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Research Article

SCREENING THE ANTIMICROBIAL ACTIVITY OF SOME MEDICINAL PLANTS AGAINST MULTIDRUG RESISTANCE *ESCHERICHIA COLI TYPE (1)*

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ABSTRACT
The increasing number of *Escherichia coli* causing mastitis and of bacteria resistant to conventional antibiotics has become a serious problem in recent years. So the search for new antibiotics and alternative products to solve this problem is the question of the age. This research aims to investigate the effectiveness of the extracts prepared from different parts of the following plants: *Olea europea Linn (Oleaceae*) ، *Myrtus communis Linn (Liliaceae)*، *Majorana syriacus Linn (Laminaceae)*، *Zingiber officinale Linn (Zingiberaceae)*، *Achillea falcata Linn (Asteraceae)* against resistant *Escherichia coli Type (1).* Investigation began for *E.coli* bacteria in 667 milk samples. The bacteria were identified culturally, morphologically and biochemically. Antibiotic susceptibility testing against *E.coli* by Kirby-Bauer disk diffusion method were conducted. Then using the blood agar, MacConkey agar, salmonella - shigella agar, and biochemical testing method [API 20 E testing Enterobacteriaceae] were made to type *E.coli*. Plants were extracted with water, absolute alcohol, then ether using a soxhlet apparatus and rotary vacuum evaporator. Then extracts susceptibility testing against antibiotic resistant *E.coli Type (1)* were studied. *E. coli* was defined as oxidase negative, indole positive, catalase positive. The studied antibiotics did not show any antibacterial effect against *E.coli* . By the results of the biochemical analysis (API20e) on resistant *E.coli* , *E.coli type (1)* was 33.35% of the total number of samples. The anti-bacterial effectiveness against *E.coli type (1)* of ethanol extracts prepared from different parts of the studied plants were variant, whereas the *Myrtus communis* extract effectively has the most powerful antibacterial effect for these bacteria, while the *Zingiber officinale* extract has the lowest influence.

KEY WORDS
Olea europea، *Myrtus communis* ,*Achillea falcata* ، *Majorana syriacus* ، *Zingiber officinale* ،*E.coli* ، resistant bacteria.

INTRODUCTION
Mastitis is the inflammation of milk gland with physical, chemical and microbiological changes characterized by an increase in somatic cells especially, leukocytes in the milk. This disease possess the risk for the transmission of zoonotic diseases like *tuberculosis, brucellosis, leptospirosis* and *streptococcal* sore throat to human beings [(1)]. The inflammation of the mammary gland is usually a consequence of invasion and colonization in the secretory tissue by one or more microorganisms, especially *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis*, and *Escherichia coli* [(2)]. *Escherichia coli* are facultative anaerobes in the normal intestinal flora of humans and animals; however, pathogenic strains of these bacteria are an important cause of bacterial infections. In humans, these strains are the foremost cause of urinary tract infections. Infection with Shiga toxin–producing E. coli (STEC) may also result in complications including thrombocytopenic purpura, severe hemorrhagic colitis, and hemolytic uremic syndrome [(3)]. Multi-drug resistant strains of *Escherichia coli* are widely distributed. Thus, it is urgent need to find out new antimicrobial agents. For this reason, researchers are increasingly turning their attention to herbal products, looking for new leads to develop better drugs against multidrug resistant microbe strains. Philip *et al.* reveled that none of the extracts of *Pereskia bleo, pereskia grandifoila, Curcuma aeruginosa Roxb., Curcuma zedoria, curcuma mangga, curcuma inodora aff.Blatter, and zingiber officinale var.* that used by Malaysia traditional health care systems, showed activity against *Escherichia coli*[(4)].

Myrtus communis Linn back to the Myrtaceae, is an evergreen, grows throughout the Mediterranean area and has been used for medicine, food and spice since ancient times. In folk medicine, the fruit of the plant is used in the treatment of various infectious diseases, including diarrhea and dysentery, whereas the leaves are used as antiseptic and anti-inflammatory agents, as well as in the therapy of urinary diseases. The leaves contain tannins, flavonoids such as quercetin, catechin and myricetin derivatives and volatile oils[(5)].Many studies have shown its antibacterial effectiveness[(6, 7)]. ***Achillea falcata Linn*** backs to the *Asteraceae*, is an herb perennial. The flowers have yellow color, which grows in the Badia region of Syria. Indigenous uses of ***Achillea*** species are diuretic, emmenagogic agents, wound healing, curing stomachache, diarrhea and antispasmodic, and are used in cosmetics.

Recent studies reported that *Achillea* species had constituents such as flavonoids (aglycones & glycosides), sesquiterpene lactones and essential oils, the major constituent are 1,8-cineole, camphor, piperitone and ascaridole [8].Some studies have pointed to its anti-bacterial effect[9]. The ***Olea europea*** Linn from the *Oleaceae*, is an evergreen long-lasting fruit tree, and is rooted in the Mediterranean region [9]. Olive leaves are a good source of several antioxidants including oleuropeoside compounds such as oleuropein and verbascoside, and flavonoid compounds such as luteolin, luteolin-7-glucoside, apigenin-7-glucoside, diosmetin, diosmetin-7-glucoside, rutin and catechin, and simple phenolic compounds such as tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid [10]. Most of these phenolic compounds have been shown to possess substantial antimicrobial and antioxidant activities *in vitro*, while other non-phenolic components such as aldehydes have also been studied for their antimicrobial properties[5]. A number of studies have shown the antibacterial, antiviral, and antifungal compounds[10, 11]. ***Zingiber officinale*** *Linn (Zingiberaceae)*، is a minor chemical irritant, and has a sialagogue action. Mature *Zingiber officinale (Ginger)* roots are fibrous and nearly dry. Its roots are used as spice in cooking [12]. *Ginger* possesses other interesting pharmacological and physiological properties. For instance, it acts as an anti-inflammatory, analgesic, antipyretic, anti-hepatotoxic and cardiotonic substance. Several studies also revealed its anti-bacterial effect [13, 14]. The main constituents of Ginger are sesquiterpenoids with zingiberene as the main component. Gingerone, shoagoles and gingerols, volatile oils [12]. Gingerol-related components have been reported to possess antimicrobial and antifungal properties, as well as several pharmaceutical properties[15]. Finally ***Majorana syriacus*** *Linn (Marjoram or Origanum syriaca L.)* (Laminaceae family). The genus Origanum majorana L. is an aromatic, perennial, herbaceous plant. The plant has been used as a flavouring and herbal spice from time immemorial. The plant is pungent, bitter, hot, stomachic, anthelmintic, alexipharmic, useful in diseases of the heart and blood, fevers, leucoderma and inflammation. The plant is reported to posse antibacterial activity[16]. The major volatiles and semi-volatiles of Palestinian wild M. syriaca are ,a-phellandrene, a-pinene, b-myrecene, o-cymene, p-cymene, c-terpinene, thymol, and carvacrol [17]. Marjoram (Origanum majorana),as well as wide use in cooking, marjoram has a long history of medicinal use, by the Creeks as an antidote to poisoning and snake venom, by the Romans for stomach disorders and more recently for digestive, antispasmodic and sedative properties[18].

MATERIAL & METHODS

1- Preparing plant extracts: *Myrtus communis, Olea europea,* and *Majorana syriacus* leaves, and *Achillea falcata* flowers, were collected in the early morning hours during the period from June to August from Damascus rural area, while the *Zingiber officinale* roots were purchased from Damascus markets, which were identified by Prof. Dr. Anwar Al-Khatib from Damauscs University. Plant parts were extracted separately by continuous extraction device (Soxhlet apparatus), adopted method described by Wang[19] for preparing plant extracts by organic solvents, which are respectively: water, absolute ethanol, and then Light Petroleum.

2- Sampling method:

667 milk samples were collected from dairy cows with clinical mastitis (as veterinary diagnosis). These samples were investigated for the presence of *Escherichia coli*. The bacteria were identified culturally, morphologically and biochemically. Microscopic examination was conducted after 24 hours of incubation on blood agar (HiMedia) plates. All of the following tests were conducted : oxidase, catalase ,Indole test.

3-A bacterial growth inhibition test of antibiotics by the disk diffusion method:

Pure cultures of udder pathogens were tested for antibacterial susceptibility by the disc diffusion method (Kirby-Bauer Disk Diffusion Susceptibility Test Protocol) using the 15 antimicrobial substances (Becton Dikinson, Microbiology Systems, MD, USA)on Mueller- Hinton agar medium. Testing was performed according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) document M100-S17 in 2009 [20, 21] .

5 mm diameter standard discs contain certain concentrations of the following antibiotics (Bioanalyse): amikacin (30 µg), Cephalexin (30 µg), cephalothin(30 µg), Doxycyclin(30 µg), Cefadroxil (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), Norfloxacin (10 µg), Oxytetracycline (30 µg), Pefloxacin (5 µg), Oxacillin (1 µg), Enrofloxacin (5 µg), tetracycline (30 µg), and Amoxicillin (25 µg). The resistance breakpoints were those defined by the National Committee for Clinical Laboratory Standards (NCCLS, 2000) for gram-positive bacteria[20, 21] .Negative controls were prepared using the same solvents as used to prepare the extracts. The size of the zones of inhibition was measured.

4–Analytical Profile Index technique (API20E):

The bacterial colonies, which were susceptibility to the antibiotic, were abandoned.

23 differential biochemical tests were performed by API20E system (Analytical Profile Index) manufactured by Bio Merieux , France:

a- Investigate the effectiveness of enzymes: 2 - nitrophenyl-βD-galactopyranoside, Arginine dihydrolase, oxidase.

b- Decarboxylation Interactions of amino acids: L-lysine, L-omithine.

c- Deaminase interaction of the amino acid: L-tryptophan.

d- Fermentation reactions of the following sugars: D-glucose, D-mannitol, Inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, Amygdaline Arabinose.

e- Production reactions: indole, Asitoen.

f- Reactions produce gases: hydrogen sulphide, nitrogen, nitrogen oxide.

g- Study the interactions of Recipes: gelatin diluted, citrate use, hydrolysis of urea (urease).
Then for stereotyping resistance *E. coli* ,the selective media according to the method of [22], were McCoonky agar, XLD agar , Salmonella – shigella agar.

5- A bacterial growth inhibition test of plant extracts by the disk diffusion method aginast E. coli type (1) that showed resistance to all antibiotics:

Bacterial suspension was emulsified with Agar-agar medium to test the susceptibility of plant extracts. Sterile filter paper discs (5 mm) were soaked with 5mcl of the diluted extracts (66 mg/ml) of pericarp, leaves, flowers ,seeds in ethanol, water ,and petroleum ether, so that each disc was impregnated with 0.33 mg / tablet. Control disks also prepared with absolute ethanol, Water, and petroleum ether. Results were expressed as the percentage of inhibition of bacterial growth, determined by comparing it with Control disks, and standard susceptibility disks[23].

RESULTS:

1- Identification of the bacteria:

Bacteria samples that gave us the following results were selected:
Staining with a gram stain: Gram-negative, non-motile, straight rods with round edges, no capsule, and non sporing bacteria that form compatible with reference[22, 24].

2- The results of biochemical tests:

Biochemical confirmation of the strains was performed and *E. coli* was defined as oxidase negative, indole positive, catalase positive. These results were depended according to [24].

3- Antimicrobial susceptibility results against E. coli :

Bacterial colonies showed resistant to all studied antibiotics, based on the criteria of NCCLS2000[25], and to the standard's leaflet of antibiotic discs from the manufacturer. These colonies were selected to test the plant extracts.

Table(1):The percentage of antimicrobial sensibility to *E.coli*

Antibiotics	**diameters zones of inhibition (mm)**	**Antimicrobial susceptibility results**	**Percentage of sensitive bacteria %**
Oxytetracycline (T)	9,2±1,09	resistant	98,89
Amoxicillin(AX)	8,4±0,8	resistant	91,59
Oxacillin(OX)	7,6±0.9	resistant	88,75
Cefadroxil(CER)	7,2±0,8	resistant	93,14
Pefloxacin(PEF)	9,2±1,1	resistant	94,09
Amikacin(AK)	8±1,2	resistant	96,92
Tetracyclin(TE)	7,9±0,7	resistant	90,11
Ciprofloxacin(CIP)	9,3±0,8	resistant	87,49
Norfloxacin(NOR)	6,9±1,4	resistant	84.04
Gentamycin(CN)	8,5±1,3	resistant	78,62
Chloramphenicol(C)	8,7±0,9	resistant	95,76
Enrofloxacin(ENR)	10,9±0,5	resistant	79,57
Doxycyclin(DO)	8,5±1,2	resistant	87,18
Cephalexin(CL)	7,4±0,8	resistant	90.64
Cephalotin(KF)	9,7±1,3	resistant	93.51

4-The results of Analytical Profile Index technique (API20E):

According to the data contained in the annex with these tests the results of these tests were depended. The results of the biochemical analysis (API20e) after the end of the incubation period, according to the method used in the directory of the test facilities, were: 5144512 as a result of this reading on *E.coli type (1).*

Table (2): shows the results of *(API20E) technique.*

Test	Test result
Urea	-
Tryptophan deaminase	-
Indol	+
Voges-proskauer eactin	-
Gelatinase	-
Glucose	+
Mannitol	+
Inositol	-
Sorbitol	+
Ramnose	+
Saccarose	-
Melibiose	-
Amygdalin	-
Arabinose	+
Oxidase	-

So as a result 223(33.43%) out of total number 667 samples were gotten of *E. coli type (1)*.

5- Colonial and cultural characters:

These results were depended according to[22, 24].

- Growth on blood agar: circular colonies, white, and smooth with a fecal odor.
- Growth on McCoonky agar: the growth of red colonies.
- Growth on XLD agar (xylose lysine desoxycholate agar) European pharmacopoeia: yellow colonies with wet strength, and with zone of yellow precipitation around the colonies.
- Growth on Salmonella – shigella agar: red colonies.

6- The results of Antibacterial Efficacy of plant extracts against E. coli type(1):

The petroleum ether, and water extract from studied plants were not active against *E. coli type I (1)* (diameters of zone of inhibition were zero).

As shown in Table (3), the ethanol extracts of various plants showed antibacterial activity against *E. coli type(1)*, with the diameters of zone of inhibition ranging between 6 and 20 mm. Mean inhibition zones indicate that *Myrtus communis leaves* extracts exert a powerful effect on *E.coli type(1),* while the *Zingiber officinale* extract has the lowest influence.

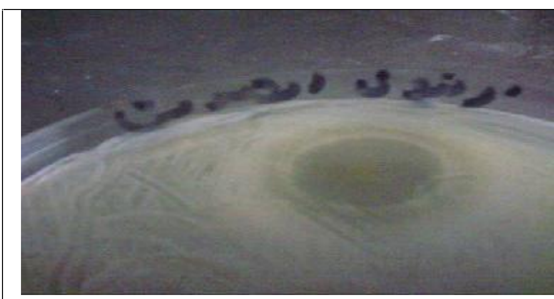


Figure 1:
Inhibition zones of *olea* extracts against *E.coli type(1)*.

Figure 2:
shows no effect of studied antibiotics against *E.coli*

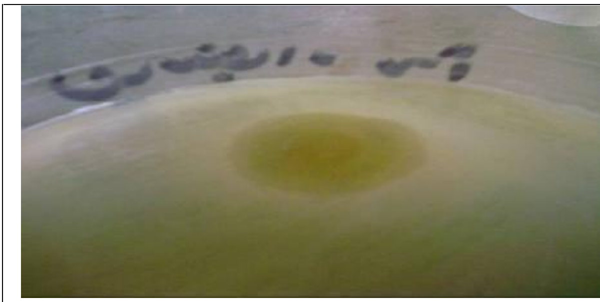
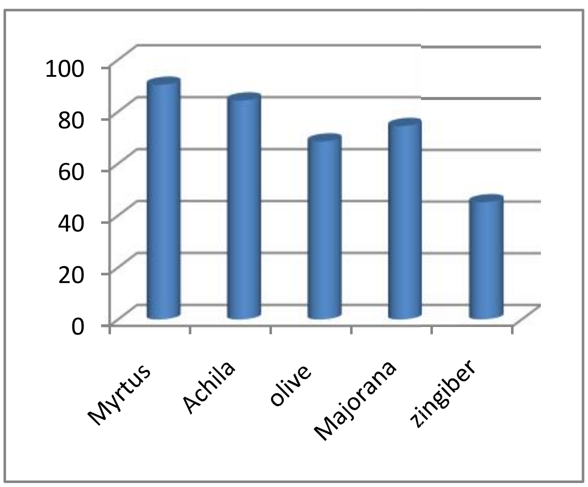


Figure 3:
Inhibition zones of *myrtus* extracts against *E.coli type(1)*

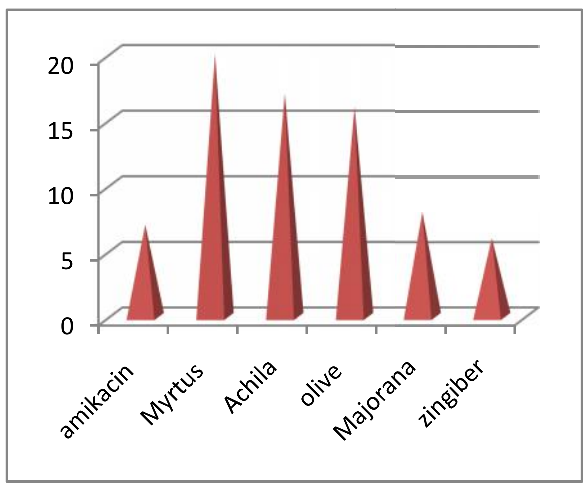
Figure 4:
Inhibition zones of *Achilla* extracts against *E.coli type(1)*

Table (3): Antibacterial activity of different extracts of studied plants

Plant	Inhibition zones of plant extracts (mm) mean ±S.D	Percentage of sensitive bacteria %
Control/5 µm	0	0
Myrtus communis leaves	20±1,3	90.4
Achillea falcata flowers	18±0,7	84.35
Olea Europea leaves	16±1,2	68.55
Majorana syriacus leaves	8±1,08	74.55
Zingiber officinale roots	6±1,1	45.36



Figure(5): Percentage of sensitive bacteria



Figure(6): Mean of inhibition diameter of plants extract and Amikacin against *E. coli type(1)*.

DISCUSSION

In the present study, antibacterial activity in the leaf extracts of various plants against multidrug resistant pathogenic bacteria *Escherichia coli type* (1) was evaluated by in vitro agar disc diffusion method, *E.coli* was resistance to all antimicrobials assayed, where the ethanol extract exhibits maximum antibacterial activity against *Escherichia coli* type (1). The present study demonstrates that ethanol extract, not aqueous, or ether petroleum extracts, exhibits an inhibitory effect on *E.coli* type (1) growth. This may in part be due to different chemical compositions between aqueous and ethanol extracts. The results showed variation in the antimicrobial properties of plant extracts (Table 3). The ethanol extracts showed strong activity (inhibition zone 20-16 mm), and weak inhibition (zone 8-6 mm). Attending to this, we arrange plants according to their effectiveness against *E.coli type(1)* to find that the major effectiveness was achieved by the ethanol extracts from *Myrtus communis* leaves, followed by *Achillea falcata* flowers, and *Olea Europea* leaves extract. While the *Majorana syriacus,* and *Zingiber officinale* leaves has the weakest's influence. Considering the large number of different groups of chemical compounds present in plants, it is most likely that their antibacterial activity is not attributed to one specific mechanism but that there are several targets in the cell. The antimicrobial potency of plants is believed to be due to, saponins, phenolic compounds, essential oils and flavonoids[26].Most of the studies on the mechanism of phenolic compounds have focused on their effects on cellular membranes. They have been seen to attack not only cell walls and cell membranes, thereby affecting their permeability and the release of intracellular constituents, but also to interfere with membrane functions such as electron transport, enzyme activity or nutrient uptake. Thus, active phenolic compounds might have several targets which could lead to the inhibition of bacteria [27]. As in khder article these compounds were found in denature proteins and block enzymes and subsequently the bacteria losses its activity[6]. *Myrtus communis* leaves contain different polyphenol classes as flavonols and galloyl derivatives, their antimicrobial mode of action was related to the presence of phenolic compounds[27]. More over *Myrtucomlone A&B* are considered as two new acylphloraglucinols identified in the leaves and fruits of *M.communis* and have shown a significant antimicrobial effect against bacteria. The inhibition effect may be regarded to present of Tannin via producing hydrogen bonds with proteins, which converted its structure and lead to block the protein synthesis, and tannins considered as a phenolic compounds of plants [6]. So the inhibition effect of *Myrtus communis* towards *E.coli type(1)* isolates may refer to the polyphenolic and Tannin they contain. Information about the antimicrobial activity of *Achillea* extracts is limited. The study of the 13 Turkish *Achillea* species showed that not all of the *Achillea* species possess antibacterial activity. *Achillea falcata* showed mild to low antibacterial activity[9]. Also water-insoluble parts of the methanolic extracts exhibited slight or no activity [28]. In contrast at Stojanović's study, extracts of several species of *Achillea* possess a broad spectrum of antimicrobial activity against tested strains *Staphylococcus aureus*, and *Escherichia coli* [29, 30], which matched our results. The composition of the extract of *Achillea clavennae* are alkanes, fatty acids, monoterpenes, guaiane sesquiterpenes, and flavonoids (apigenin and centaureidin) [29]. The genus *Achillea* has been extensively studied in regard to its flavonoids and sesquiterpene lactones. Flavonoids possess antimicrobial properties and several investigations have examined the relationship between flavonoid structure and antibacterial activity. Promising evidence has clearly shown that sesquiterpene lactones derived from several different plant species have significant antimicrobial activity in vitro[9]. Researchers have published numerous studies concluding that *olive* leafs active ingredient, oleuropein and its derivative hydroxytyrosol act as a natural antibiotic agent. Oleuropein has been shown to have strong antimicrobial activity against both Gram-negative and Gram-positive bacteria, as well as mycoplasma. Oleuropein and other phenolic compounds completely inhibit the development of *Klebsiella pneumoniae, Escherichia coli* and *B.* cereus[31].

Phenolic structures similar to oleuropein seem to produce its antibacterial effect by damaging the bacterial membrane and/or disrupting cell peptidoglycans[32]. However, the exact mechanism of the antimicrobial effect of oleuropein is still not completely confirmed, although some authors have suggested proposed that it is due to the presence of the ortho-diphenolic system (catechol)[31]. Other researchers concluded that oleuropeins can enhance nitric oxide production in mouse macrophages. By increasing nitric oxide production, oleuropein appears to arm the macrophages against endotoxins. Interestingly, oleuropein only increased nitric oxide production when endotoxins were present[33]. Ethanol extract of *Ginger* inhibited *E.coli* showing 9 mm diameter[34], as our study. *Ginger* extract found to have moderate antibacterial properties against *E. coli* serogroups O8 and O88, while it did not show any antibacterial activity against all other serogroups of *E. coli* [35].In contrast, Sofia at el. Reported that the *Ginger* extract showed negligible antibacterial activity against *Escherichia coli, staphylococcus aureus* and *Bacillus cereus*[36],and no effect not even by hexane, ethyl acetate, water, and methanol extracts(13). Also aqueous extracts of *Zingiber officinale* did not exhibit *in vitro* any inhibition on the growth of *E. coli* [37], which confirmed the result of our study. The methanol extract of *Origanum majorana* against the *E.coli* has 18 mm diameter Zone of inhibition [16], while our study showed 8 mm . The methanol extract of shade dried aerial parts of *O.majorana* had showed variable activity against *Bacillus subtilis,B. megaterium, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus*[16]. The antibacterial activity exhibited by *Origanum majorana* essential oil, and The n-hexane extract of Four sweet marjoram *(Origanum majorana)* fractions have been demonstrated [38]. In contrast, at Joshi's study the most resistant bacteria to *Origanum majorana* extract were *E.coli* [39].

We believe that this is the first study on the antibacterial effect of *Achillea*, and majorana on this type of bacteria, *Escherichia coli type(1)*.

CONCLUSION

The ethanol extracts of *Myrtus communis, Achillea falcata, Olea Europea, Majorana syriacus,* and *Zingiber officinale* showed variant antibacterial activity against resistant *Escherichia coli Type(1),* which causes mastitis. *Myrtus, Achillea,* and *olive* had beaten out all the strongest antibiotics in possession the antibacterial effect on these bacteria.

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