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Antibacterial Activity Of Some Medicinal Plants Oils Against Multiresistant Acinetobacter Strains Isolated From Nosocomial infections.

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ABSTRACT

A big problem in intensive care units (ICU) are caused by antibiotic-resistant bacterial nosocomial infections. Acinetobacter is among the most challenging bacterial pathogens and to help in formulating antibiotic policy for better management of patients with bacterial diseases.The data showed that 93.3% of bacterial isolates were resistant to ampicillin while 80% and 66.7% of bacterial isolates were resistant to ceftazidime and sulphamethoxazole/trimethoprim, respectively. Identification of multi-resistant isolates according to morphological and biochemical characteristics, Acinetobacter baumannii found to be the most frequent pathogen representing 66.7% followed by Acinetobacter calcoaceticus and Acinetobacter lwoffii with 20% and 13.3% percentages, respectively. The main objective of this study was studying the susceptibility of multi-drug resistant isolates to different ten essential oils derived from different parts of ten medicinal plant species traditionally used in Egyptian folk medicine. The essential oils of Clove, Thyme and Rosemary were the most active oils respectively. Followed by Marjoram, Black seed, Lemongrass, Fennel, Peppermint, Chamomile, and Anise respectively. The identification of the most frequent and multi-drug resistant Acinetobacter baumannii isolate (5) was confirmed by using 16S rRNA gene.

Keywords: Intensive care units, Antibiotic resistance; Essential oils; Antimicrobial activity; Acinetobacter sp.

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INTRODUCTION

Nosocomial infections (NIs) are frequent complications of hospitalizations. The quality of healthcare and a principal source of adverse outcomes are critical problems, which have been affected by nosocomial [1].

Currently, the clinicians are facing the most challenging bacterial pathogens which are Acinetobacter species. Acinetobacter spp.demonstrate high rates of resistance to multiple antimicrobial agents [2]. A. baumannii, A. calcoaceticus, A. haemolyticus and A. lwoffiiare are the most important species in clinical practices [3].

Acinetobacter spp. cause a wide range of healthcare-associated infections such as ventilator-associated pneumonia, bloodstream infections, urinary tract infections, surgical site infections, meningitis, cholangitis, peritonitis, skin and wound infections, ventriculitis, and infective endocarditis [4].

Widespread use of antimicrobials within hospitals resulted in the emergence and increase of antibiotics resistance among Acinetobacter strains, in particular, the wide use of extended-spectrum cephalosporins and quinolones. Acinetobacter species are more resistant to antimicrobial agents than other representatives from the family Enterobacteriaceae [5,6, 7and 8].

Unlike antibiotics, which are chemotherapeutic drugs used, mostly, internally to control infections with specific structures [9].Treatment of infections continues to be a problem in modern time because of severe side effects of some drugs and growing resistance to antimicrobial agents. Hence, search for newer, safer and more potent antimicrobials is a pressing need [10].

The plant essential oils are the rich source of scents and used in food preservation and aromatherapy. These possess multiple antimicrobial (antibacterial, antifungal & antiviral), anticancer and antioxidant properties [11, 12, 13 and 14].

Essential oils and extracts from aromatic plants have long been used for a wide variety of medicinal and domestic purposes. The combined effect of both their active and inactive compounds was the result of their action, several active components might have the synergistic effect [11 and 15]. Some plant extracts were more active than commercial antibiotics [16].

The activity of chamomile (Matricaria chamomilla) toward all of the Areobacter strains tested was significantly enhanced by using methanol as a solvent [17]. Oil extracted from chamomile was active in low concentrations against Streptococcus pyogenes, S. mutans, S.salivarius and S. faecalis [18].Black seed used a traditional medicine for treatment of various diseases such as skin infection [19]. The essential oil of the seeds has antibacterial effects on Gram-positive and Gram-negative bacteria [20]. Cymbopogon citratus (Lemongrass)has quite a high degree of antimicrobial activity against oral pathogen [21]. Essential oil of clove showed strong antibacterial activity against E.coli, Proteus mirabilis, P. aeruginosa and K. pneumonia [22].Ethanolic extracts of clove showed inhibitory activity against all the six food-associated bacteria[23]. Rosmarinus officinalis essential oil has immense medicinal worth for its powerful antimutagenic, antiphlogistic, antioxidant, chemopreventive and antibacterial properties [24]). Thyme has been extensively used as herbal tea, tonic, carminative, antitussive, expectorant, and antiseptic, as well as for treating colds [25]. Thyme oil considered as the potent food preservative due to its antimicrobial properties The mode action of essential oils and extracts on bacterial cells. They can degrade the cell wall, disrupt the cytoplasmic membrane, cause leakage of cellular compounds, change fatty acids and phospholipids constituents and influence the synthesis of DNA, RNA and cell protein [26 and 27].The aim of the work, Studying the antibacterial effect of some famous medicinal plants essential oils used in Egypt to introduce new alternatives without side effects against multi-drug resistant Acinetobacter infections

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MATERIALS AND METHODS

Clinical specimens:

Samples were collected during a period from May to December 2013 from Zagazig University hospitals. Under aseptic conditions, the samples were collected from patients with different infections [28]. Clinical samples were investigated to find the distribution of nosocomial pathogens in causing different opportunistic infections and their antibiotic resistance profile.

Antibiotic susceptibility tests:

Ten different antibiotics were selected for carrying out the antimicrobial susceptibility test.The antibiotic disks (Oxoid Ltd., England) were placed onto the surface of the inoculated agar medium and then plates were incubated at 30° C for 48h and the standard evaluation of inhibition zones according to [29]. The antibiotics which were tested were Amikacin 30 µg (AK), Ampicillin 10 µg (AM), Ampicillin/ sulbactame10/10µg (SAM), Ceftazidime 30 µg (CAZ), Ciprofloxacin 5 µg (CIP), Gentamicin 10 µg (GM), Imipenem 10 µg (IPM), Polymyxin B 300 µg (PB),Tetracycline 30 µg (Te) and Sulphamethoxazole/ trimethoprim 23.75/1.25 µg (SXT).

Isolation, purification, and Identification of bacterial isolates:

The inoculated and streaked MacConkey agar plates were incubated at 37^{o}C/ 24hrs. Selected bacterial isolates were streaked for several consecutive times on nutrient agar medium until pure single colonies were obtained. The purified selected isolates were identified morphological characters and biochemical tests as Gram staining, capsule staining, motility, oxidase and catalase tests according to [30 and 31].

Antibacterial activity of essential oils against the selected multi-drug resistant Acinetobacter strains:

Different concentrations of volatile oils (diluted by Tween 80) from medicinal plants shown in Table (1) were tested against the selected multiresistant Acinetobacter isolates using disc diffusion method according to [32 and 33]. By using sterilized Muller-Hinton agar medium and inoculated with the multi-resistant.The density of the bacterial suspension equivalent to that of standard barium sulfate (0.5 McFarland).Sterile filter paper discs were saturated by diluted volatile oils, the disks were allowed to dry for one hour and then placed on the surface of agar plates and incubated for 24 hours at 37ºC then, the diameter of the inhibition zone was measured including the diameter of the disk (6mm).

Table (1): Family, scientific, English and parts used from each plant in preparing essential oils:

Family	Scientific name	English name	Parts used
Asteraceae (Compositae)	Matricaria chamomilla	Chamomile	Flowers
Lamiaceae)Labiatae(	Origanum vulgare	Marjoram	Aerial parts
Lamiaceae)Labiatae(	Mentha piperita	Peppermint	Aerial parts
Lamiaceae)Labiatae(	Rosmarinus officinalis	Rosemary	Aerial parts
Lamiaceae)Labiatae(	Thymus vulgaris	Thyme	Aerial parts
Apiaceae)(Umbelliferae	Pimpinella anisum	Anise	Seeds

Apiaceae (Umbelliferae)	Foeniculum vulgare	Fennel	Seeds
Myrtaceae	Syzygium aromaticum	Clove	Floral buds
Poaceae (Gramineae)	Cymbopogon citratus	Lemongrass	Whole plant
Ranunculaceae	Nigella sativa	Black seed	Seeds

Molecular identification of the most resistant and frequent strain:

By investigation of 16S rRNA gene sequences, identification of the most resistant and frequent strain, Acinetobacter baumannii (5), was confirmed. The genes coding for (16S rRNA) are referred to as 16S rDNA and are used in phylogenetic studies [34]. The 16S rDNA gene was amplified by polymerase chain reaction (PCR) using universal primers, 8F (5'-GAGTTTGAT CCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3').

Statistical analysis

Statistical comparisons between the groups were performed using the one-way analysis of variance (ANOVA). Data were expressed as means, Student's f-test and using the one-way analysis of variance (ANOVA) in SPSS® Statistics software. All parameters were considered significantly different if $P < 0.05$.

RESULTS AND DISCUSSION

Distribution of collected isolates

A total number 955 samples were collected from different medical specimens of different patients (male &female), total collected samples (580) produced positive growth while 375samples produced negative growth. Acinetobacter spp. bacteria isolated from respiratory tract infections represented by 46.7%of total Acinetobacter spp. isolates, while those from urinary tract infections, wound infections, bloodstream infections represented by 26.7%, 13.3%, and 13.3%, respectively, Table (2). These results are in line with that of who demonstrated that 584 Acinetobacter strains isolated from 420 patients at 12 different hospitals over a 12-month period, 426 (72.9%) strains were identified as A. baumannii, with 208 A. baumannii isolates being recovered from respiratory tract specimens, 113 being recovered from blood cultures and central venous lines, 70 being recovered from wound swabs [35].

Table (2): Distribution of Acinetobacter spp. Isolates according to source of isolation

source of isolation	No.	%
Respiratory tract infections	7	46.7
Urinary tract infections	4	26.7
Wound infections	2	13.3
Bloodstream infections	2	13.3
Total Acinetobacter spp. isolates	15	100

Antibiotic susceptibility tests:

This experiment was carried out to study the susceptibility of the different bacterial isolates towards different ten antibiotics by using a standardized disc diffusion method. The results in **(Fig.,1)** revealed that the tested isolates were highly susceptible to polymyxin B with susceptibility percentage (86.6%) so that it represented the most effective antibiotic followed by imipenem, amikacin, ampicillin/sulbactam and

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tetracycline with 66.7%, 60%, 46.7% and 40% susceptibility, respectively. On the other hand, the data showed that 93.3% of bacterial isolates were resistant to ampicillin while 80%and 66.7% of bacterial isolates were resistant to ceftazidime and sulphamethoxazole/ trimethoprim, respectively.

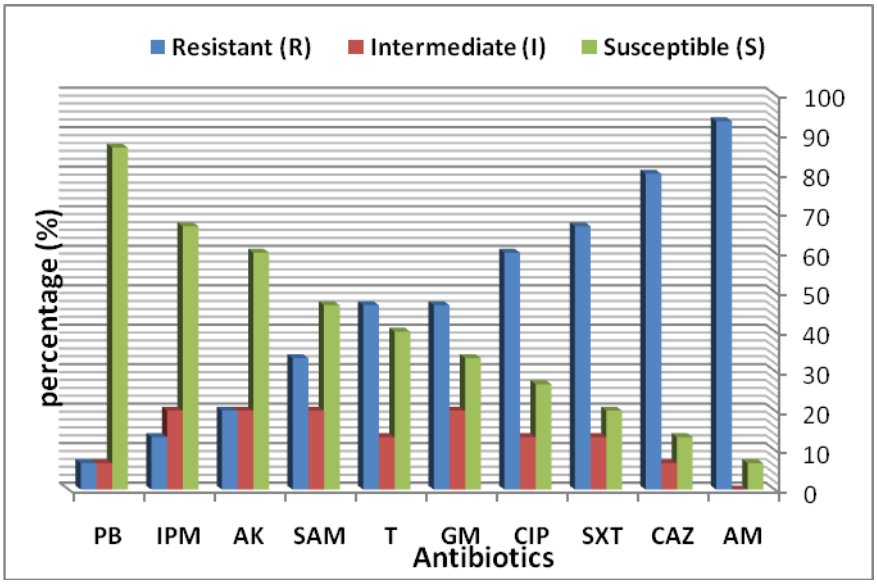

Figure (1): Susceptibility of Acinetobacter spp. isolates against different antibiotics.

Identification of Acinetobacter spp. by morphological, physiological and biochemical tests:

Different morphological, physiological and biochemical tests were conducted to identify multidrug resistance of Acinetobacter spp. isolates. The obtained results are tabulated in (Table 3).

Table (3): Morphological characteristics, biochemical tests and confirmatory tests for identification of the suspected Acinetobacter spp. isolates:

Test	Group I	Group II	Group III
Morphological characters:			
Gram's stain	- ve	- ve	- ve
Shape	coccobacilli	coccobacilli	coccobacilli
Colonies characters	circular, convex, smooth, and slightly opaque with entire margins; colonies are 0.5–1.5 mm in diameter after 24 h and 2.5–3.5 mm in diameter after 48	circular, convex, smooth, and slightly opaque with entire margins and sometimes have a butyrous aspect. Colonies are 1.5–2.0 mm in diameter after 24 h and 3.0–4.0 mm in diameter after 48 h	circular, convex, smooth, and slightly opaque with entire margins; colonies are 1.0–1.5 mm in diameter after 24 h and 3.0–4.0 mm in diameter after 48 h
Motility	**- ve**	**- ve**	**- ve**
Growth at 44 °c	**+ ve**	**- ve**	**- ve**
Physiological characters:			
Oxidase	**- ve**	**- ve**	**- ve**
Catalase	**+ ve**	**+ ve**	**+ ve**
Coagulase	**- ve**	**- ve**	**- ve**

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Blood hemolysis	**- ve**	**- ve**	**- ve**
Indole	**- ve**	**- ve**	**- ve**
Methyl Red	**- ve**	**- ve**	**- ve**
Voges-Proskauer	**- ve**	**- ve**	**- ve**
Citrate	**+ ve**	**+ ve**	**- ve**
H2S production	**- ve**	**- ve**	**- ve**
Nitrate reduction	**- ve**	**- ve**	**- ve**
Gelatin Liquification	**- ve**	**- ve**	**- ve**
Acid from:			
Glucose	**+ ve**	**+ ve**	**- ve**
Galactose	**+ ve**	**+ ve**	**- ve**
Lactose	**+ ve**	**+ ve**	**- ve**
Xylose	**+ ve**	**+ ve**	**- ve**
Sucrose	**- ve**	**- ve**	**- ve**
D-Malate	**+ ve**	**- ve**	**- ve**
Glycerate	**- ve**	**+ ve**	**- ve**
Identification:	**Acinetobacter baumannii**	**Acinetobacter calcoaceticus**	**Acinetobacter lwoffii**

According to the keys of identification protocols, the tested isolates were divided into three groups as follows:

Group I: **Acinetobacter baumannii**
Group II: **Acinetobacter calcoaceticus**
Group III: **Acinetobacter lwoffii**

The frequency of different Acinetobacter species within the collected isolates was shown in Fig. (2). Acinetobacter baumannii found to be the most frequent pathogen representing 66.7% of Acinetobacter species isolates followed by Acinetobacter calcoaceticus and Acinetobacter lwoffii with 20% and 13.3% percentages, respectively.

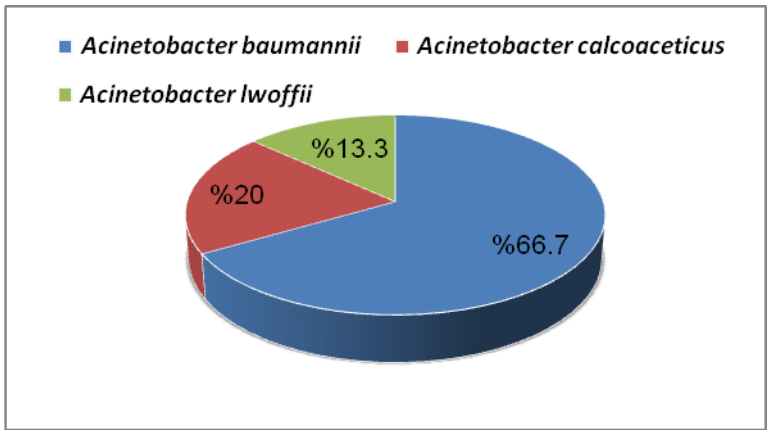

Figure (2): Frequency of different Acinetobacter species within the collected isolates.

Antibacterial activity of selected essential oils against the multi-drug resistant (MDR) Acinetobacter strains:

Essential oils have traditionally been used to treat infections and diseases around the world for centuries. In recent years there has been extensive research to explore and determine the antimicrobial activity of essential oils. Previous studies have shown that plant essential oils possess the capacity to inhibit microorganisms [36 and 37]. Also, essential oils are aromatic and volatile oily liquids obtained from plant materials as secondary metabolites. They are normally found in leaves, stems, barks, and fruits [38]. Thymol, Carvacrol, Linalool, and Eugenol are main constituents of some plant essential oils that have been shown to have a wide spectrum of activity against microbes [39].

The present study determined the antibacterial activity of ten essential oils in Tables (4, 5, and 6). The results revealed that all Acinetobacter isolates are sensitive to clove even at low concentration. This match with [40] who found that clove oil was active against gram-negative bacteria. This due to eugenol, the major constituent of clove oil; eugenol exhibits pharmacological effects on almost all systems in the body. Eugenol possesses significant antioxidant and anti-inflammatory properties, in addition to having analgesic and local anesthetic activity [41].

Rosemary was active againstAcinetobacter baumannii (5), Acinetobacter calcoaceticus (12) and Acinetobacter lwoffii (2). Our results match with [42] who reported that rosemary plants are rich sources of phenolic compounds with high antimicrobial activity against both Gram-positive and Gram-negative.

lemongrass had the high effect on Acinetobacter baumannii (5), Acinetobacter calcoaceticus (12) and Acinetobacter lwoffii (2). Our result match with [43] who reported that lemongrass showed the high inhibitory effect on Gram-negative and does not match with [44].

Marjoram oil was active against Acinetobacter baumannii (5), Acinetobacter calcoaceticus (12) and Acinetobacter lwoffii (2). The present results were in agreement with [45] showed that concentrations of marjoram oil for gram-negative bacteria were lower than gram-positive bacteria. Moreover, it was reported that volatile aromatic components in plant kingdoms exhibit more antimicrobial potential than those of components nonaromatic volatile essential oils [46].

Anise had intermediate effect against Acinetobacter baumannii (5), Acinetobacter calcoaceticus (12) and Acinetobacter lwoffii (2).These results go in line with [47] as gram-negative bacteria. Thyme had the higher effect on all tested bacterial isolates. Our results are in agreement with [48] as gram-negative bacteria. Peppermint oil had the intermediate effect on the growth of all bacterial isolates due to the active constituents in peppermint oil, which was prepared through distillation of the ground parts of the peppermint plant, include methanol, menthone, cineol, and several other volatile oils [49].

Nigella sative (black seed) was active against Acinetobacter baumannii (5),Acinetobacter calcoaceticus (12) and Acinetobacter lwoffii (2).The antibacterial activity of rosemary oil may due to one of the active ingredients was Thymoquinone (volatile oil of these seeds) and Melanine (fixed oil).This result match with [50]as gram-negative bacteria.

Fennel had significant antibacterial activity against Acinetobacter baumannii (5), Acinetobacter calcoaceticus (12) and Acinetobacter lwoffii (2).This result goes in the line with [51 and 52] as gram-negative bacteria. But don't match with [53] who reported that gram-negative strains of bacteria have less sensitivity to fennel essential oil.

Chamomile oil had intermediate effect on the growth of all bacterial isolates and this result match with [54] who illustrated antimicrobial activity may be due to numerous free hydroxyl ions that have the capability to combine with the carbohydrates and proteins in the bacterial and fungal cell wall they may get attached to enzyme sites rendering them inactive.

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Table (4): Antibacterial activity of selected essential oils against Acinetobacter baumannii(5):

Essential oils	Chamomile	Anise	Fennel	Rosemary	Peppermint	Thyme	Marjoram	Clove	Lemongrass	Black Seed
20%	0d	0d	0d	0e	0d	0e	0e	0e	0c	0d
40%	0d	0d	0d	8d	0d	9d	8d	10d	0d	0d
60%	8c	7c	11c	15c	8c	16c	13c	18c	10c	12c
80%	12b	10b	13b	18b	10b	20b	16b	22b	15b	15b
100%	15a	13a	17a	22a	13a	24a	21a	25a	19a	20a
L.S.D	0.45	0.30	1.45	1.08	0.45	1.87	0.98	1.91	0.91	0.86

Means with same letters within column non-significant difference.
Means with different letters within column significant difference, P ≤ 0.05-0.01
L.S.D.: Least significant difference at P ≤ 0.05-0.01

Table (5): Antibacterial activity of selected essential oils against Acinetobacter calcoaceticus (12):

Essential oils	Chamomile	Anise	Fennel	Rosemary	Peppermint	Thyme	Marjoram	Clove	Lemon grass	Black Seed
20%	0d	0d	0e	0e	0e	0e	0e	9e	0e	0e
40%	0d	0d	9d	11d	8d	12d	10d	12d	7d	10d
60%	11c	10c	14c	18c	10c	19c	15c	21c	12c	13c
80%	15b	14b	18b	22b	13b	22b	18b	26b	18b	17b
100%	17a	17a	21a	26a	17a	28a	24a	30a	22a	23a
L.S.D	0.55	0.48	0.96	1.53	0.51	1.88	095	2.21	0.97	1.72

Means with same letters within column non-significant difference.
Means with different letters within column significant difference, P ≤ 0.05-0.01
L.S.D.: Least significant difference at P ≤ 0.05-0.01

Table (6): Antibacterial activity of selected essential oils against Acinetobacter lwoffii (2):

Essential oils	Chamomile	Anise	Fennel	Rosemary	Peppermint	Thyme	Marjoram	Clove	Lemongrass	Black seed
20%	0d	0d	0e	0e	0d	0e	0e	0e	0e	0d
40%	0d	0d	8d	9d	0d	11d	8d	12d	7d	0d
60%	8c	9c	12c	16c	9c	18c	13c	19c	11c	12c
80%	12b	12b	14b	18b	13b	22b	16b	22b	16b	15b
100%	15a	15a	17a	24a	15a	26a	22a	29a	19a	21a
L.S.D	1.03	0.84	0.98	1.25	0.47	1.86	0.93	1.89	1.03	0. 97

Means with same letters within column non-significant difference.
Means with different letters within column significant difference, P ≤ 0.05-0.01
L.S.D.: Least significant difference at P ≤ 0.05-0.01

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Molecular identification of the most frequent and multi-drug resistant Acinetobacter baumannii isolate:

The identification of the most frequent and multidrug-resistant Acinetobacter baumannii isolate (5) was confirmed identification by using 16S rDNA sequencing. Sequences data were submitted to GenBank at NCBI web site (www.ncbi.nlm.nih.gov) with determined accession numbers. This was carried out using Sequencing Technology: Sanger dideoxy sequencing using specific universal forward and reverse primers, the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers designed to amplify 1.5 K base pair fragment of the conserved 16s rDNA region [55]. Purification, cycle sequencing, and analysis also occurred. Sequence analysis and comparison to published sequences (based on an alignment of 16s rDNA available in gene bank) made using website http://blast.ncbi.nlm.nih.gov/ Blast.cgi commonly shown as phylogenetic trees or dendrogram (to visualize the result of a hierarchical clustering calculation) and linear alignments [56]. Results confirmed that the multidrug-resistant bacteria isolates were Acinetobacter baumannii (5) as gram-negative bacteria (Table,7 and Fig.3).

Table (7): Sequences producing significant alignments to isolate (No. 5):

Description	Max score	Total score	Query cover	E value	Ident	Accession
Acinetobacter baumannii strain XH386, complete genome	1234	3234	100%	0.0	98%	NZ_CP010779.1
Acinetobacter haemolyticus CIP 64.3 genomic scaffold acLrj-supercont1.2, whole genome shotgun sequence	1184	1184	100%	0.0	97%	NZ_KB849805.1
Acinetobacter calcoaceticus PHEA-2 chromosome, complete genome	1179	2325	100%	0.0	97%	NC_016603.1
Acinetobacter junii CIP 64.5 genomic scaffold acLZZ-supercont1.1, whole genome shotgun sequence	1175	2353	100%	0.0	97%	NZ_KB849653.1
Acinetobacter oleivorans DR1, complete genome	1173	2999	100%	0.0	97%	NC_014259.1
Acinetobacter schindleri CIP 107287 genomic scaffold acLsx-supercont1.18, whole genome shotgun sequence	1157	1157	100%	0.0	96%	NZ_KB849580.1
Acinetobacter parvus DSM 16617 = CIP 108168 genomic scaffold acLZw-supercont1.4, whole genome shotgun sequence	1154	1861	100%	0.0	96%	NZ_KB849210.1
Acinetobacter sp. Ver3 contig00147, whole genome shotgun sequence	1151	1151	100%	0.0	96%	NZ_JFYL01000147.1

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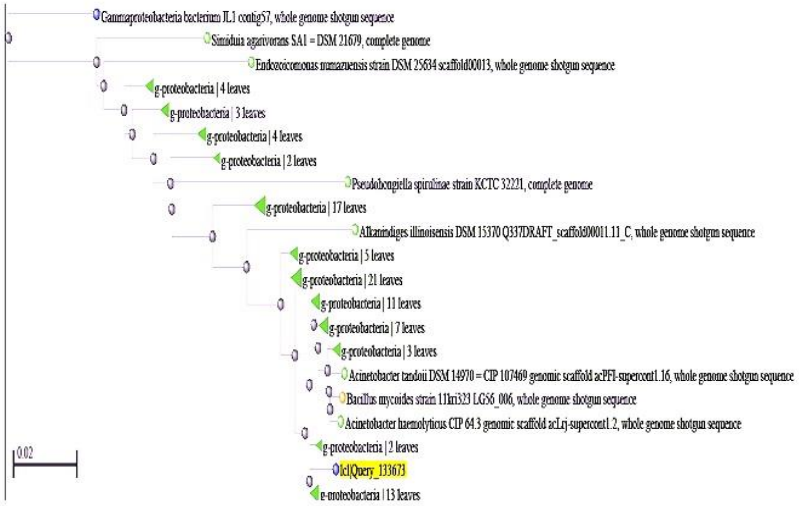

Figure (3): Phylogenetic tree of Acinetobacter baumannii (isolate no. 5)

CONCLUSIONS

The obtained results clearly demonstrated that all bacterial isolates showed resistance to essential oils in low concentrations but are sensitive to high concentration. The essential oils of Clove, Thyme and Rosemary were the most active oils respectively. Followed by Marjoram, Black seed, Lemongrass, Fennel, Peppermint, Chamomile, and Anise respectively. It is clear from the results the importance of medicinal plant oils, which is offered as an alternative and safe solution for the treatment of antibiotic-resistant organisms

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