

Antimicrobial Activity by Solvents Extracted from *Ocimum Basilicum* Herb Against Multidrug Resistant Gram-Negative Rods

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ABSTRACT

Background: Failure of treatment with antibiotics occurs due to increase in number of multidrug resistant gram-negative bacteria, worldwide. The objective of this study was to find out the antimicrobial activity of crude ethanolic extract and its further three fractions by *Ocimum basilicum* leaves against multi drug resistant gram-negative rods.

Material and Methods: This descriptive study was conducted in the Department of Microbiology, University of Health Sciences, Lahore from 1st July 2016 to 30th June 2017. A total of 80 multidrug resistant gram-negative rods were included in this study. Agar dilution method was performed to determine minimum inhibitory concentration of crude ethanolic extract and different fractions i.e., n-hexane, chloroform and ethyl acetate of *Ocimum basilicum* leaves against multidrug resistant gram-negative rods i.e., extended spectrum beta lactamases and carbapenemase producers. Multi-inoculator was used for inoculation.

Result: The mean MICs of crude ethanolic extract, n-hexane fraction, chloroform fraction, and ethyl acetate fraction of *Ocimum basilicum* against ESBLs were 100.0±8.00, 168.13±8.00, 176.88±8.00 and 41.75±8.00 respectively. Similarly, the mean MICs of crude ethanolic extract, n-hexane fraction, chloroform fraction, and ethyl acetate fraction of *Ocimum basilicum* against carbapenemase producers were 77.50±8.00, 113.75±8.00, 132.50±8.00 and 29.50±8.00 respectively.

Conclusion: Ethyl acetate fraction and crude ethanolic extract from leaves of *Ocimum basilicum* showed good antibacterial effectiveness against ESBLs and carbapenem resistant organisms than other fractions. This finding may also promote the effective use of *O. basilicum* herb and its components in modern medicine.

Key words: Multi-drug Resistance, Minimum Inhibitory Concentration (MIC), *Ocimum basilicum*

Authors' Contribution:

¹Conception; Literature research; manuscript design and drafting; ²Critical analysis and manuscript review; ³Data analysis; ⁴Manuscript Editing.

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Article info:

Received: February 1, 2021
Accepted: December 13, 2021

Cite this article. Ikram A, Saleem S, Imran M, Ghazal A. Antimicrobial Activity by Solvents Extracted from *Ocimum Basilicum* Herb Against Multidrug Resistant Gram-Negative Rods. *J Islamabad Med Dental Coll.* 2021; 10(4):

Funding Source: Nil

Conflict of Interest: N

Introduction

Failure of treatment with antibiotics occurs due to increase in number of MDR bacteria worldwide.¹ One of the most significant problems in hospital settings is multidrug resistant gram-negative rods (MDR-GNR), which includes MDR *Pseudomonas aeruginosa*, *Acinetobacter*

baumannii, carbapenemase-producing *Klebsiella pneumonia* and extended-spectrum-beta-lactamase (ESBL) producing *Escherichia coli*.² Medicinal plants are a wealthy source of antimicrobial components. A wide range of medicinal plant extracts were used to treat several

infections before the progression of western medicine, due to their possible antimicrobial activity.³ In Pakistan, 80% of the people (mostly rural) depend upon the traditional medicines.⁴

Ocimum basilicum is a common pharmaceutical plant that is traditionally used for the treatment of various diseases worldwide.⁴ It is a cultured plant widespread in the tropics of Africa and Asia.⁵ It belongs to the family Lamiaceae and it is the most abundant of the genus *Ocimum*.⁶ *O. basilicum* is a culinary herb, commonly called basil, also called sweet basil. Aerial parts which include leaves and flower of *O. basilicum* are conventionally used as galactagogue, carminative, digestive, stomachic, aromatic, antispasmodic and tonic agent.⁷

Ethanol, methanol, hexane and watery extracts of *O. basilicum* were assessed in a previous study and out of all these extracts of *O. basilicum*, hexane extract showed the strongest spectrum of antimicrobial activity.⁸ Extract of leaves of basil show antimicrobial activity against human dental plaque pathogens and reduced *Streptococcus mutans* and *Lactobacillus acidophilus* colony count.⁹

The present study was conducted to find out the antimicrobial activity of *O. basilicum* herb against MDR-GNR. This study will provide an adequate knowledge about possible advantage of this natural therapeutic product. This study may also promote the effective use of *O. basilicum* herb and its components in modern medicine.

Methodology

This descriptive study was conducted in the Department of Microbiology, University of Health Sciences, Lahore over a period of 1 year, from 1st July 2016 to 30th June 2017. WHO calculator was used for the calculation of sample size with anticipated population proportion to be 70%, significance level 95%, and margin of error 10%.¹⁰ Eighty (80) multidrug resistant gram-negative rods including 40 ESBLs and 40 carbapenem resistant

gram-negative rods were collected conveniently from microbiology section of CMH Lahore, Jinnah Hospital Lahore and Post Graduate Medical Institute (PGMI) Lahore. They were re-confirmed at the department of Microbiology, University of Health Sciences Lahore on the basis of morphology, cultural characteristics and biochemical identifications. All carbapenemase producing gram negative rods were reconfirmed for meropenem resistance. Modified Hodge test (MHT) was performed to confirm the organisms for carbapenemase production. To reconfirm ESBLs producing organisms, double disk diffusion test was performed. The results were interpreted according to clinical laboratory standard institute guidelines (2017).¹¹ Organisms sensitive to cephalosporins were excluded (because we used ESBLs which are resistant to cephalosporin), confirmed MDR strains were preserved in 16% (v/v) glycerol in brain heart infusion and stored in refrigerator at -70°C till use. Ethical approval was taken from Ethical Review Committee of institute.

Ocimum basilicum (Basil) leaves were collected from local nursery of Lahore. Plant was confirmed by taxonomist who worked at Botany Department, University of the Punjab (Lahore). After collection, the leaves were washed first with tap water and then with sterilized distilled water and air dried at room temperature.¹² Dried leaves of the plant were ground by using electric grinder. Two kilogram of powdered material was soaked in ten liter of ethanol for two weeks. After two weeks, whatmann (1) filter paper was used for filtration. As a result, ethanolic extract of *O. basilicum* was obtained which was dried under vacuum in a rotary evaporator. The ethanolic extract was concentrated by using a rotary evaporator at temperature 40°C.¹³ Crude ethanolic extract of *O. basilicum* was further partitioned according to increasing polarity into n-hexane, chloroform and ethyl acetate fraction. Separating funnel was used for further successive partitioning by three solvents in order of increasing polarity, first by n-hexane, second by chloroform

and third by ethyl acetate. The layers that were formed in a flask were separated. Rotary evaporator was used to evaporate respective solvent and fractions of *O. basilicum* dried. Air tight containers were used to store all solvent extracts at 4°C till further investigation.¹⁴

Agar dilution method was used for the determination of MIC. The stock solution was prepared using the extract and fractions to be tested. DMSO (Dimethyl sulfoxide) (universal solvent having no antibacterial activity) was used for the preparation of stock.¹⁵

The organisms were streaked on nutrient agar medium. After incubation at 37°C for 24h, 4 or 5 pure colonies were selected. They were shifted in a sterile normal saline tube and carefully rotated. Prepared suspension was equal to the 0.5 McFarland's standards. Further, diluted this suspension in 1:10 sterile normal saline. Inoculums in this dilution had 10⁶CFU/ml concentration. Within 15min of preparation, this suspension was used.¹⁶

Multi inoculator was used for inoculation of the prepared extract plates. After inoculation, plates were incubated at 35°C for 24 hours. After 24 hours of incubation, the plates were seen for any kind of growth. Already identified by PCR, ESBL producing strain and Modified hodge test, positive strains were used as positive control for ESBLs and carbapenemase producing organisms respectively from Microbiology department of University of Health Science Lahore. DMSO incorporated in plate

containing mueller-hinton agar was used as negative control.²

All statistical analysis was done by using SPSS software (version 17.0, SPSS Inc). All MICs results of crude extract and different solvent fractions of *Ocimum basilicum* leaves were expressed as mean ± S.D. For comparison of mean among MICs of crude extract and different solvent fractions of *Ocimum basilicum* leaves, ANOVA test was applied followed by Post hoc tukey test.

Result

Out of 40 carbapenemase producing gram negative rods, there were 22(55%) *Acinetobacter baumannii*, and 18(45%) *Pseudomonas aeruginos*. Similarly out of 40 ESBL producing organisms, there were 16(35%) *Klebsiella pneumoniae* and 24(65%) *Escherichia coli*. Minimum inhibitory concentration (MIC) of crude extract and three different fractions of *Ocimum basilicum* leaves against MDR gram negative rods is shown in (Table I). Mean MICs of crude extract and different fractions of *O.basilicum* leaves against multidrug resistant gram negative rods is shown in (Table II).

The minimum inhibitory concentration of extract and all fractions of *Ocimum basilicum* leaves against cabapenemase producing organisms and ESBLs are shown in (Figure I, II).

ANOVA test was applied to compare mean MICs of crude extract and different solvent fractions of *Ocimum basilicum* leaves, for multiple comparison post hoc tukey test was applied. All the results were statistically significant (Table II).

| Organisms | Crude ethanolic extract | | | n-hexane fraction | | | Chloroform fraction | | Ethyl acetate fraction | | |
|-------------------|-------------------------|----------|----------|-------------------|----------|----------|---------------------|----------|------------------------|----------------------|---------|
| ESBLs (n=40) | 75mg/ml | 100mg/ml | 125mg/ml | 125mg/ml | 150mg/ml | 175mg/ml | 175mg/ml | 200mg/ml | 40mg/ml | 50mg/ml | |
| | 2 | 36 | 2 | 2 | 7 | 31 | 37 | 3 | 31 | 9 | |
| Carbapenem (n=40) | Crude ethanolic extract | | | n-hexane fraction | | | Chloroform fraction | | | Ethylcetate fraction | |
| | 75mg/ml | 100mg/ml | | 75mg/ml | 100mg/ml | 125mg/ml | 150mg/ml | 100mg/ml | 125mg/ml | 150mg/ml | 30mg/ml |
| | 36 | 4 | | 7 | 11 | 15 | 7 | 7 | 13 | 19 | 40 |

| Organism | Crude ethanolic extract | N-hexane fraction | Chloroform fraction | Ethyl acetate fraction | P-value |
|-------------------------------------|-------------------------|-------------------|---------------------|------------------------|---------|
| MIC against Carbapenemase producers | 77.50±8.00 | 113.75±8.00 | 132.50±8.00 | 29.50±8.00 | <0.05* |
| MIC agaist ESBL producers | 100±8.00 | 168.13±8.00 | 176.88±8.00 | 41.75±8.00 | |

Analysis of the mean MICs of crude extract and different fractions of *O.basilicum* leaves against multidrug resistant gram negative rods show significant difference.

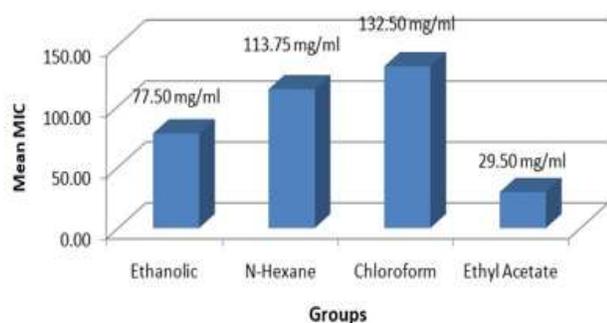


Figure 1: Comparative analysis of Mean MICs against Carbapenemase Procedures

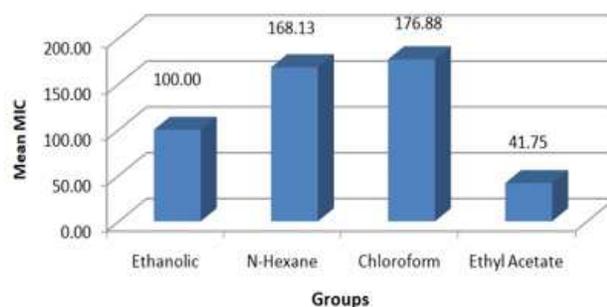


Figure 2: Comparative analysis of Mean MICs against ESBL procedures

Ethyl acetate fraction has least value of MIC and shows greatest antibacterial activity against carbapenemase producing organisms and ESBLs followed by crude ethanolic extract, n-hexane fraction and then chloroform fraction.

Discussion

Phytochemical active compounds like flavonoids, tannins, alkaloids and phenolic compounds in *O.basilicum* plant extract are potent inhibitors of microbial growth.¹⁷⁻¹⁸In the present study, crude ethanolic extract and its further three fractions i-e

n-hexane, chloroform and ethyl acetate were used for evaluating its antimicrobial activity. Out of these fractions, ethyl acetate fraction exhibited best antimicrobial activity against MDR-GNR. Ethyl acetate fraction of basil leaves contain polyphenolics, flavonoids and alkaloids.¹⁹The highest antimicrobial action of ethyl acetate fraction could be due to these bioactive compounds.¹⁷These findings support the idea that ethyl acetate fraction of *O.basilicum* leaves could be used as antimicrobial with broad-spectrum antimicrobial properties.

Crude ethanolic extract showed second best antimicrobial activity. N-hexane and chloroform fractions showed less activity as compared to the fraction of ethyl acetate. Ad khalil 2013²⁰ in his study discussed that *O.basilicum* ethanolic extract contain compounds which have strong antibacterial activity against gram positive (*Staph aureus*) and gram negative organisms (*E.coli*). It is known that ethanol is a highly polar solvent which is able to extract phytochemicals efficiently; greater number of active constituents could have been produced by extraction of ethanol which are responsible for good antibacterial activity.

Abubutain I 2019²¹ used gas chromatography coupled with mass spectrometry (GC/MS) to

analyze ethanolic extract of *O.basilicum*, results of his study showed that extract contains phenols, terpene, esters, steroids and fatty acids. These compounds showed antimicrobial activity against some gram positive, gram negative and fungal stains that were used in his study. Issazadeh k 2012¹⁷ also reported that crude ethanolic extract exhibited antibacterial activities more than the aqueous extract. This is consistent with findings of this study.

N-hexane fraction obtained from crude ethanolic extract of *O.basilicum* herb exhibited a high range of MIC against multidrug resistant gram negative rods. In a previous research, Patil D ⁷ reported that n-hexane extract of *O.basilicum* showed antimicrobial activity against bacterial (gram positive and gram negative) and fungal strains that were used in their study. The results revealed that hexane extract had broad-spectrum activity followed by ethanolic extract against all tested bacteria. Bilal A. 2012²² also reported that hexane extract of *O.basilicum* has strong, broad spectrum antimicrobial and anticandidal activity than ethanolic extract. In contrast, the results obtained from current study showed that crude ethanolic extract had strong antibacterial activity than n-hexane fraction. Variations between these results could be due to MDR strains used in current study. Tabassum S 2016¹² reported the effect of hexane extract of *O.basilicum* on selected bacterial strains. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E coli*, *Proteus mirabilis* and *Klebsiella pneumoniae* were included in their study. The results of their study revealed that antimicrobial activity of crude ethanolic and hexane extracts for gram negative bacteria was much higher than gram positive bacteria. In current study, no gram-positive bacteria were used so, in future a study could be designed against MDR-gram positive bacteria. Useful phytochemicals of *O. basilicum* can be a great scope for future researches. Moreover, isolation and purification of pure compounds should be carried out. HPLC analysis should be

done to identify active compounds, because a variety of multifunctional compounds is present in the extract and fractions of *Ocimum basilicum*, which can be used for the production of novel antibiotics.

Chloroform fraction obtained from crude ethanolic extract of *O.basilicum* herb exhibited high range of MIC against multidrug resistant gram negative rods than all other fractions. Aruna K 2015²³ showed the effect of *O.basilicum* leaf extracts against ESBL and MBL producing uropathogens. Methanol, Ethanol, Chloroform, Acetone and Butanol extracts of *O.basilicum* were analyzed against these organisms. Chloroform extract showed best strong and broad-spectrum antibacterial activity against tested strains than others. The difference in results might be due to difference in extraction technique e.g, the plant extract used in the current study being fractionated from crude, whereas others used pure active chloroform extract. Further GC-MS analysis of the chloroform extract of *O.basilicum* also done by ArunaK 2015²³ showed that eugenol and estragole are major contributors. These compounds express good antibacterial activity in the chloroform extract of *O.basilicum*. Gebrehiwot H 2016¹³ also reported that major constituent of chloroform extract of leaves of *O. basilicum* was estragole (38.22%). Estragole is a potent phytochemical compound which has the ability to inhibit all tested bacteria and fungi.¹³

It can be assumed that the extracts or its components affect some key processes in the organism's growth. Antimicrobial activity of the different extracts of *O.basilicum* could be due to some active components (flavonoids, alkaloids, estragole, phenolics, tannins, linalools) which have the ability to combine with extra cellular and soluble protein and to make a complex with bacterial cell wall disrupting membrane of microbes.²⁴

Conclusion

Ocimum basilicum crude ethanolic extract and different solvent fractions had potent antibacterial activity against all tested strains of ESBLs and carbapenemase producing gram negative rods. *O.basilicum* can be used as alternative antimicrobial drug. The medicinal value of *O.basilicum* extract might be due to natural bioactive phytochemicals.

Acknowledgement

We are thankful to University of Health Sciences, Lahore for full technical and financial support to perform this research activity.

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