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GC-MS and *in vitro* antibacterial potential of *Cinnamomum camphora* essential oil against some clinical antibiotic-resistant bacterial isolates

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Abstract. – OBJECTIVE: Antibiotic resistance is increasing alarmingly in all parts of the world. *Cinnamomum camphora* (Linn.) Presl (*C. camphora*) is one of the earliest herbal remedies still in use today in traditional medicine. This study aimed to analyze the component of *C. camphora* grown widely in Saudi Arabia (Qassim region) using GC-MS. Also, this study evaluates the *in vitro* antibacterial properties of *C. camphora* against certain clinical bacteria obtained from hospitals, including multi-drug resistant pathogens.

MATERIALS AND METHODS: Leaves of *C. camphora* tree were collected and essential oil was extracted for this study. The extract was subjected to GC-MS analysis. Eight clinical antibiotic-resistant pathogens were used in this study for the following assays: antibiotics susceptibility assay, determination of minimum inhibitory concentration (MIC), determination of minimum bactericidal concentration (MBC) and MIC index (MBC/MIC).

RESULTS: The results show that the main components of the essential oil (EO) from the leaves of *C. camphora* were Eucalyptol. The EO had good antibacterial activity against eight clinical antibiotic-resistant pathogens, namely, *Pseudomonas aeruginosa, Acinetobacter baumannii* (two strains), *Klebsiella pneumonia* (two strains), *Escherichia coli* (one strain), *Staphylococcus aureus* (two strains).

CONCLUSIONS: These findings may lead to a more complete knowledge of this aromatic plant's antibacterial action against antibiotic-resistant pathogens (*in vitro*).

Key Words:
Cinnamomum camphora, Eucalyptol, GC-MS, Antibacterial potential, Antibiotic-resistant bacteria, MIC, MBC.

Introduction

Infectious diseases are the leading causes of morbidity and mortality worldwide, and in recent years, infection by antibiotic-resistant microorganisms has become more widespread[1]. Antibiotic resistance in bacteria has developed from the overuse and abuse of antibiotics in medical treatments, resulting in the buildup of antibiotic residues in hospital effluent, domestic effluent, wastewater treatment plants, and sewage sludge[2]. This critical situation requires the innovation of a new series of antibacterial drugs with minimal side effects on humans and the environment. One of the renewable and tremendously diverse sources of antimicrobial agents is medicinal plants[3,4] and nearly 80% of the world's entire population, particularly in third world countries, is estimated to rely on traditional medicine for their health care needs[5].

Plants produce a wide range of secondary metabolites, many of which have antimicrobial properties, including alkaloids, phenols, polyphenols, flavonoids, tannins, quinones, coumarins, terpenes, polypeptides, lectins, and saponins. These

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secondary molecules are involved in antagonistic relationships between plants and other organisms and their activities, with various modes of action on prokaryotic cells[6,7].

Cinnamomum camphora (*C. camphora*), commonly known as the camphor tree, is an evergreen tree with a distinguished aromatic scent. The genus *Cinnamomum* belongs to the family Lauraceae, which includes up to 250 species[8]. Camphore tree has a long history in traditional medicine and has been used as an analgesic, stimulant and tonic, antispasmodic and muscle pain, anti-toothache, anti-cold and lung diseases, sedative, anti-rheumatic, anthelmintic, cardiotonic, carminative, and many other things[8,9].

Scientific studies revealed that the essential oils extracted from *C. camphora* (*C. camphora*) have significant *in vitro* antibacterial activities against an array of microorganisms such as various Gram-positive and Gram-negative bacteria[10], oral microorganisms[11], food related bacteria[12], *E. coli* biofilm formation[13] and inhibit the expression of quorum-sensing-regulated virulence genes[14]. The present study aimed to evaluate the antibacterial properties (*in vitro*) of *C. camphora* grown widely in Saudi Arabia (Qassim region) against certain clinical bacteria obtained from hospitals, including multi-drug resistant pathogens.

Materials and Methods

Plant Material

Approximately 300 g powder from the leaves of plant material of *Cinnamomum camphora* tree, was collected in May 2020 from Al-Rass province, Kingdom of Saudi Arabia. The specimens were identified, and a voucher specimen of this plant material was deposited in the herbarium of the College of Sciences and Arts at Al-Rass, Qassim University (QU), Saudi Arabia. The plant specimens were authenticated by Prof. Gamal E. Elghazali, College of Science and Arts, Al Rass, Qassim University, Saudi Arabia.

Plant Extraction

The leaves of the plant samples were washed by flushing firstly with tap water and secondly with distilled water and dried under shade for 3 days in a dark place at room temperature. The dry leaves of the plant were grounded to a fine powder with a commercial blender, then percolated in warm distilled water and left for overnight at room temperature (25-35°C)[15-18]. The simple distillation system and separating funnel used for extracted essential oils from percolate, which was collected in a good vials glass sample tube. The 300 grams of the leaves produced 18 mL (3.6% w/v).

GC-MS Analysis

GC-MS analysis was conducted using A Shimadzu QP ultra plus 2010. Column: Thermo (TR5MS), (25 m x 250 µm i.d.), with the following conditions: HE (32 kPa), 1.6 mL/min.; initial inlet temperature 50°C, after a 16 s delay injection volume, 1 µL of the oil (LVI) liner velocity, oven temperature program, 50°C/min. Mass detector: Shimadzu 2010 ultra plus. The MS instrument transfer line temperature was 250°C; split ratio 1:40 MS Program; Library used: NIST Version-2015; Mass scan: (m/z): 30-450; Total MS running time: 20 min. The individual constituents showed by GC were identified by comparing their MS with standard compounds of the NIST library[1,3].

Microorganisms

Eight clinical antibiotic-resistant pathogens were collected from Buraidah Central Hospital in Saudi Arabia, consisting of six Gram-negative and two Gram-positive bacterial strains. Identified samples were provided on Petri plates anonymously (without identifying patients) using the usual protocols described by Cheesbrough[19]. Each sample was assigned a voucher number, preserved, and documented at the medical microbiology lab, Buraidah Central Hospital. *Pseudomonas aeruginosa* 89WS (wound swab), *Acinetobacter baumannii* 28TA (trachea aspiration), *Acinetobacter baumannii* 21SP (sputum), *Klebsiella pneumonia* 192U (Urine), *Klebsiella pneumonia* 81TA (trachea aspiration), *Escherichia coli* 324U (urine), *Staphylococcus aureus* 24ABS (abscess) and *Staphylococcus aureus* 418BLD (blood) were the clinical bacteria provided and used in this investigation.

Antibiotics Susceptibility Assay

The antibiotic susceptibility profile of isolated clinical pathogenic bacterial strains was determined using the referenced broth microdilution method as recommended by the Clinical and Laboratory Standards Institute[20] and the MicroScan WalkAway system (96 plus, Bekman Coulter, Brea, CA, USA) in order to determine the isolated clinical strains' possible susceptibility or resistance to antibiotics. Antibiotics used include the majority of commonly used antibiotics from

various antibiotic classes. To summarize, an overnight culture broth of bacterial isolates was adjusted to 0.5 McFarland (BSS 0.5, Roth, Germany), the adjusted bacterial suspension was inoculated into the panel, and the desired antibiotics were also loaded. The seeded panel was incubated in the MicroScan WalkAway system for 24 hours for Gram-positive bacteria and 8 hours for Gram-negative bacteria. The MicroScan WalkAway system read and expressed bacteria as resistant or sensitive to antibiotics automatically. The machine's automated results sheets were collected, revealing the susceptibility of microorganisms to antibiotics.

Determination of Minimum Inhibitory Concentration (MIC)

C. camphora essential oil's minimum inhibitory concentration (MIC) against eight bacterial strains was evaluated using the broth microdilution technique described by Wang et al[13], with minor modifications. Bacteria were cultured in Mueller-Hinton (MH) broth at 37°C, shaking, until the exponential growth phase was attained. A diluted bacterial solution was applied to a 96-well microtiter plate to achieve a final concentration of 1×10^5 CFU/mL using a turbidity comparator (DensiCHEKplus, BioMerieux SA, France). Serial twofold dilutions of the essential oil were made and applied to each well (200 µL) in order to achieve a final concentration of between 0.52 and 50% (v/v). To increase the solubility of the essential oil, all wells included 10% DMSO (v/v). Additionally, there was a positive control (Chloramphenicol 2.5 mg/mL), a solvent control (test bacteria and MH broth containing 10% DMSO), a bacterial control (test bacteria and MH broth), a blank control (MH broth comprising 10% DMSO and matched essential oil concentrations), a blank solute control (MH broth comprising 10% DMSO), and a blank medium (MH broth). All plates were then incubated at 37°C for 24 hours, and growth was determined using a Bio-Rad SmartSpec 3000 Spectrophotometer (Bio-Rad, Hercules, CA, USA). The minimum inhibitory concentration (MIC) was defined as the essential oil concentration at which no visible growth was detected (optical density at 600 nm (OD_{600} nm) $p\leq 0.05$).

Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) of *C. camphora* essential oil against the investigated bacterial strains was determined using the procedure described by Kwieciński et al[21]. 10 µL of the test solutions were withdrawn from the wells representing the MIC and the three next highest concentrations and plated on MH agar. The plates were then incubated at 37°C for 24 hours. Finally, the colonies on the agar plate were counted. The MBC concentration was determined as the lowest at which no colonies were found. Three replicates were used to determine the MBC value.

MIC Index

To assess if the plant extract has bactericidal or bacteriostatic characteristics, the MIC index (MBC/MIC) was determined. When the MBC/MIC ratio is less than or equal to four, the extract is bactericidal; when the MBC/MIC ratio is more than four, the extract is bacteriostatic[22].

Results

GC-MS Analysis

In *Cinnamomum camphora*, GC-MS analysis revealed the presence of forty chemicals identified as essential oil compounds. These essential oil compounds were identified using the retention time, molecular formula, compound name, and mass spectrum. Twenty-five essential chemical ingredients were identified (Table I), Eucalyptol (62.13 2,6,6-trimethylbicyclo (3,1,1) hept-2-ene (12.20%), D-Limonene (6.42%), Bicyclo [3.1.1] heptane-3-ol, 6,6-dimethyl-2-methylene (2.18%), alpha-Terpineol (1.51%) and 3-Cyclohexene-1-ol ، 4-methyl-1- (1-methyl ethyl) - (R) (1.22%). These components make up (85.67%) of the *Cinnamomum camphora* oil's total relative content. Eucalyptus oil, which is rich in *Cinnamomum camphora*, was also found to be a good source of these compounds in this study.

Eucalyptol (molecular weight: 154.25) is an essential oil found in significant amounts in a variety of plants and is generally used in cosmetics to increase drug penetration through the body, as a nasal decongestant and anti-cough agent, in aromatherapy, and in dentistry bronchitis, sinusitis, and chronic rhinitis have already been handled with eucalyptol, as well as asthma. These actions relate to an anti-infection strategic approach[18,23].

Antibiotic Profile

The *in vitro* antibiotic profile of the tested bacterial strains revealed that all of these pathogens can be classified as multi-drug resistant and pre-

Table I. Chemical composition of the essential oil in Cinnamomum camphora leaves analyzed by GC-MS.

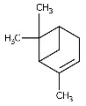
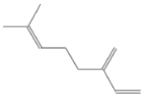
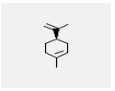
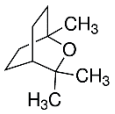
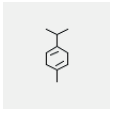
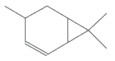
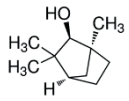
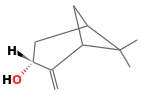
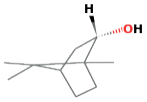
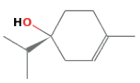
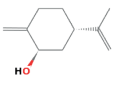
Peak	Identification	Area (w/w)%	RT	M wt.	Structure
1	2,6,6-trimethylbicyclo (3,1,1) hept-2-ene	12.20	4.716	$C_{10}H_{16}$	
2	Camphor	0.30	5.425	$C_{10}H_{16}O$	
3	Beta-myrcene	0.40	5.579	$C_{10}H_{16}$	
4	D-Limonene	6.42	6.317	$C_{10}H_{16}$	
5	Eucalyptol	62.13	6.424	$C_{10}H_{18}O$	
6	Gamma-Terpinene	0.28	6.748	$C_{10}H_{16}$	
7	(+)-4-Carene	0.67	7.236	$C_{10}H_{16}$	
8	Bicyclo[2.2.1]heptan-2-ol, 2,3,3-trimethyl	0.29	7.875	$C_{10}H_{18}O$	
9	Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene	2.18	8.256	$C_{10}H_{16}O$	
10	Pinocarvone	0.59	8.622	$C_{10}H_{14}O$	
11	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-	0.35	8.763	$C_{10}H_{18}O$	
12	3-Cyclohexen-1-ol ‹ 4-methyl-1- (1-methylethyl)-, (R) -	1.22	8.680	$C_{10}H_{18}O$	
13	trans-p-Mentha-1(7),8-dien-2-ol	0.64	8.970	$C_{10}H_{16}O$	

Table I. ***(Continued).*** Chemical composition of the essential oil in Cinnamomum camphora leaves analyzed by GC-MS.

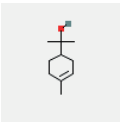
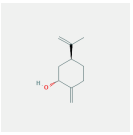
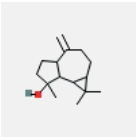
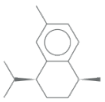
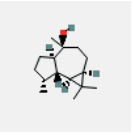
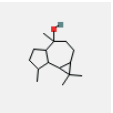
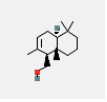
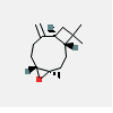
Peak	Identification	Area (w/w)%	RT	M wt.	Structure
14	alpha-Terpineol	1.51	9.097	$C_{10}H_{18}O$	
15	cis-p-Mentha-1(7),8-dien-2-ol	0.60	9.636	$C_{10}H_{16}O$	
16	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aalpha,4aalpha,7beta,7abeta,7balpha)]-	1.09	12.675	$C_{15}H_{24}O$	
17	Alloaromadendrene	0.34	12.981	$C_{15}H_{24}$	
18	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)	0.32	13.686	$C_{15}H_{24}$	
19	Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-	0-34	13.779	$C_{15}H_{22}$	
20	Epiglobulol	0.41	14.356	$C_{15}H_{26}O$	
21	Globulol	0.20	14.458	$C_{15}H_{26}O$	
22	(-)-Globulol	2.64	14.691	$C_{15}H_{26}O$	
23	Ledol	0.66	14.810	$C_{15}H_{26}O$	
24	Drimenol	0.23	14.940	$C_{15}H_{26}O$	
25	Caryophyllene oxide	0.22	15.010	$C_{15}H_{24}O$	

Table II. The antibiotics susceptibility profile of the clinical bacterial strains.

Clinical isolates	Gram	Source	Resistant to:
Staphylococcus aureus 24ABS	+	Abscess	Ampicillin and Penicillin (Beta-lactamase positive)
Staphylococcus aureus 418BLD	+	Blood	Ampicillin and Penicillin (Beta-lactamase positive)
Pseudomonas aeruginosa 89WS	-	Wound swab	Aztreonam and Pip/Tazo
Acinetobacter baumannii 28TA	-	Trachea aspiration	Amikacin, Cefepime, Ceftazidime, Ciprofloxacin, Gentamicin, Imipenem, Levofloxacin, Meropenem, Tobramycin and Trimeth/Sulfa
Acinetobacter baumannii 21SP	-	Sputum	Amikacin, Amp/Sulbactam, Cefepime, Ceftazidime, Ciprofloxacin, Gentamicin, Imipenem, Levofloxacin, Meropenem, Tobramycin and Trimeth/Sulfa
Klebsiella pneumonia 192U	-	Urine	Amikacin, Amox/K Clav, Amp/Sulbactam, Ampicillin, Cefazolin, Cefepime, Cefuroxime, Ciprofloxacin, Ertapenem, Gentamicin, Imipenem, Leovofloxacin, Norfloxacin, Pip/Tazo, Tobramycin and Trimeth/Sulfa.
Klebsiella pneumonia 81TA	-	Trachea aspiration	Amikacin, Amox/K Clav, Amp/Sulbactam, Ampicillin, Aztreonam, Cefepime, Cefotaxime, Cefoxitin, Ceftazidime, Cefuroxime, Ciprofloxacin, Ertapenem, Gentamicin, Leovofloxacin, Meropenem, Moxifloxacin, Pip/Tazo, Tobramycin and Trimeth/Sulfa.
Escherichia coli 324U	-	Urine	Ciprofloxacin, Levofloxacin and Tigecycline

sented a health risk (Table II). In the current study, beta-lactamase was detected in both Gram-positive strains (S*taphylococcus aureus* 24ABS and 418BLD). Globally, beta-lactamase bacteria have been shown to have a substantial impact on antibiotic treatment and will continue to be the primary factor in clinically significant resistance to penicillins and cephalosporins [24]. Clearly, the four *Acinetobacter baumannii* and *Klebsiella pneumonia* bacterial strains shown resistance to 10-19 antibiotics, with some strains being very resistant.

Antibacterial Activities

C. camphora essential oil was examined for *in vitro* antibacterial activity against the mentioned resistant strains, and the MIC, MBC, and MIC index values were computed as shown in (Table III). The essential oil was shown to be effective against all bacterial strains tested. The MIC ranged between 1.04 and 6.25% (v/v), while the MBC values were between 3.13 and 12.5% (v/v), with an MIC index of 2-3.

Discussion

In qualitative analysis, the GC-MS can provide more reliable data. Long-chain, branched-chain hydrocarbons, alcohols, acids, esters, and also other volatile matter constituents can be identified using gas chromatography with a mass detector, or GC-MS[18]. Twenty-five essential chemical ingredients were identified and out of these compounds, eucalyptus oil was rich in *Cinnamomum camphora* in this study.

Multidrug–resistant bacteria are those that exhibit *in vitro* resistance to three or more antibiotic classes or that develop extended-spectrum beta-lactamases or carbapenemases[25]. Therefore, seven of the eight bacteria were classed as multidrug resistant strains, while just one (*Pseudomonas aeruginosa* 89WS) was classified as non-multidrug resistant. The four *Acinetobacter baumannii* and *Klebsiella pneumonia* bacterial strains shown resistance to 10-19 antibiotics, with some strains being very resistant. In developed countries over the last several decades, a unique hypervirulent clinical strain of *Klebsiella pneumonia* has steadily evolved into one capable of causing community-acquired, invasive, and life-threatening infection in young and healthy persons[26]. As well, *Acinetobacter baumannii* has emerged as a significant nosocomial pathogen, creating a substantial health concern to immunocompromised individuals and because this bacterium is extremely capable of acquiring antibiotic resistance, it has attracted international interest[27].

A low MIC value indicates that the substance has a strong antibacterial activity[28]. *Staphylococcus aureus* 418BLD and *Acinetobacter bau-*

Table III. MIC, MBC and MIC index of *C. camphora* essential oil against the investigated bacterial strains.

Microorganism	MIC (%)	MBC (%)	MIC index
Staphylococcus aureus 24ABS	6.25	12.5	2
Staphylococcus aureus 418BLD	1.04	3.13	3
Pseudomonas aeruginosa 89WS	6.25	12.5	2
Acinetobacter baumannii 28TA	1.04	3.13	3
Acinetobacter baumannii 21SP	6.25	12.5	2
Klebsiella pneumonia 192U	6.25	12.5	2
Klebsiella pneumonia 81TA	6.25	12.5	2
Escherichia coli 324U	6.25	12.5	2

mannii 28TA were the most sensitive strains (MIC=1.04 and MBC=3.13%, respectively). The fact that it was effective against Gram-positive and Gram-negative bacteria demonstrates its broad-spectrum potency. Since the MIC index values were between 2 and 3, less than 4, the essential oil has bactericidal activity against all tested bacterial strains. The MIC index (MBC/MIC ratio) is an important metric that indicates a compound's bacteriostatic or bactericidal potential[29]. Earlier studies appear to confirm our findings, *C. camphora* EO had potent *in vitro* antibacterial activity against all bacteria tested, including *Chromobacterium violaceum, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*, with MIC values ranging from 2.5 to 10% (v/v)[14]. It was also reported that *C. camphora* essential oil was highly effective against *Enterococcus faecalis*, *Salmonella gallinarum, Escherichia coli, Bacillus subtilis* and MRSA *Staphylococcus aureus*. The MIC and MBC ratios were between 0.8 mg/mL and 3.2 mg/mL, respectively[30]. The EO of *C. camphora* was shown to have broad antimicrobial effects (*in vitro*) against Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*), Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), yeasts (*Hansenula anomala* and *Saccharomy cescerevisiae*), and two moulds (*Aspergillus niger* and *Chaetomium globo*) and MIC values were in the range of 31.25 to 125 µg/ml[31].

Conclusions

These findings may lead to a more complete knowledge of this aromatic plant's antibacterial action (*in vitro*) against antibiotic-resistant pathogens. The essential oil (EO) of this plant could be a source of effective natural supplements to treat infections caused by multi-drug resistant bacteria in both community and hospital settings. The findings also suggest that the EO of this plant could be utilized in combination therapy to treat illnesses caused by the bacteria examined. However, *in vivo* antibacterial evaluation using experimental animals and the toxicity tests of the active ingredients (Eucalyptol in particular), and pharmacokinetic features must be investigated.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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