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ANTIBACTERIAL ACTIVITY OF *NIGELLA SATIVA* EXTRACTS AGAINST EXTENDED SPECTRUM B-LACTAMASE PRODUCING *ESCHERICHIA COLI* ISOLATES

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Keywords:

Nigella sativa, Supercritical fluid carbon dioxide extraction, Extended-spectrum β-lactamase, Antibacterial activity, *Escherichia coli*

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ABSTRACT: The most striking feature of natural products in connection to their long-lasting importance in drug discovery is their structural diversity that is still largely untapped. Most natural products are not only sterically more complex than synthetic compounds but also differ in regards to the statistical distribution of functionalities. The chemical diversity and unique biological activities of a wide variety of natural products have propelled many discoveries in chemical and biological sciences and provided therapeutic agents to treat various diseases as well as offered leads for the development of valuable medicines. The present study has been designed to screen the phytochemicals and to evaluate the antibacterial potential of *Nigella sativa* L. seeds against ESBL positive *Escherichia coli* isolates. The crude extracts of *Nigella sativa* L. seeds were prepared by utilizing two methods namely Soxhlet extraction and Supercritical Fluid Carbon dioxide (SCFE-CO_2) extraction. Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, phenols, tannins, saponins, and terpenoids which are known to exhibit medicinal as well as physiological activities. The Supercritical Fluid Carbon dioxide (SCFE-CO_2) extracts of *Nigella sativa* L. seeds exhibited high activity against ESBL *E. coli* as compared to other crude extracts. These antibacterial properties would assure for further studies on the clinical applications of *Nigella sativa* L. against multidrug clinical isolates.

INTRODUCTION: In recent years, strains of multidrug-resistant organisms have become quadrupled worldwide [1]. Presently, antimicrobial resistance to synthetic drugs poses significant threat to patient's treatment as it leads to increased morbidity and mortality [2, 3].

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Synthetic drugs also block receptor sites, and hence attempts are being made to control the use of synthetic drugs and develop new drugs from natural resources like medical plants. Medical plants are important therapeutic aids for various ailments, and the use of those that are native to India in the different traditional system of medicine are awe-inspiring [4].

Nigella sativa seeds, as a nutritional and medicinal plant, have traditionally been used for thousands of years as folk medicine and some of its active compounds were reported against many ailments. Different pharmacological effects such as anti-

cancer activity [5], anti-antioxidant activity [6], anti-inflammatory [7], anti-diabetic and anti-glycating property [8, 9, 10], antimicrobial, antiepileptic and hepatoprotective activity [11, 12, 13], potential remedial action on dental and oral illness [14], protection against cardiovascular health problem [15], a potentate drug against neurogenerative disorders and brain damage [16], a remedy for renal diseases including nephrolithiasis and renal damage [17], protection against kidney damage due to morphine toxicity [18] and as a curative drug for stress-induced gastric ulcers [19], have been reported for this medicinal plant. The objective of this study is to screen the phytochemicals and to evaluate the antibacterial potential of *Nigella sativa* L. seeds against ESBL positive *Escherichia coli* isolates. The crude extracts of *Nigella sativa* L. seeds are obtained by Supercritical Fluid Carbon dioxide (SCFE-CO_2) extraction and conventional solvent extraction using soxhlet apparatus. The *Escherichia coli* isolates included in this study are multidrug resistant, resistant to third generation Cephalosporins namely Ceftriaxone (CTR), Cefotaxime (CTX) and Ceftazidime (CAZ) and harbors ESBL genes particularly ^{bla}TEM, ^{bla}SHV, and ^{bla}CTXM.

MATERIALS AND METHODS:

Collection of Plant Material: *Nigella sativa* L. seeds were procured from Lab Chemicals, India. The seeds were dried and pulverized into a fine powder using a mechanical blender and packed in an airtight container for further analysis. The plant species were identified and registered (Reg. no. PARC/2017/ 3547) by Dr. P. Jayaraman, Director of Plant Anatomy Research Centre.

Supercritical Fluid Carbon Dioxide Extraction (SCFE-CO_2): SCFE-CO_2 extraction was carried through Thar SFC extractor, Department of Food Process Engineering, SRM University, India. Extraction of pounded *Nigella sativa* L. seeds was done at 120 bar pressure at 40 °C. The flow rate of CO_2 and Co-Solvent was 10g/min and 11g/min respectively. The yield of extracts was inspected, dried in a rotary vacuum evaporator and stored at 4 °C in the dark until analysis.

Soxhlet Extraction: Crude extracts of *Nigella sativa* L. seeds were prepared using seven solvents namely hexane, diethyl ether, ethyl acetate, acetone, ethanol, methanol and water. 50 gm of dried powdered seeds were treated with 250 ml of solvents in a Soxhlet apparatus. The extracts procured were filtered with Whatman filter paper 1, and the solvents are removed by rotary vacuum evaporator (40 °C) and dried in a vacuum oven at 30 °C for 1h.

Preliminary Phytochemical Screening: Phytoconstituent analysis of crude extracts was determined by the following methods [20, 21, 22].

Test for Alkaloids (Wagner's Reagent): 1.27 gm of iodine and 2 gm of potassium iodide was dissolved in 5 ml of water, and the solution was diluted to 100 ml with water. Few drops of Wagner's reagent was added to the test solution and observed for brown flocculent precipitate.

Test for Flavonoids: 10 ml of ethyl acetate was mixed with a test sample and heated over a steam bath for 2 min. The mixture was then filtered with Whatman filter paper 1, and 1 ml of dilute ammonia was added to 4 ml of filtered test solution. The appearance of a yellow color indicates the presence of flavonoids.

Test for Phenols (Ferric Chloride Test): 1 ml of test solution was heated over a steam bath for 2 min. 2 ml of ferric chloride ($FeCl_3$) was added slowly to the test solution and observed for green and blue color formation which indicates the presence of phenolic compounds.

Test for Tannins (Ferric Chloride Reagent): Ferric chloride solution was prepared by adding 5% w/v ferric chloride in 90% of alcohol. Few drops of ferric chloride solution were added to the warmed, filtered test solution carefully and observed for dark green color which indicates the presence of tannins.

Test for Glycosides: 5 ml of the test sample was treated with 2 ml of glacial acetic acid and one drop of ferric chloride solution. The test sample was overlayed with 1 ml of concentrated sulphuric acid (H_2SO_4). Formation of brown ring indicates the presence of Glycosides.

Test for Steroids (Salkowaski Reaction): 2 ml of chloroform and 2 ml of concentrated sulphuric acid was mixed with 500 mg of the test sample in a

clean, dry test tube. The test tube was shaken carefully and observed for the formation of red color in the chloroform layer which indicates positive for steroids.

Test for Saponins (Foam Test): Small amount of sodium bicarbonate and water was mixed with 0.5 mg of the test sample in a clean, dry test tube and shaken vigorously; the formation of froth indicates the presence of saponins.

Test for Terpenoids: 5 ml of the test sample was mixed with 2 ml of chloroform and 3 ml of concentrated sulphuric acid (H_2SO_4) and observed for reddish brown color at the interface which indicates the presence of terpenoids.

Preparation of Extracts for Antibacterial Activity: The known quantities of solvent-free crude extracts and Supercritical Fluid Carbon dioxide (SCFE-CO_2) extracts of *Nigella sativa* L. seeds were dissolved in Dimethyl Sulfoxide (DMSO) for the evaluation of antibacterial activity.

Test Bacteria: The bacterial strains included in this study are as follows, *Staphylococcus aureus* MTCC 9542, *Bacillus substilis* MTCC 10224, *Escherichia coli* MTCC 1563, *Klebsiella pneumonia* MTCC 3384, *Pseudomonas aeruginosa* MTCC 14676 and 44 ESBL positive *Escherichia coli* clinical isolates. The *Escherichia coli* clinical isolates included in this study are multidrug resistant, resistant to third generation Cephalosporins namely Ceftriaxone (CTR), Cefotaxime (CTX) and Ceftazidime (CAZ) and harbors ESBL genes particularly ^{bla} TEM, ^{bla} SHV and ^{bla} CTX-M.

Preparation of Bacterial Inoculums: The test bacteria are maintained at 4 °C on slopes of nutrient agar. Active cultures for investigation was prepared by transferring a loop full of cells from stock cultures to the sterile nutrient broth and incubated at 37 °C for 24 h. The turbidity of culture medium was adjusted to 0.5 McFarland standard or an O.D value of 0.08 to 0.1 at 600 nm wavelength which corresponds to 1.5×10^8 CFU/ml.

Antibacterial Assay: Antibacterial activities of all eight extracts namely hexane, diethyl ether, ethyl acetate, acetone, ethanol, methanol, water and Supercritical Fluid Carbon dioxide (SCFE-CO_2) extract of *Nigella sativa* L. seeds was determined by agar well diffusion method [23]. The inoculants of the bacterial strains were swabbed on to the Mueller Hinton agar plate using Himedia swabs to ensure the uniform lawn of growth. 6 mm diameter wells were punched on to MHA plates using sterile cork borer. The solvent-free extracts were diluted with dimethyl sulfoxide (DMSO) to the concentration of 4 mg/ml, 2 mg/ml, 1 mg/ml, 400 µg/ml and 200 µg/ml. The wells were filled with 75 µl of above-mentioned plant extracts. Gentamycin at the concentration of 100 µg/ml and dimethyl sulfoxide (DMSO) was included as positive and negative controls respectively. Finally, the plates were incubated at 37 °C for 24 h, and the resulting diameter of zone inhibition was measured and tabulated. The results were calculated as the mean diameter of zone inhibition in mm ± standard deviation (mm ± SD).

RESULTS AND DISCUSSION: Conventional solvent extraction using soxhlet apparatus and Supercritical fluid carbon dioxide extraction (SCFE-CO_2) of *Nigella sativa* L. seeds was successfully done and the solvent-free crude extracts were tested for bioactive phytoconstituents using standard protocols. The results revealed the presence of alkaloids, flavonoids, phenols, tannins, glycosides, steroids and terpenoids **Table 1**.

TABLE 1: PHYTOCHEMICAL ANALYSIS OF CRUDE EXTRACTS

Phytochemicals	Water	Methanol	Ethanol	Acetone	Ethyl acetate	Diethyl ether	Hexane	$SCCO_2$
Alkaloid	+	+	+	-	+	+	-	+
Flavanoid	+	+	+	-	+	-	-	+
Phenol	+	+	+	-	-	+	+	-
Tannins	+	-	-	-	+	-	-	+
Glycosides	-	+	+	+	-	+	+	-
Steroids	-	+	+	+	+	+	+	+
Saponins	-	-	-	-	+	+	-	+
Terpenoids	-	-	-	-	+	-	-	+

+ Present, - absent

World health organization estimated that about 80% of the world population relies on traditional medicine for their primary health care. Most of these therapy regimes involved the use of alkaloids, flavonoids, phenols and terpenoids, all traditionally known as secondary metabolites [24].

TABLE 2: ANTIBACTERIAL ACTIVITY OF EIGHT EXTRACTS AGAINST MTCC BACTERIAL STRAINS

S. no.	Extract	Concentration	*S. aureus* MTCC 9542	*Bacillus subtilis* MTCC 10224	*E. coli* MTCC 1563	*K. pneumo* MTCC 3384	*P. aeruginosa* MTCC 14676
1	Hexane	4mg	13	12	10	12	11
		2mg	10	9	-	9	10
		1mg	-	7	-	-	-
		400µg	-	-	-	-	-
		200µg	-	-	-	-	-
2	Diethyl ether	4mg	-	-	13	10	12
		2mg	-	-	10	9	10
		1mg	-	-	11	-	-
		400µg	-	-	-	-	-
		200µg	-	-	-	-	-
3	Ethyl acetate	4mg	15	13	16	14	15
		2mg	13	12	14	12	13
		1mg	10	9	11	10	10
		400µg	-	-	10	-	7
		200µg	-	-	-	-	-
4	Acetone	4mg	12	11	13	12	13
		2mg	10	9	11	10	10
		1mg	-	-	10	-	9
		400µg	-	-	-	-	-
		200µg	-	-	-	-	-
5	Ethanol	4mg	13	12	12	11	11
		2mg	-	10	11	9	10
		1mg	-	-	9	-	-
		400µg	-	-	-	-	-
		200µg	-	-	-	-	-
6	Methanol	4mg	12	13	12	14	13
		2mg	10	11	11	12	10
		1mg	-	-	9	10	11
		400µg	-	-	-	-	-
		200µg	-	-	-	-	-
7	Water	4mg	10	10	13	-	14
		2mg	9	-	10	-	11
		1mg	-	-	-	-	9
		400µg	-	-	-	-	-
		200µg	-	-	-	-	-
8	$SCCO_2$	4mg	16	14	16	15	16
		2mg	14	12	15	14	14
		1mg	11	10	13	11	12
		400µg	-	-	11	10	10
		200µg	-	-	-	-	-
9	Negative control (DMSO)	4mg	-	-	-	-	-
		2mg	-	-	-	-	-
		1mg	-	-	-	-	-
		400µg	-	-	-	-	-
		200µg	-	-	-	-	-

In the current study the solvent-free crude extracts were evaluated for its antibacterial activity against MTCC strains as follows, *Staphylococcus aureus* MTCC 9542, *Bacillus substilis* MTCC 10224, *Escherichia coli* MTCC 1563, *Klebsiella pneumonia* MTCC 3384, and *Pseudomonas aeruginosa* MTCC 14676. Out of eight extracts tested Supercritical fluid carbon dioxide extract (SCFE-CO_2) of *Nigella sativa* L. seeds showed an active antibacterial activity followed by Ethyl acetate extract **Table 2**. The present work goes in agreement with a similar work done by Suresh

Kumar *et al*., 2010 who had found that $SCCO_2$ extract was more effective to inhibit the growth of tested bacteria which was followed by ethyl acetate.

The SCFE-CO_2 and ethyl acetate extract of *Nigella sativa* L. seeds were further exploited for its antibacterial activity against 44 ESBL *Escherichia coli* isolates. The *Escherichia coli* clinical isolates included in this study are multidrug resistant, resistant to third generation Cephalosporins namely Ceftriaxone (CTR), Cefotaxime (CTX) and Ceftazidime (CAZ) and harbors ESBL genes particularly ^{bla}TEM, ^{bla}SHV, and ^{bla}CTX-M.

In the current study, SCFE-CO_2 extract exhibited effective antibacterial activity against ESBL *E. coli* than the conventional solvent extracts. The SCFE-CO_2 extract was more effective to inhibit the growth of tested bacteria which was followed by ethyl acetate extract. The antibacterial activity of extracts was concentration dependent, and the results (zone of inhibition) were calculated as the mean diameter of zone inhibition in mm ± standard deviation (mm ± SD). The SD value of SCFE-CO_2 extract was highest with 15.5 ± 1.6 followed by 13.8 ± 1.8 for ethyl acetate extract respectively **Table 3**.

TABLE 3: ANTIBACTERIAL ACTIVITY OF CRUDE EXTRACT AGAINST ESBLS (TOTAL NO. OF ISOLATES TESTED – 44)

S. no.	Extract	4mg	2mg	1mg	400µg	200µg
1	Ethyl acetate	13.8 ± 1.8	11.7 ± 1.8	7.5 ± 5.0	3.4 ± 4.8	1.0 ± 3.0
2	$SCCO_2$	15.5 ± 1.6	13.3 ± 1.6	11.2 ± 2.2	7.0 ± 4.9	2.3 ± 4.1

CONCLUSION: In the present study the SCFE-CO_2 extract of *Nigella sativa* L. seeds exhibited effective antibacterial activity against ESBL *Escherichia coli* than the conventional solvent extracts and the preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phenols, tannins, glycosides, steroids and terpenoids. Thus, *Nigella sativa* L. seeds can be further analyzed for its bioactive phyto-constituents for the development of natural remedies against multidrug-resistant bacterial infections.

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