resultant zone of inhibition against Gram negative bacteria; *P. mirabilis* (8.2±0.5mm), *S. typhi* (4.3±0.4mm) and Gram positive bacteria; *S. aureus* (5.5±0.2mm), *B. polymyxa* (4.7±0.3mm) and *B. pumilis* (4.1±0.3mm) of aqueous extract were observed at 50 µg/mL where as both Gram negative bacteria; *P. aeruginosa*, *E. coli*, *V. cholera*, *S. dysenteriae*, *P. alcalifaciens* and Gram positive bacteria; *B. subtilis*, *B. megaterium*, *B. cereus* and *S. pyogenus* were found not susceptible at this concentration. The concentration (100 µg/mL) was found suitable for Gram negative and Gram positive bacteria viz *S. typhi* (7.2±0.5mm), *P. mirabilis* (11.2±0.5mm), *P. alcalifaciens* (5.7±0.5mm), *S. aureus* (10.8±0.62m), *B. megaterium* (10.1±0.6mm), *B. pumilis* (7.1±0.4mm), *B. cereus* (3.4±0.4mm), *B. polymyxa* (6.2±0.3mm) respectively though no zone of inhibition was observed with Gram negative bacteria; *P. aeruginosa*, *E. coli*, *V. cholera*, *S. dysenteriae* and Gram positive bacteria; *B. subtilis*, *S. pyogenus* respectively. Two Gram negative bacteria (*E. coli* and *V. cholerae*) were not susceptible while all Gram positive bacteria exhibited considerable margin of zone of inhibition at 250 µg/mL with aqueous extract. Low zone of inhibition was observed with methanolic extract against Gram negative bacteria *P. mirabilis* (6.2±0.8mm) and Gram positive bacteria *B. pumilis* (3.2±0.6mm) while a surprising result was observed with *P. aeruginosa*, *E. coli*, *V. cholera*, *S. typhi*, *S. dysenteriae*, *P. alcalifaciens*, *S. aureus*, *B. subtilis*, *B. megaterium*, *B. cereus*, *S. pyogenus* and *B. polymyxa* although it was not able to inhibit the bacterial growth at 50 µg/mL. It was confirmed that the concentration (100 µg/mL) was

found suitable for Gram negative bacteria *P. mirabilis* (8.7±0.6mm) and Gram positive bacteria *S. aureus* (7.3±0.4mm), *B. pumilis* (6.5±0.8mm) and *B. polymyxa* (3.2±0.8mm) although it gave negative results for *P. aeruginosa*, *E. coli*, *V. cholera*, *S. typhi*, *S. dysenteriae*, *P. alcalifaciens*, *B. subtilis*, *B. megaterium*, *B. cereus* and *S. pyogenus*. Clear zone of inhibition was observed against Gram negative; *P. aeruginosa* (10.1±0.6mm), *S. typhi* (10.5±0.8mm), *P. mirabilis* (11.1±0.8mm), *P. alcalifaciens* (13.0±0.8mm) and Gram positive bacteria; *S. aureus* (8.1±0.6mm), *B. subtilis* (8.4±0.4mm), *B. megaterium* (10.0±0.8mm), *B. pumilis* (12.4±0.5mm), *B. cereus* (4.4±0.4mm) and *B. polymyxa* (6.4±0.6mm) though no zone of inhibition was ascertained with *E. coli*, *V. cholera*, *S. dysenteriae* and *S. pyogenus* at 250 µg/mL. All Gram negative and Gram positive bacteria were found susceptible at very high concentration (500 µg/mL–1000 µg/mL). The results ascertained two Gram negative bacteria (*S. typhi* and *P. mirabilis*) and three Gram positive bacteria (*S. aureus*, *B. pumilis* and *B. polymyxa*) were found susceptible at very low concentration (50 µg/mL) with aqueous extract whereas only one Gram negative bacteria; *P. mirabilis* and one Gram positive bacteria; *B. pumilis* was found susceptible (50 µg/mL) with methanolic extract of EOFE [Figure 4].

In Tables 5 and 6 results of antifungal activity of EOFE (aqueous and methanolic) are reported against seven pathogenic fungi to recognize which fruit extracts produced maximum zone of inhibition. Similarly, the inhibition zones formed by standard antifungal (AMT) are shown in [Table 7]. AMT was susceptible at very low concentration (50 µg/mL) against *C. albicans* and showed maximum zone of inhibition (5.2±0.3mm) and this concentration was found not susceptible against *A. niger*, *N. crassa*, *P. chrysogenum*, *T. viridae*, *A. brasileinsis* and *C. oxysporum* while zone of inhibition was observed at 100 µg/mL against *A. niger* (2.1±0.2mm), *N. crassa* (4.1±0.1mm), *A. brasileinsis* (3.1±0.2mm) and *C. albicans* (9.3±0.2mm). The pathogenic fungi; *P. chrysogenum*, *T. viridae* and *C. oxysporum* not exhibited any zone of inhibition at 100 µg/mL. At high concentration (250 µg/mL) AMT exhibited more remarkable zone of inhibition with all pathogenic fungi. Maximum resultant zone of inhibition of aqueous extract (100 µg/mL) were observed against

**Table 4: Antibacterial activity of standard (Ciprofloxacin) on Gram negative and Gram positive bacteria.**

Types and name of organisms	Concentration of CPF ( $\mu\text{g/mL}$ ) and Zone of inhibition (mm) <sup>a</sup> $\pm$ SD							
	25 $\mu\text{g}$	50 $\mu\text{g}$	100 $\mu\text{g}$	250 $\mu\text{g}$	500 $\mu\text{g}$	1000 $\mu\text{g}$	MIC $\mu\text{g}$	
Gram negative	<i>E. coli</i>	26.0 $\pm$ 0.4	29.2 $\pm$ 0.3	32.1 $\pm$ 0.3	34.1 $\pm$ 0.2	38.3 $\pm$ 0.2	*	25
	<i>P. aeruginosa</i>	30.1 $\pm$ 0.2	32.4 $\pm$ 0.1	34.3 $\pm$ 0.3	35.47 $\pm$ 0.2	38.5 $\pm$ 0.2	*	25
	<i>V. cholerae</i>	30.2 $\pm$ 0.5	31.1 $\pm$ 0.2	34.4 $\pm$ 0.2	36.2 $\pm$ 0.3	38.0 $\pm$ 0.8	*	25
	<i>S. typhi</i>	27.2 $\pm$ 0.3	31.4 $\pm$ 0.2	35.4 $\pm$ 0.2	38.3 $\pm$ 0.4	40.3 $\pm$ 0.2	*	25
	<i>S. dysenteriae</i>	17.3 $\pm$ 0.4	21.4 $\pm$ 0.2	25.1 $\pm$ 0.2	28.2 $\pm$ 0.4	30.3 $\pm$ 0.3	*	25
	<i>P. mirabilis</i>	16.3 $\pm$ 0.3	19.2 $\pm$ 0.2	22.2 $\pm$ 0.2	26.3 $\pm$ 0.1	31.4 $\pm$ 0.2	*	25
	<i>P. alcalifaciens</i>	20.4 $\pm$ 0.5	22.3 $\pm$ 0.1	26.3 $\pm$ 0.2	29.5 $\pm$ 0.2	32.2 $\pm$ 0.1	*	25
Gram positive	<i>S. aureus</i>	25.1 $\pm$ 0.2	28.1 $\pm$ 0.2	31.4 $\pm$ 0.1	34.3 $\pm$ 0.2	36.3 $\pm$ 0.2	*	25
	<i>B. subtilis</i>	20.4 $\pm$ 0.5	24.3 $\pm$ 0.2	27.2 $\pm$ 0.3	30.3 $\pm$ 0.1	36.3 $\pm$ 0.2	*	25
	<i>B. megaterium</i>	19.2 $\pm$ 0.2	26.1 $\pm$ 0.2	28.3 $\pm$ 0.4	30.3 $\pm$ 0.3	36.2 $\pm$ 0.2	*	25
	<i>B. pumilis</i>	18.2 $\pm$ 0.1	20.1 $\pm$ 0.3	22.1 $\pm$ 0.2	24.3 $\pm$ 0.2	26.3 $\pm$ 0.2	*	25
	<i>B. cereus</i>	15.4 $\pm$ 0.2	18.1 $\pm$ 0.3	21.1 $\pm$ 0.2	26.2 $\pm$ 0.2	28.2 $\pm$ 0.2	*	25
	<i>S. pyogenus</i>	19.2 $\pm$ 0.3	24.4 $\pm$ 0.2	26.1 $\pm$ 0.3	29.3 $\pm$ 0.3	34.2 $\pm$ 0.1	*	25
	<i>B. polymyxa</i>	21.1 $\pm$ 0.2	25.2 $\pm$ 0.1	28.0 $\pm$ 0.7	32.4 $\pm$ 0.1	35.4 $\pm$ 0.2	*	25
DMSO Negative control	-	-	-	-	-	-	-	

MIC  $\mu\text{g}$ : Minimum inhibitory concentration in microgram; NS: Not susceptible (did not exhibit any zone of inhibition); SD: Standard deviation; DMSO: Dimethyl sulfoxide; CPF: Ciprofloxacin; Values are expressed as mean $\pm$ SD from three experiments (\*n=3); \* Zones could not be measured due to margining.

**Table 5: Antifungal activity of methanolic fruit extract of *E. officinalis*.**

Name of organisms	Concentration of extract ( $\mu\text{g/mL}$ ) and Zone of inhibition (mm) <sup>a</sup> $\pm$ SD						
	25 $\mu\text{g}$	50 $\mu\text{g}$	100 $\mu\text{g}$	250 $\mu\text{g}$	500 $\mu\text{g}$	1000 $\mu\text{g}$	MIC $\mu\text{g}$
<i>A. niger</i>	NS	NS	NS	NS	3.1 $\pm$ 0.3	6.0 $\pm$ 0.4	500
<i>N. crassa</i>	NS	NS	NS	NS	5.0 $\pm$ 0.4	8.0 $\pm$ 0.4	500
<i>P. chrysogenum</i>	NS	NS	NS	NS	2.3 $\pm$ 0.2	4.6 $\pm$ 0.2	500
<i>T. viridae</i>	NS	NS	NS	NS	2.3 $\pm$ 0.3	3.3 $\pm$ 0.2	500
<i>A. brasileinsis</i>	NS	NS	NS	2.5 $\pm$ 0.1	3.3 $\pm$ 0.2	7.1 $\pm$ 0.3	250
<i>C. albicans</i>	NS	NS	NS	4.2 $\pm$ 0.1	13.2 $\pm$ 0.2	14.3 $\pm$ 0.2	250
<i>C. oxysporum</i>	NS	NS	NS	NS	NS	10.4 $\pm$ 0.3	1000
DMSO	-	-	-	-	-	-	-

MIC  $\mu\text{g}$ : Minimum inhibitory concentration in microgram; NS: Not susceptible (did not exhibit any zone of inhibition); SD: Standard deviation, DMSO: Dimethyl sulfoxide; Values are expressed as mean  $\pm$  SD from three experiments (\*n=3).

**Table 6: Antifungal activity of aqueous fruit extract of *E. officinalis*.**

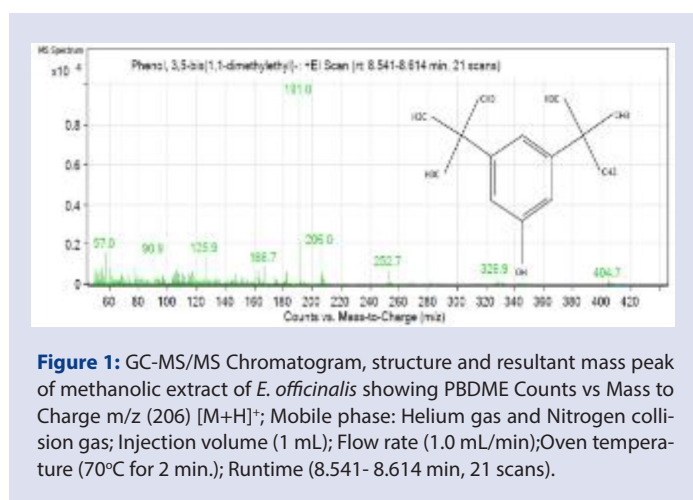
Name of Organism	Concentration of extract ( $\mu\text{g/mL}$ ) and Zone of inhibition (mm) <sup>a</sup> $\pm$ SD						
	25 $\mu\text{g}$	50 $\mu\text{g}$	100 $\mu\text{g}$	250 $\mu\text{g}$	500 $\mu\text{g}$	1000 $\mu\text{g}$	MIC $\mu\text{g}$
<i>A. niger</i>	NS	NS	NS	3.2 $\pm$ 0.1	4.0 $\pm$ 0.1	7.1 $\pm$ 0.3	250
<i>N. crassa</i>	NS	NS	2.1 $\pm$ 0.1	3.5 $\pm$ 0.1	5.7 $\pm$ 0.6	8.7 $\pm$ 0.5	100
<i>P. chrysogenum</i>	NS	NS	NS	NS	3.0 $\pm$ 0.3	5.6 $\pm$ 0.2	500
<i>T. viridae</i>	NS	NS	NS	NS	3.2 $\pm$ 0.1	4.3 $\pm$ 0.2	500
<i>A. brasileinsis</i>	NS	NS	NS	3.0 $\pm$ 0.2	4.0 $\pm$ 0.3	9.5 $\pm$ 0.5	250
<i>C. albicans</i>	NS	NS	2.8 $\pm$ 0.2	5.0 $\pm$ 0.2	15.3 $\pm$ 0.3	16.6 $\pm$ 0.7	100
<i>C. oxysporum</i>	NS	NS	NS	NS	7.2 $\pm$ 0.1	12.4 $\pm$ 0.4	500
DMSO	-	-	-	-	-	-	-

MIC  $\mu\text{g}$ : Minimum inhibitory concentration in microgram; NS: Not susceptible (did not exhibit any zone of inhibition); SD: Standard deviation, DMSO: Dimethyl sulfoxide; Values are expressed as mean  $\pm$  SD from three experiments (\*n=3).

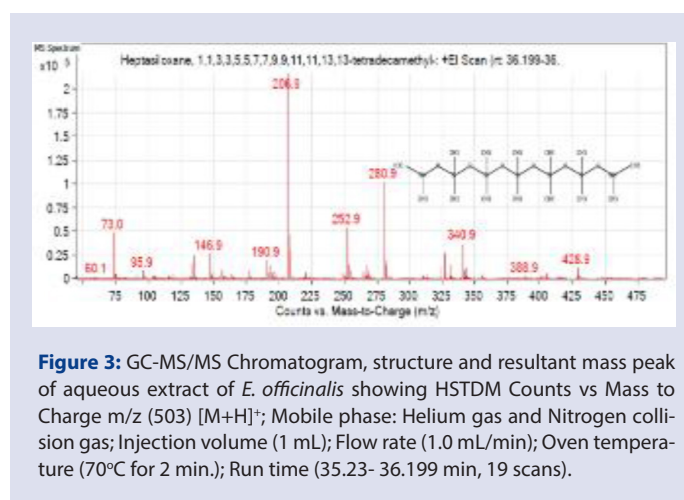
**Table 7: Antifungal activity of Amphotericin.**

Name of Organism	Concentration of AMT (µg/mL) and Zone of inhibition (mm) <sup>±</sup> SD						
	25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	MIC µg
<i>A. niger</i>	NS	NS	2.1±0.2	5.2±0.2	8.2± 0.2	10.4±0.1	100
<i>N. crassa</i>	NS	NS	4.1±0.1	6.5±0.1	12.2±0.3	19.1±0.4	100
<i>P. chrysogenum</i>	NS	NS	NS	4.1±0.2	10.4±0.1	15.3±0.2	250
<i>T. viridae</i>	NS	NS	NS	5.1±0.2	9.4±0.1	14.3±0.2	250
<i>A. brasileinsis</i>	NS	NS	3.1±0.2	5.4±0.3	8.1±0.2	13.2±0.3	100
<i>C. albicans</i>	NS	5.2±0.3	9.3±0.2	18.5±0.1	20.1±0.3	22.7±0.7	50
<i>C. oxysporum</i>	NS	NS	NS	9.3±0.2	15.2±0.1	17.4±0.4	250
DMSO	-	-	-	-	-	-	-

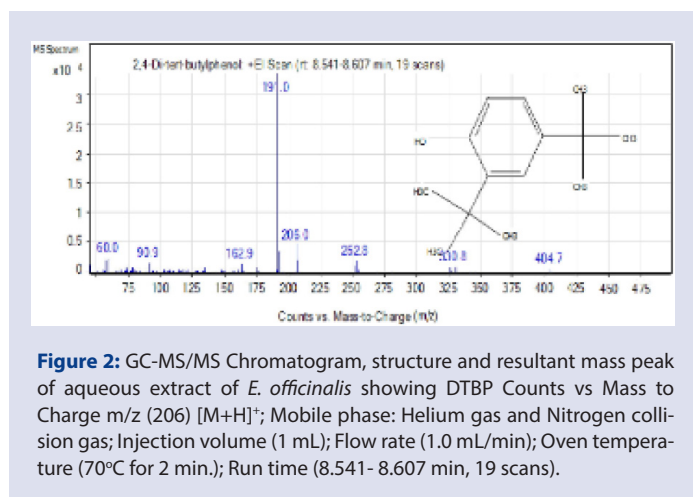
MIC µg: Minimum inhibitory concentration in microgram; NS: Not susceptible (did not exhibit any zone of inhibition); SD: Standard deviation; DMSO: Dimethyl sulfoxide; AMT: Amphotericin; Values are expressed as mean± SD from three experiments (\*n=3).



**Figure 1:** GC-MS/MS Chromatogram, structure and resultant mass peak of methanolic extract of *E. officinalis* showing PBDME Counts vs Mass to Charge m/z (206) [M+H]<sup>+</sup>; Mobile phase: Helium gas and Nitrogen collision gas; Injection volume (1 mL); Flow rate (1.0 mL/min); Oven temperature (70°C for 2 min.); Runtime (8.541- 8.614 min, 21 scans).



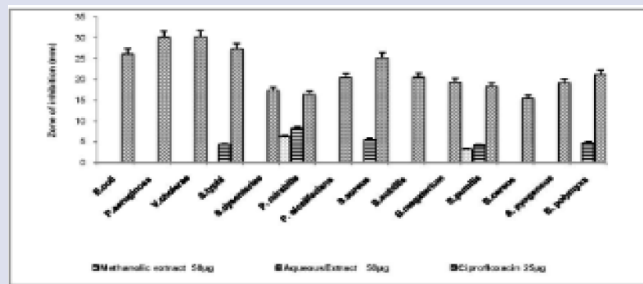
**Figure 3:** GC-MS/MS Chromatogram, structure and resultant mass peak of aqueous extract of *E. officinalis* showing HSTDME Counts vs Mass to Charge m/z (503) [M+H]<sup>+</sup>; Mobile phase: Helium gas and Nitrogen collision gas; Injection volume (1 mL); Flow rate (1.0 mL/min); Oven temperature (70°C for 2 min.); Run time (35.23- 36.199 min, 19 scans).



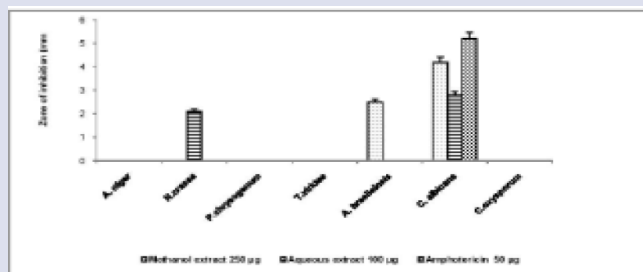
**Figure 2:** GC-MS/MS Chromatogram, structure and resultant mass peak of aqueous extract of *E. officinalis* showing DTBP Counts vs Mass to Charge m/z (206) [M+H]<sup>+</sup>; Mobile phase: Helium gas and Nitrogen collision gas; Injection volume (1 mL); Flow rate (1.0 mL/min); Oven temperature (70°C for 2 min.); Run time (8.541- 8.607 min, 19 scans).

*C. albicans* (2.8±0.2mm) and *N. crassa* (2.1±0.1mm) accept *A. niger*, *P. chrysogenum*, *T. viridae*, *A. brasileinsis* and *C. oxysporum* while no zone of inhibition of methanolic extract was observed at 100 µg/mL with all pathogenic fungi. The zone of inhibition was observed for *A. niger* (3.2±0.1mm), *N. crassa* (3.5±0.1mm), *A. brasileinsis* (3.0±0.2mm) and *C. albicans* (5.1±0.2mm) at 250 µg/mL whereas the pathogenic fungi;

*P. chrysogenum*, *T. viridae* and *C. oxysporum* were not susceptible at this concentration with aqueous extract. Zone of inhibition of methanolic extract against *C. albicans* (4.2±0.1mm) and *A. brasileinsis* (2.5±0.1mm) were observed at 250 µg/mL while no zone of inhibition was observed at 250 µg/mL against *A. niger*, *N. crassa*, *P. chrysogenum*, *T. viridae* and *C. oxysporum*. The fungi *A. niger*, *N. crassa*, *P. chrysogenum*, *T. viridae*, *A. brasileinsis*, *C. albicans* and *C. oxysporum* were found susceptible with aqueous extract at high concentration (500 µg/mL) whereas only *C. oxysporum* was not susceptible with methanolic extract at this concentration. In our study, it was marked that aqueous extract of EOFE has good antifungal activity at low concentration (100 µg/mL) while methanolic extract exhibited minimal antifungal activity at (250 µg/mL) [Figure 5]. It was amazing that aqueous extract showed a potent inhibitory effect for Gram negative bacteria such as (*P. mirabilis*) with MIC value of (50 µg/mL) and zone of inhibition (8.2±0.5 mm); two Gram positive bacteria (*B. pumilis* and *B. polymyxa*) with MIC value of MIC of (50 µg/mL) and zone of inhibition (4.1±0.3 mm) and (4.7±0.3 mm) respectively in comparison to methanolic extract at the same MIC. Methanolic extract confirmed low antifungal activity, However broad antifungal effect of aqueous extract was observed with *N. crassa*. All these results confirmed aqueous extract as broad spectrum antimicrobial agent and inhibit the growth of wide range of Gram negative, Gram positive bacteria and fungi.



**Figure 4:** Standard (Ciprofloxacin) and *E. officinalis* extracts (methanolic and aqueous) graph which displayed the most comprehensive minimum inhibitory concentration (MIC) and number of bacteria against which they were active at different doses. Standard is more susceptible at 25 µg concentration against all Gram negative and Gram positive bacteria and give maximum zone of inhibition (30.2±0.5mm with *V. cholerae*). Maximum resultant zone of inhibition (8.2±0.5mm, 4.3±0.4mm, 5.5±0.2mm, 4.7±0.3mm and 4.1±0.3mm) of aqueous extract is observed at 50 µg concentration against Gram negative (*P. mirabilis*, *S. typhi*) and Gram positive (*S. aureus*, *B. polymyxa* and *B. pumilis*) bacteria. Low margin of zone of inhibition of methanolic extract is observed (6.2±0.8mm and 3.2±0.6mm) at 50 µg concentration with Gram negative (*P. mirabilis*) and Gram positive (*B. pumilis*), while no margin of zone of inhibition of methanolic extract is observed with *S. typhi*, *S. aureus* and *B. polymyxa*.



**Figure 5:** Standard (Amphotericin) and *E. officinalis* extracts (Methanolic/Aqueous) graph which displayed the most comprehensive minimum inhibitory concentration (MIC) and the number of fungi against which they were active at different concentration. Standard is more susceptible at 50 µg concentration against *C. albicans* and give maximum zone of inhibition (5.2±0.3mm). Maximum resultant zone of inhibition (2.8±0.2mm and 2.1±0.1mm) of aqueous extract is observed at 50 µg concentration against *C. albicans* and *N. crassa* fungi while no margin of zone of inhibition of methanolic extract is observed at 50 µg concentration with all fungi tested. Zone of inhibition of methanolic extract is observed (4.2±0.1mm and 2.5±0.1mm) at 250 µg concentration against *C. albicans* and *A. brasiliensis*.

## DISCUSSION

UAE is a novel technique used to extract bioactive compounds from medicinal plants. The same technique was employed in this study for the extraction of bioactive compounds with different solvent (methanol and water) from the fruit of *E. officinalis*. This is an efficient method, that requires very short time, less solvent, reduced solvent exposure, eco-friendly and is comparatively simple to operate. It minimizes the risk of oxidation, disintegration and decomposition of chemical constituents

of plant.<sup>6</sup> In this method, cavitations and bubbles are produced that exerts strong shear significant to fragment and disrupt plant tissues and cell walls leading to enhanced mass transfer of the cell contents. During the UAE process, extraction efficiency is improved due to increase in surface areas, rate of mass transfer and production of cavitations and bubbles.<sup>12-15</sup> optimization and demonstration of an ultrasonic assisted extraction (UAE) Because of these advantages, the UAE techniques have become one of the best techniques for the extraction of bioactive constituents from medicinal plants. In this study, UAE was efficiently used for the preparation of aqueous and methanolic extracts of *E. officinalis* fruits. The extraction was carried out at 20°C, for 20 min using a solid-to-solvent ratio of 1: 60 w/v. The percentage yield was found to be 41.33% w/v and 23.0 % w/v with water and methanol, respectively. On the basis of these results, the study concludes that UAE is suitable for the extraction of phytoconstituents from medicinal plants. GC-MS/MS is a frequently used analytical technique for the quantification of phytoconstituents including polyphenols from plant extracts due to its accuracy, reliability and repeatability. In an earlier study, DTBP was detected by GC-MS analysis and was found to be a potential antifungal agent. Another report indicated that 100 µg/mL of DTBP prevent spore germination of *F. oxysporum* and was detected by GC-MS analysis. Moreover, PBDME, DTBP and HSTDM were also reported to possess antibacterial, antifungal, antioxidant and anticancer potential.<sup>16-17</sup> Recently, PBDME, DTBP and HSTDM have been reported to decrease the growth of *S. marcescens*, a Gram negative bacteria.<sup>18</sup> Furthermore, the antifungal activities exhibited by DTBP was investigated by Varsha *et al.*<sup>17</sup> their finding was in total harmony with the previous report, in which the phenolic nature of PBDME, DTBP was found to alter the cell membrane integrity of bacteria.<sup>19</sup> The result clearly showed that the antimicrobial activity of EOFE was due to presence of PBDME, DTBP and HSTDM and confirmed these compounds as an ideal antimicrobial agent. This comprehensive study supports and confirms previous results on antifungal potential of EOFE.<sup>20</sup> The results of the study demonstrated that volatiles present in EOFE inhibited the congregation of spindle microtubules and modified the chromosomal arrangement at the metaphase leading to chromatid loss, which led to reduce the germination of spores. The findings confirmed the previous reports as well as explained the efficiency of these compounds in prevention of fungal growth. Still, to the best of our knowledge; these compounds have hardly ever been documented for antibacterial and antifungal activity against such collection of microbes. The mode of action of phenolic compounds is not fully clear and noticeably speculated to involve membrane distraction by means of active compound.<sup>21</sup> Antibacterial and antifungal activity of the EOFE was quantitatively assessed on the basis of zone of inhibition. In the current study, the inhibitory potential of EOFE was evaluated against seven Gram negative bacteria; *P. aeruginosa*, *E. coli*, *V. cholera*, *S. typhi*, *S. dysenteriae*, *P. mirabilis*, *P. alcalifaciens* and seven Gram positive bacteria; *S. aureus*, *B. subtilis*, *B. megaterium*, *B. pumilis*, *B. cereus*, *S. pyogenus* and *B. polymyxa*. A remarkable antibacterial activity with CPF and EOFE was observed against pathogenic bacteria for which they were active at different concentration taking DMSO as negative control. All the Gram positive bacteria were susceptible to CPF at 25 µg/mL and exhibited maximum zone of inhibition with *V. cholerae*. Both Gram negative and Gram positive bacteria were found susceptible to EOFE at concentration (500 µg/mL–1000 µg/mL). The results ascertained two Gram negative bacteria (*S. typhi* and *P. mirabilis*) and three Gram positive bacteria (*S. aureus*, *B. pumilis* and *B. polymyxa*) were found susceptible at very low concentration (50 µg/mL) to aqueous extract whereas only one Gram negative bacteria; *P. mirabilis* and one Gram positive bacteria; *B. pumilis* was found susceptible (50 µg/mL) to methanolic extract of EOFE. For antifungal activity the inhibitory potential of EOFE was evaluated against seven pathogenic fungi strains; *A. niger*, *N. crassa*,



*P. chrysogenum*, *T. viridae*, *A. brasileinsis*, *C. albicans* and *C. oxysporum*. Here, the potency of EOFE was compared with AMT. Among the tested fungal strains, only *N. crassa* and *C. albicans* showed moderate inhibition with aqueous extract at 100 µg/mL while no inhibition was observed with methanolic extract at 100 µg/mL. *C. albicans* and *A. brasileinsis* were found to be susceptible to methanolic extract at 250 µg/mL. The fungi *A. niger*, *N. crassa*, *P. chrysogenum*, *T. viridae*, *A. brasileinsis*, *C. albicans* and *C. oxysporum* were found susceptible to aqueous extract at high concentration (500 µg/mL) whereas only *C. oxysporum* was found to be not susceptible with methanolic extract at this concentration. The results of the current study revealed that there was marked antibacterial and anti-fungal inhibition with the aqueous extract of EOFE at low concentration (100 µg/mL) while methanolic extract exhibited minimal antifungal activity at 250 µg/mL. The aqueous extract also inhibited the growth of *N. crassa*. The existence of antifungal potential only in crude extracts may be due to the synergistic power of various bioactive constituents in the both extracts. The antimicrobial activities of EOFE can be explained on the basis of its GC-MS/MS analysis. In this study, the results revealed the broad spectrum of antibacterial and antifungal activity in crude extract of EOF may be due to the synergistic influence of various bioactive compounds in EOFE. In a nutshell, EOFE showed their potential antimicrobial activities against the bacterial and fungal pathogens and provides an ample opportunity to plant based drug designing based on ethnomedicine. The possibility of using EOFE in folk medicine for the management of infectious diseases is validated by this study.

## CONCLUSION

In summary, the results of this study showed that both the methanolic and aqueous extracts exhibit antibacterial and antifungal properties due to their bioactive compounds. To our knowledge, this is the first report of its activity against such collection of Gram negative bacteria; *P. alcalifaciens* and Gram positive bacteria; *B. pumilis*, *B. polymyxa* and fungal strains; *N. crassa*, *A. brasileinsis* and *C. oxysporum*. Furthermore, use of UAE to extract vital bioactive extract as EOFE worth mentioning. The considerable inhibitory effect of EOFE against various microbial species was mainly due to the presence of phenolic compounds and other phytochemicals including PBDME, DTBP and HSTDME compound. With this milieu, the present study was focussed towards analysing the value of EOFE against various pathogens microbes. The results revealed excellent antimicrobial activity of aqueous extract and methanolic extracts of *E. officinalis*.

## ACKNOWLEDGEMENT

The authors gratefully acknowledge the Bioactive Natural Compound Laboratory (BNCL), Department of Pharmacognosy and Phytochemistry, Glocal School of Pharmacy, Glocal University, Saharanpur 247121, U.P, India providing the experimental facilities. The authors would like to thank Ms. Kahkashan for their support and valuable suggestions to improve the quality of the manuscript.

## FUNDING

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

## CONFLICT OF INTEREST

The authors declare no conflict interest.

## ABBREVIATIONS

**BNCL:** Bioactive Natural Compound Laboratory; **UAE:** Ultrasonic assisted extraction; **EOFE:** *Emblca officinalis* fruit extract; **RT:** Retention Time; **Mol.Wt.:** Molecular weight; **MIC µg:** Minimum inhibitory concentration in microgram; **NS:** Not susceptible (did not exhibit any zone of inhibition); **SD:** Standard deviation; **DMSO:** Dimethyl sulfoxide; **CPF:** Ciprofloxacin; **AMT:** Amphotericin; **DTBP:** 2,4-Di-tert-butylphenol; **PBDME:** Phenol,3,5-bis(1,1dimethylethyl); **HSTDME:** Heptasiloxane1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl.

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