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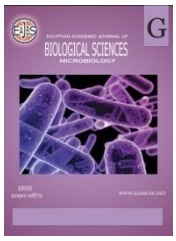

Antibacterial Activity of Some Essential Plant Oils Against Opportunistic Pathogenic Bacteria

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ABSTRACT

Corynebacterium stationis is a facultative anaerobic gram-positive bacterium. It is an opportunistic pathogen that is one of more than 42 *Corynebacterium* species and subspecies found in humans, the majority of which have been linked to opportunistic illness. Human excrement, blood, and seawater are commonly used to isolate it. Mastitis in cattle is caused by the bacteria *C. stationis* and is characterized by inflammation of the mammary gland. Milk from the infected cows is contaminated with this bacterium, which makes it unsafe for human consumption and causes bacterial diseases in humans. In this study, the antimicrobial activity of some essential plant oils was investigated against *C. stationis* bacteria isolated from human feces. One bacterial isolate was identified biochemically, then characterized by 16S rRNA genotyping, and was designated as *Corynebacterium stationis* E. Antibiotic susceptibility testing showed resistance of *C. stationis* E to three antibiotics (erythromycin, clindamycin and azithromycin). Thirteen Egyptian essential plant oils were screened for their antibacterial activity; the result showed that the efficacy of black seed oil and rosemary oil against *C. stationis* E had an inhibition zone of 18.00 ±1.3 mm. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of black seed and rosemary oils against *C. stationis* E were found to be 19.5 mg/L and 39 mg/L, respectively. The killing times of *C. stationis* E upon growth with 200 mg/L of black seed and rosemary were 6 and 7 hours, respectively.

INTRODUCTION

"club-shaped" Gram-positive, medically relevant species of the genus *Corynebacterium* and the "irregular" Gram-positive coryneform mostly but not exclusively from the suborder Micrococcineae. Those coryneforms were chosen because they phenotypically most closely resemble Corynebacterium species and can be difficult to distinguish from members of that genus. Coryneforms described here are rare, opportunistic human pathogens, and they are Gram-positive bacilli or coccobacilli. Nearly all are catalase-positive, and they express a range of pigments and metabolic processes (Kathryn Bernard, 2012).

These coryneform bacteria are increasingly being recognised as causing opportunistic disease in specific circumstances, such as in patients who are immunocompromised, have prosthetic devices, or have spent extended periods of time in hospitals or nursing homes.

Corynebacterium stationis is a gram-positive, facultative anaerobic organism. It is an opportunistic pathogen that represents one of more than 42 species and subspecies of Corynebacterium recovered from humans, most of which have been associated with opportunistic disease (Bernard, 2016). It is usually isolated from human stool, blood, and seawater (Kathryn Bernard, 2012). Mastitis in bovines is caused by C. stationis and is characterized by inflammation of the mammary gland. It is a complex and costly disease in dairy herds. The bacterial contamination of milk from the affected cows makes it unhealthy for human consumption and causes human bacterial infections (León-Galván *et al.,* 2015). Interestingly, *C. stationis* is resistant to some commonly used antibiotics, such as penicillin and clindamycin (León-Galván *et al.,* 2015). Despite the organism's prevalence, only a few papers dealing with this opportunistic pathogen have been published worldwide, and none have produced any reliable alternative therapy to treat these infections. Accordingly, we have designed this study to isolate this bacterium from human clinical specimens and to shed light on the efficacy of using the essential oils of some of the Egyptian native medicinal herbs as a safe alternative treatment.

MATERIALS AND METHODS

1. Sample Collection and Processing:

Twenty-two stool specimens were collected from a private laboratory at Benha, Egypt, and inserted into nutrient broth tubes as a transport medium, then transported, according to Murray Baron Baron and Ellen Jo (2007), under aseptic conditions to the microbiology laboratory at the Faculty of Science at Benha University, Egypt, where the study was carried out.

2. Isolation and Cultivation of Bacteria:

By using a micropipette, 100 µl of nutrient broth containing a stool sample was inoculated on trypticase soy agar enriched with 10% human blood. The initial growth of bacteria was obtained after 24 h of culture in anaerobic conditions at 37 °C.2.3. Biochemical identification and 16S rRNA gene sequencing

Only five out of the 22 collected samples, grown anaerobically, showed bacterial growth on the blood-selective media. The biochemical properties of these five grown bacteria identified a single type of anaerobic bacterial species. These bacterial species were found to be polymorphic (gram-positive, non-motile bacilli are commonly found in short rods, occur singly, in pairs, and in "V" forms). The biochemical profile of this anaerobic bacteria was positive (catalase, urease, glucose, fructose, nitrate reduction) and negative (oxidase, mannitol, trehalose). This profile was consistent with the bacteria formerly known as Corynebacterium stationis.

The biochemical identification was confirmed using partial 16S rRNA gene sequencing. Universal 16s rRNA primers 8F (50 -AG AGTTTGATCCTGGCTCAG-30) and 1492R (50 -GACGGGCGGTGTGTRC A-30) (Turner *et al.,* 1999) were used to amplify the 16S rRNA of anaerobic bacteria. Sanger sequences were generated at the Sigma Genomics Core Facility (Cairo, Egypt) using an ABI PRISM 3730xl DNA Analyzer (Life Technologies Corporation, CA). Sequence similarity searches were conducted using the 98 BLASTn search at the NCBI website, and phylogenetic and molecular evolutionary analyses were conducted using MEGA software version X (Kumar *et al.,* 2018).

3.Isolates Accession Numbers:

Corynebacterium stationis E was deposited in the Genebank database and was assigned the accession number MW543939.1.

4. Antibiotic Susceptibility Test:

Antibiotic susceptibility testing was performed using the disc diffusion method (Biemer, 1973) for the following antibiotics

(Oxoid, UK); The antibiotics tested in this study include Amoxicillin (25µg), Tetracycline (30µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Norfloxacin (10 µg), vancomycin (30µg), Gentamycin (10 µg), Rifampin (5µg), Linezolid (30µg), Ceftazidime (30µg), Cefotaxime (30µg), Clindamycin (2 µg), Azithromycin (15 µg), Erythromycin (15 µg)(Wayne, 2019).

Table 1. The antibiotic discs have been involved in our study.

Antibiotic agent	Symbol	Concentration (µg/disk)
Azithromycin	AZM	15
Erythromycin	E	15
Clindamycin	DA	2
Ceftazidime	CAZ	30
Cefotaxime	CTX	30
Linezolid	LZD	30
Chloramphenicol	C	30
Rifampin	RA	5
Vancomycin	VA	30
Amoxicillin	AX	25
Tetracyclin	TEC	30
Ciprofloxacin	CIP	5
Norfloxacin	NOR	10
Gentamycin	GEN	10

5. Antibacterial Activity of Some Plant Oils:

Agar well diffusion method (Magaldi *et al.,* 2004; Valgas *et al.,* 2007; Balouiri *et al.,* 2016) was used to evaluate the antimicrobial activity of the following plant oils: tea tree, cinnamon, rosemary, cactus, lavender, basil, lemon, thyme, parsley, almond, sage, sage black seed, and lupine. The crude oils were purchased from the commercial markets in Egypt. The nutrient agar plates were inoculated with 100 l of actively growing bacteria. A sterile cork was used to punch holes with a diameter of 6–8 mm, and 10 µl of the tested oils (200 mg/L) were introduced into the well. The inoculated agar plates were incubated at 37 ºC for 24 h. The inhibition zones' diameters were measured in millimetres (mm).

6. Mics and Mbcs of Black Seed and Rosemary Oils:

The MICs of black seed and rosemary oils were determined using a broth assay (Klannik *et al.,* 2010) in 96-well microtiter plates (Sigma Aldrich, USA). Fresh cultures of the tested isolates were prepared in nutrient broth; inoculate of concentrations between 2 × 107 cfu/mL were used. A two-fold dilution series of black seed and rosemary oils were prepared in 1% DMSO to yield final concentrations ranging from 5000 mg/L to 9.7 mg/L. Chloramphenicol was employed as a positive control. After 18 and 48 hours for *Corynebacterium stationis*, the optical density at 600 nm was measured with a microplate reader (680 XR reader, Bio-Rad). Bacterial growth was confirmed by adding 10 µl of a sterile 0.5% aqueous solution of triphenyl tetrazolium chloride (TTC, Sigma–Aldrich) and incubating at 36 ºC for 30 min. The viable bacterial cells reduced the yellow TTC to pink/ red 1, 3, and 5-triphenyl formazan (TPF). All assays were performed in triplicate. Subcultured on Blood Agar media, streaks were taken from the two lowest concentrations of each oil concentration exhibiting invisible growth. The plates were incubated at 37 ºC for 24-48 h, then inspected for bacterial growth in the areas corresponding to both oils. MBC was taken as the concentration of the oil that did not exhibit any bacterial growth.

7. Time-Kill Kinetics:

This experiment was performed as described previously (May *et al.*, 2000). Prior to the experiment, bacteria were incubated on nutrient broth (Oxoid: England, CM0003) for 90 min at 37 ºC to ensure that all the bacteria were in the logarithmic growth phase. The initial bacterial concentration was measured as cfu/mL. The isolate was inoculated into three flasks, one as a growth control and one for each type of oil. All flasks were incubated at 37 ºC while shaking at 150 rpm. Aliquots were taken at 0, 1, 2, 3, 4, 6, and 7, and viable colony counts on blood agar were calculated as cfu/ml.

RESULTS AND DISCUSSION

1. Isolation, Biochemical Characterization and 16S rRNA typing of *Corynebacterium stationis*:

The collected samples were grown anaerobically; five out of the 22 stool samples showed bacterial growth on the blood-selective media. Biochemical properties identified one type of bacterial species. This species was found to be non-motile, gram-positive bacilli that are found in short rods and occur singly, in pairs, and in club shapes. The biochemical profile of this anaerobic bacteria was consistent with what was previously known as Corynebacterium *stationis* (Table 2).

BlASTn alignments and phylogenetic tree analysis (Fig. 1) of the assembled 16S rRNA gene sequences showed the highest similarity with the previously partially sequenced 16S rRNA of *Corynebacterium stationis* on the NCBI website. Corynebacterium stationis strain E was given to the bacteria.

Table 2. Morphological, cultural and biochemical characteristics of the bacterial isolate

Isolate no	Morphological & cultural characters				Biochemical tests								Presumptive isolate
	Gram stain	Shape	Motility	Blood hemolysis	catalase	Oxidase	urease	nitrate reduction	Glucose	Fructose	Mannitol	Trehalose	
101	(+)	Bacilli	Non motile	No hemolysis	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	Corynebacterium stationis

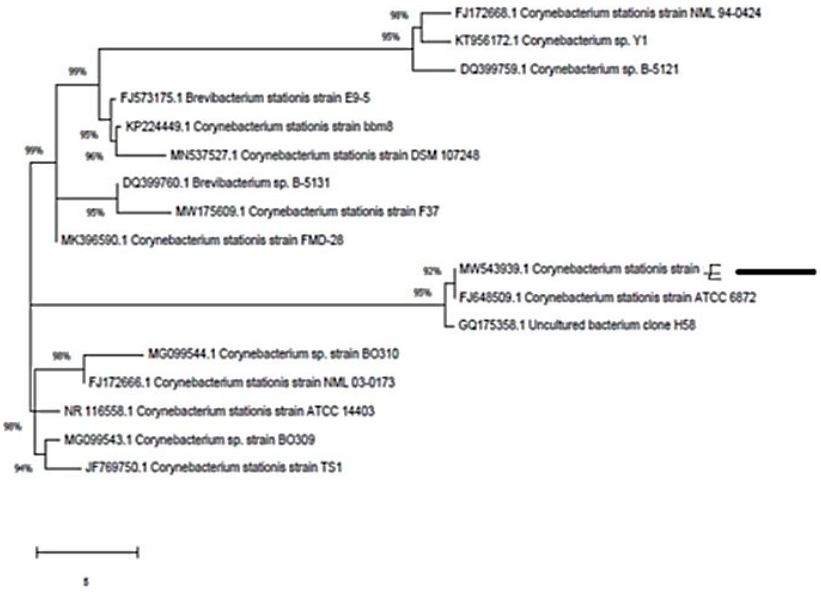

Fig. 1. Molecular phylogenetic analysis of *Corynebacterium stationis* strain E by Maximum Likeliho Model of MEGA 10.0 package

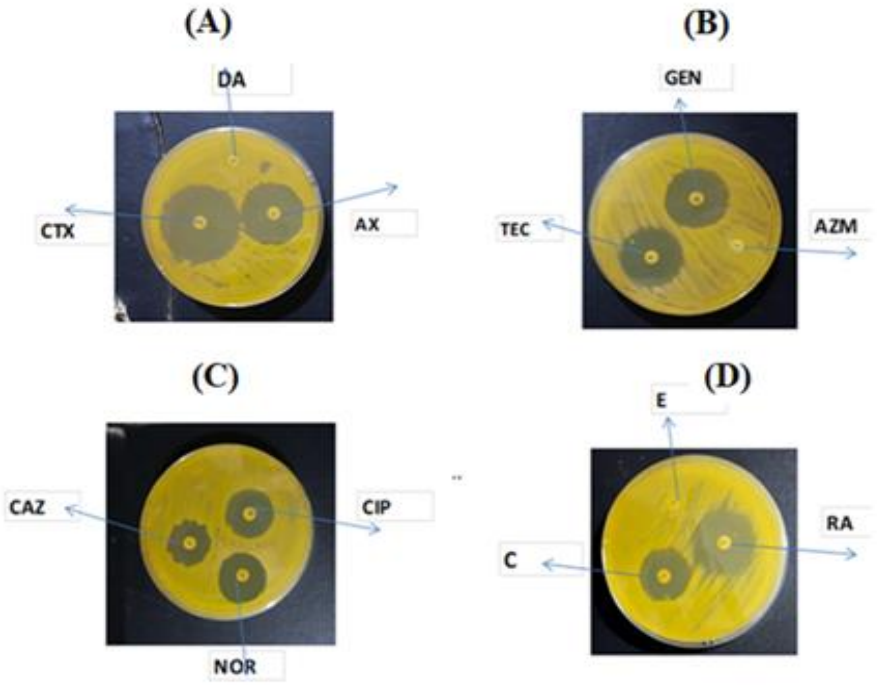

Fig. 2. Sensitivity test of different antibiotics on *Corynebacterium stationis* E

2. Antibiotic Susceptibility Testing:

Antibiotic resistance to *Corynebacterium stationis* E was found to be 21.4% based on qualitative antibiogram results (Table 3). All of the *C. stationis* isolates were resistant to three antibiotics (Clindamycin, Erythromycin, and Azithromycin) (Fig. 2). This is in contrast to a previous study that showed no resistance to all antibiotics tested.

Table 3. Antimicrobial susceptibility test against isolated bacteria

Antibiotic group	Antimicrobial agent	Symbol	Concentration (µg/disk)	*Corynebacterium stationis* E	
Macrolides	Azithromycin	AZM	15	4	R
	Erythromycin	E	15	4	R
Fluoroquinolones	Ciprofloxacin	CIP	5	21	S
	Norfloxacin	NOR	10	19	S
Cephalosporines	Ceftazidime	CAZ	30	15	I
	Cefotaxime	CTX	30	23	S
Lincosamides	Clindamycin	DA	2	4	R
Oxazolidinone	Linezolid	LZD	30	21	S
Chloramphenicol	Chloramphenicol	C	30	18	S
Rifamycins	Rifampin	RA	5	17	S
Glycopeptide	Vancomycin	VA	30	25	S
β-lactam	Amoxicillin	AX	25	19	S
Tetracycline	Tetracyclin	TEC	30	17	S
Aminoglycoside	Gentamycin	GEN	10	18	S

* Indicates resistance (R), intermediate (I), and susceptibility (S). Ax=Amoxicillin (25 µg),Tec= Tetracycline(30 µg), C= Chloramphenicol (30 µg), Cip=Ciprofloxacin (5 µg), Nor= Norfloxacin (10 µg), VA= vancomycin (30 µg), GEN=Gentamycin (10 µg), RA=,Rifampin (5 µg), LZD=inezolid (30 µg), CAZ= Ceftazidime (30 µg), CTX=Cefotaxime (30 µg), DA= Clindamycin (2 µg), AZM= Azithromycin (15 µg), E=Erythromycin (15 µg).

3. Antibacterial activity of some plant oils against the isolated bacteria

Corynebacterium stationis E showed resistance to 11 out of 13 selected essential plant oils (Table 4). Rosemary and black seed were the only two oils that were effective against this tested bacterium. With an inhibition zone of 18.00 1.3 mm, black seed oil outperformed rosemary against *Corynebacterium stationis* E. Unfortunately, there was no previous published paper to compare with our results.

The evolution of multidrug-resistant microorganisms is accelerating at an alarming rate. Antibiotics may be acquired straight from drugstores, which is one of the main reasons. According to the Centers for Disease Control and Prevention, at least 2 million people in the United States get infected with antibiotic-resistant bacteria every year, with at least 23,000 of them dying. Effect of Cefotaxime, Clindamycin, Amoxicillin (A), Azithromycin, Gentamycin, Tetracycline (B), Ceftazidime, Ciprofloxacin, Norfloxacin (C), Cholramphenicol, Rifampin, and Erythromycin (D) on *Corynebacterium stationis* E

Table 4. Antimicrobial activity of different plant oils on the bacterial isolate. Each value is the mean of three readings (mm) standard error (SE).

Bacteria	Mean diameter of Inhibition zone(mm) /original zone diameter 5mm												
	Sage	Black seed	Lupine	Almond	Parsley	Thyme	Lemon	Basil	Lavender	Cactus	Rosemary	cinnamon	Tea tree
Corynebacterium stationis E	5	18.50±0.50	5	5	5	5	5	5	5	5	16.0±0.45	5	5

4. Minimum Inhibitory (MICs) and Minimum Bactericidal Concentrations (MBC):

The MIC and MBC of the most effective oils (black seed and rosemary oils) were examined to evaluate their bacteriostatic and bactericidal properties (Table 5). MBC of the most effective oils (black seed and rosemary oils) were examined to evaluate their bacteriostatic and bactericidal properties. Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of the black seed and rosemary oils against the isolated bacteria. The MICs/MBCs of black seed oil against *Corynebacterium stationis* E were found to be 19.5 mg/L, while those of rosemary oil's MICs/MBCs against Corynebacterium stationis E were 39 mg/L.

Black seed oil was more potent than Rosemary oil against *Corynebacterium stationis* E. (Fig. 3**).**

Table 5. Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of the black seed and Rosemary oils against the isolated bacteria**.**

	***Corynebacterium stationis* E**	
Time	**Black seed cfu/ml**	**Rosemary cfu/ml**
0	500	500
1	410	450
2	340	400
3	250	350
4	100	200
5	30	90
6	0	50
7	0	0

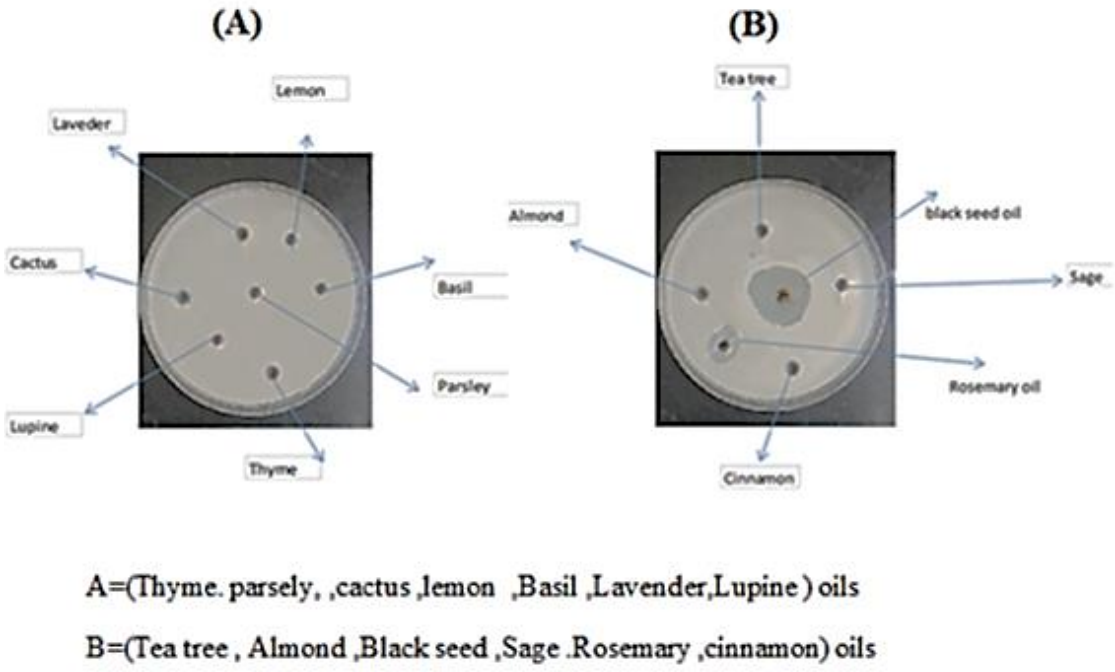

Fig. 3. Sensitivity test of 13 essential oil on *Corynebacterium stationis* E.

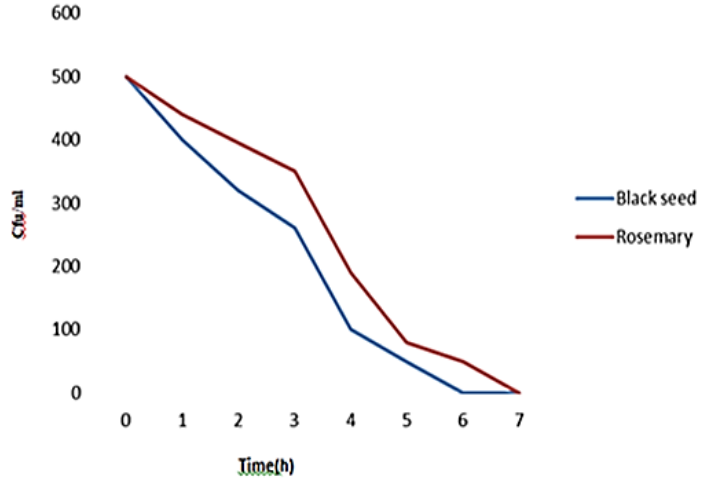

Fig. 4. Time-kill experiment of *Corynebacterium stationis* E.

5. Time-Kill Kinetics of Black Seed And Rosemary Oils:

Measurements of killing time by the black seed and Rosemary oils were made for each oil individually Table (6). Black seed oil showed greater activity than Rosemary oil against *Corynebacterium stations* strain E. Black seed oil killed this bacterial isolate after 6 hours however rosemary oil killed this bacterial isolate after 7 hours (Fig. 4).

Table 6. Killer time of *Corynebacterium stationis* E.

	Black seed oil		**Rosemary oil**	
	MIC	MBC	MIC	MBC
Corynebacterium stationis E	19.5	19.5	39	39

The relative viable count of this bacterial isolate was measured for 6 hours and calculated as cfu/ml against black seed oil and rosemary oil. Prior to the experiment, this bacterial isolate was in the logarithmic phase. Aliquots were taken at 0, 1, 2, 3, 4, 5, 6, and 7, and viable colony counts on blood agar were calculated as cfu/ml. **Conclusion:**

In this work, *Corynebacterium stationis* E was resistant to conventional antibiotics such as Azithromycin, Erythromycin, and Clindamycin. Consequently, many current trends involve exploring new antimicrobials that are both safe and effective. Therefore, this study was designed to screen the antibacterial effect of thirteen essential native oils against the opportunistic bacterium mentioned above. This study proves the efficacy of black seed oil and rosemary oil at definite concentrations as good choices for the eradication of *Corynebacterium stationis* bacteria.

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