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Research Article

Antibacterial effectiveness of many plants extracts against the resistant *Negative Coagulase Staphylococcus* that cause Clinical Mastitis in Cows

*Al Laham SH, Al Fadel F

Pharmacology & Toxicology Department - Faculty of pharmacy- Damascus University- Damascus-Syria.

ABSTRACT

Clinical mastitis caused by *negative coagulase staphylococcus* is considered to be the most dangerous diseased cases in cows and it can cause great economic loses. The aim of this study was to investigate the effectiveness of extracts prepared from different parts of the following plants: *Olea europea Linn (Oleaceae*) , *Myrtus communis Linn (Myrtaceae)* , thymus vulgaris *Linn (Laminaceae), Rosmarinuis officinalis Linn (Laminaceae) Ficus carica Linn (Moraceae),*and *Achillea falcata Linn (Asteraceae),* against resistant, *negative coagulase staphylococcus* in 1371 Samples of milk. The presence of *negative coagulase staphylococcus* in 1371 Samples of milk by using blood agar, Manitol agar, and some bio-chemical tests that were investigated .Secondly, the antibacterial activity of many antibiotics on these bacteria by using disc diffusion method were determined . The plants were extracted with water, absolute alcohol, then ether by using soxhlet apparatus and rotary vacuum evaporator. The antibacterial activity of the plants extracts were determined by using disc diffusion method. This study showed the presence of different antibacterial effectiveness of the alcoholic extracts prepared from different parts of those plants against resistant *coagulase staphylococcus*. The extract of thymus vulgaris has the strongest effectiveness, whereas the extract of *Olea europea* has the weakest effectiveness against resistant *coagulase staphylococcus*. The alcoholic extracts of the studied plants showed the antibacterial effectiveness against resistant *coagulase staphylococcus*.

Key words: *Olea europea*, *Myrtus communis* , thymus vulgaris ,*Rosemery***,** *Ficus carica, Achillea falcate, mastitis, negative coagulase staphylococcus.*

INTRODUCTION

Mastitis is a serious disease in dairy animals causing great economic losses due to reduction in milk yield as well as lowering its nutritive value. Generally mastitis occurs in two forms clinical or sub-clinical. In addition to causing colossal economic losses to farmers, the disease is important from consumers and processors' point of view. The milk from the affected animals may harbour the organisms potentially pathogenic for humans. Mastitis affects the milk quality in terms of decrease in protein, fat, milk, sugar (lactose) contents and increase in somatic cell count[1]. In the clinical mastitis all the five cardinal signs of udder inflammation (redness, heat, swelling, pain and loss of milk production) are present, while the sub-clinical form is bereft of any obvious manifestation of inflammation [2]. Many factors can influence the development of mastitis; however, Coagulase-negative staphylococci (CNS) were the most frequently isolated bacteria [3], and Increased resistance for it was reported[4].This what guided our attention to the plant kingdom which might be a substitute cure when synthetic chemical compounds are unable to perform their role. We believe that our research is the first that mentioned the activity of six syrian plants against multidrug-resistant Coagulase-negative staphylococci.

The *Olea europea Linn* from the *Oleaceae*, is an evergreen long-lasting fruit tree, and is rooted in the Mediterranean region[5]. The leaves are opposite, entire, stiff, coriaceous, nairoVF-elliptical to lanceolate or eordate with thorny tips. The upper surface is dark green, glabrous or covered with scattered scutiform hairs; the underside shimmers silver with scuitform hairs[6]. It is worth mentioning that many studies have proven its antimicrobial activity [7]. *Myrtus communis Linn* back to the Myrtaceae, is an evergreen, bushy shrub or a small tree growing up to 5 m high with opposite branches and quadrangular cane-shaped, initially delicately glandular, downy branches. The dark green leaves are glossy, glabrous, coriaceous, opposite-paired or whorled, ovate to lanceolate, entire-margined, acuminate and 1-3cm long[6]. Many studies have shown its antibacterial effectiveness[8].*Thymus vulgaris* from the *Lamiaceae*, is a dwarf shrub that grows up to 50 cm high with an erect, woody and very branched-bushy and downy stem, which never roots. The leaves are short-petioled, linear or oblong-round, acute, glandular-punctate with an involute margin and a tomentose under surface[6]. There are many studies that pointed to its anti-bacterial effect [9, 10]. As a member of the Laminaceae family, *Rosmarinuis officinalis Linn* (*Rosemary.L)*, grows wild in Mediterranean countries . The plant is an evergreen, branched subshrub, 50 to 150 cm high with erect, climbing or occasionally decumbent brown branches. The leaves are linear, coriaceous, entire-margined, light green

*Author for correspondence: E-mail: Lahamshaza@hotmail.com

and somewhat rugose above. They are tomentose, 15 to 40 mm by 1.2 to 3.5 mm[6]. Many studies have shown its anti-bacterial effect [11]. *Achillea falcata Linn* backs to the *Asteraceae*, is a herb perennial, ranging in height from 20 to 70 cm and sometimes higher. The stem is coated hairs with aromatic odor. The leaves are light green, small, long, and gear. The flowers have yellow color, which grows in the Badia region of Syria. Some studies have pointed to its anti-bacterial effect[12]. Finally *Ficus carica Linn (Moraceae)* is a deciduous, heavily branched tree growing to 4 m or more. The leaves are downy A. beneath and are 10 to 20 cm long, broad-ovate to orbicular with 3 to 5 deep lobes [6]. Fruits, leaves and roots of *Ficus carica*(fig) have been used in traditional medicine to treat various digestive disorders,respiratory, inflammatory, cardiovascular diseases and cancer, gingivitis, diabetes and constipation [13]. Some studies have pointed to its anti-bacterial effect[14].

MATERIAL AND METHODS

Collection of plant material: *Olea europea*, *Myrtus communis*,*Thymus vulgaris* and *Ficus carica* 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 *Rosmarinuis officinalis* leaves were purchased from Damascus markets, which were identified by Prof. Dr. Anwar Al-Khatib from Damauscs university. The plant were washed with cold water, distilled water, then dried with hot air at a temperature not exceeding 60°C in shadow . Then were crushed properly by metal mortar until a fine homogeneous powder was obtained, kept in paper bags with free humidity conditions [15].

Preparing plant extracts: Plant parts were extracted separately by continuous extraction device (Soxhlet apparatus), adopted method described by Wang[16] for preparing plant extracts by organic solvents. 50 g of plant powder were placed by an electric mortar, inside the thimble-holder of Soxhlet apparatus, with 500 ml of each organic solvent (rate 1:10 weight: volume). Three different polar solvents have been selected to extract the components of the plants, which are respectively: water, absolute ethanol, then Light Petroleum. Extraction period was 4 hours, until the solvent that comes out of thimble became colorless. Then to concentrate the extracts, the ethanol and petroleum ether extracts, were dried using rotary vacuum evaporator at a temperature not exceeding 40°C, while the aqueous extract was dried using lyophilizer (freeze dryer). The thick layer of the bottom was stored in sterile bottles at 4°C for further experiments. All extracts were filter-sterilized using a 0.45µm membrane filters (whatman,UK)[15].

Sampling method: During a 1 year period (2010–2011), 1371 milk samples were collected from dairy cows with clinical mastitis (as veterinary diagnosis) sent daily to the Central Laboratory of Veterinary in Damascus. The samples were preserved at 2-4°C and transported to the laboratory in sterile tubes, fitted with a strap closure, and card number includes name of the sample, place, and date of collection. These samples were investigated for the presence of *staphylococcus*.

Cultured Method of pathological sample: Media cultures were Prepared according to the manufacturer 's instructions . Then sterilized by autoclaving at 121˚C and under the pressure of 15 pounds for 15 minutes.

The following information were Registered on the bottom of the Petri-dishes : the number, name of the sample, place, and date of collection. Then milk samples were centrifuged for 20 minutes at 3000 cycles / minute, then the serum was poured and the sediment was taken. Then the platinum rod after sterilizing by flame lamp were planted into the sediment, then were spread on a blood agar plate, and incubated for 24 hours at 37°C . All the samples were planted within two hours from the time of sampling.

Identification Method of the bacteria*:* The bacteria were identified culturally, morphologically and biochemically.

Microscopic examination:Microscopic examination was conducted after 24 hours of incubation on blood agar (HiMedia), India plates. The staining and cellular morphological features of organisms were ascertained by microscopic examination of Gram stained smears.

Biochemical tests: All of the following tests were conducted : oxidase, catalase ,Indole, urease hydrolysis, coagulated test.

Fermentation reactions of the following sugars: D-Glucose, D-Mannitol, Sucrose, Maltose, Lactose , Arabinose, Sorbitol, Trihalose.

Microbial selective cultures: Once they had been isolated and identified, the growing colonies on blood agar media were cultured on the selective media Mannitol Salt agar (Haimedia) ,which was used in order to distinguish pathogenic Staphylococcus colonies with ferment d-mannitol, by using the sterilized platinum rod, then incubated for 40 to 48 hours at 35-37 °C at an aerobic culture incubator.

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 18 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 [17, 18] .

5 mm diameter standard discs contain certain concentrations of the following antibiotics (Bioanalyse): amikacin (30 µg), ampicillin (10 µg), Cephalexin (30 µg), cephalothin(30 µg), Doxycyclin(30 µg), Cefadroxil (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (15 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[17, 18].

Table 1: the results of planting Staphylococcus type on culture medium

Staphylococcus type	Staphylococcus aureus	coagulase-negative staphylococci
Culture on blood agar	convex colonies with yellowish pigment and porcelain-like surface	white rounded colonies
Culture on Mannitol Salt agar	+ (With a change in the media's color)	+ (Without a change in the media's color)

4-5 colonies of bacteria were suspended (after pure isolation and identification) to the test tube in 2 ml of physiological solution, Mixed thoroughly until a turbid homogeneous obtained. Sterile swab sticks immersed in suspension, and spread onto the surface of the Muller Hinton agar plates, then the agar plates were covered partly with lids to dry before proceeding to the next step. The antibiotic discs were placed and gently pressed, by sterilized forceps, onto the middle plates (Forceps was sterilized after each antibiotic), finally the agar plates were covered and incubated in aerobic incubator at 37 ° C for 24 hours. The result was recorded on the result sheet and sent back to the documentation accompanying by the manufacturer. Negative controls were prepared using the same solvents as used to prepare the extracts.

The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of the zone inhibition in millimeters.

Bacterial growth inhibition test of plant extracts by the disk diffusion method against coagulase-negative staphylococci that showed resistance to all antibiotics : Sterile filter paper discs (5 mm) were soaked with 5µl of the diluted extracts (66 mg/ml) of leaves, and flowers 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. The Disks were placed in Petri dishes containing Mueller Hinton agar and incubated for 17 hours at 37 ˚C. After incubation, all dishes were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters with a ruler. Results were expressed as the percentage of inhibition of bacterial growth, determined by comparing it with Control disks, and standard susceptibility disks [16]. After completing the work the Petri dishes were settled by using the autoclave.

RESULTS

Identification of the bacteria: Bacteria samples that gave us the following results were selected:

Microscopy with Gram staining: small spherical cells, non-motile, Gram-positive, found in grapelike clusters, compatible with kayser [19].

Colonial and cultural characters: The results of the plants of *Staphylococcus* type on selective media are shown in Table 1, These results were depended according to Ibrahim[20].

The results of biochemical tests: The results were shown in Table 2, These results were depended according to[20].

Table 2: the results of biochemical tests

Test	*Staphylococcus aureus*	*coagulase-negative staphylococci*
Oxidase	-	-
Catalase	+	+
coagulase test	+	-
Urease hydrolysis	+	+
Glucose	+	+
Lactose	+	Variable
Mannitol	+	Variable
Maltose	+	-
Sucrose	+	-
Arabinose	-	-

Antimicrobial susceptibility results against coagulase-negative staphylococci: Bacterial colonies showed resistant to the studied antibiotics, where all the diameter zones of inhibition were lower than the required values of each antibiotics, based on the criteria of NCCLS2000[18], and to the standard's leaflet of antibiotic discs from the manufacturer. The percentage of antimicrobial sensibility of *coagulase-negative staphylococci* isolates to the antibiotics are shown in (Table 3).

The results of Antibacterial Efficacy of plant extracts against only the resistant coagulase-negative staphylococci: As shown in Table 2, the extracts from different studied plant showed antibacterial activity against resistant *coagulase-negative staphylococci* , with the diameters of zone of inhibition ranging between 20 and 6 mm. Of the studied plants, the most active extracts were those obtained from the leaves of thymus and *Ficus*, while the less active one were *Olea* leaves and *Achillea* flowers. The organic solvent petroleum Ether, and water extract from all parts of the plants were not active (diameters of zone of inhibition) were zero .

DISCUSSION

Coagulase-negative staphylococci (CNS) have become the most common bovine mastitis isolate in many countries and could therefore be described as emerging

Table 3: shows the number of samples infected and their percentage

the total number of samples infected=1371 The infected samples of the total number of samples	Number of samples infected	Percentage of samples infected
By staphylococcus bacteria	596	43,47%
By *coagulase-negative staphylococci*	444	32,38%
By resistant *coagulase-negative staphylococci* to all antibiotic	165	12,03%

Table 4: The percentage of antimicrobial sensibility to *coagulase-negative staphylococci* isolates.

Antibiotic	Susceptible%	Intermediate sensitive%	Resistant %
Oxytetracycline(T)	19,46	23,35	57,19
Amoxicillin(AX)	11,03	21,8	67,17
Oxacillin(OX)	13,79	29,56	56.65
Cefadroxil(CER)	13,41	34,41	52,18
Pefloxacin(PEF)	17,02	24,56	58,42
Amikacin(AK)	8,21	56,67	35,12
Tetracyclin(TE)	9,1	37,97	52,93
Ciprofloxacin(CIP)	36,23	29,34	34,43
Norfloxacin(NOR)	10,46	19,07	70,47
Gentamycin(CN)	19	26,4	54,8
Chloramphenicol(C)	12,93	35,78	51,29
Enrofloxacin(ENR)	46,82	21,73	31,45
Doxycyclin(DO	19,55	25,74	54,71
Cephalexin(CL)	13,32	17,4	69,28
Cephalotin(KF)	14,53	26,35	59,12

mastitis pathogens. CNS infection can damage udder tissue and lead to decreased milk production [(21)].

Among the major mastitis pathogens in dairy cows, *Staphylococci aureus* and *Streptococcus uberis* had the highest prevalence. *Coagulase-negative staphylococci* (CNS) were the most frequently isolated bacteria [(22)],the percentage was (57.8%)in myllys study [(3)].while it was 32,38% in our study, and 24.2% in another research [(4)].

Staphylococci aureus, a microorganism extensively studied due to its ability to produce enterotoxins and exceptionally resistant to a number of antimicrobial agents.

It is well known that the defense mechanisms of *Streptococcus* and *Staphylococcus* against macrolides and lincosamines are plasmid or transposons encoded that can be strongly induced, resulting in high resistance levels. Martín revealed the highest level of resistance to penicillin, lincomycin, amoxicillin, and ampicillin [(4)] .*CNS* were resistant to trimethoprim-sulphonamide, ampicillin and erythromycin [(3)]. However, the present study demonstrates that The highest rate of resistant CNS observed was to norofloxacin (70.47%) and Cephalexin (69.28%), while the highest rate of sensitivity was to Enrofloxacin (46.82%),followed by Ciprofloxacin (36.23%),as the sensitivity to enrofloxacin 100% in Moniri result[(23)].

The present study demonstrates that ethanolic extract, nor aqueous nither ether petroleum extracts exhibits an inhibitory effect on *coagulase-negative staphylococci* growth. This may in part be due to different chemical compositions between aqueous and ethanolic rosemary extracts. The results showed variation in the antimicrobial properties of plant extracts (Table 5). The ethanolic extracts showed strong activity (inhibition zone 20 mm), and weak inhibition (zone 8-6 mm). According to this, the major effectiveness was achieved by the ethanolic extracts from *thymus vulgaris*, *Ficus carica*, *Rosemery*,and *Myrtus communis leaves*, followed by *Achillea falcata* flowers, and the less effectiveness was *Olea Europea* leaves extaract.

As we concluded in our research, the ethanolic leaves extract of *Thymus vulgaris* had an excellent inhibitory effect against *Staphylococcus aureus* and *Escherichia coli* [(9)], also the ethanolic leaves extract of *Thymus capitatus* , while the aqueous extraction gave less antibacterial activities [(10)]. The phenolics (thymol and carvacrol) showed the inhibitory effect against *Staphylococcus aureus* [(24)].

Thyme (*Thymus vulgaris* L.) volatile phenolic oil has been reported to be among the top 10 essential oils, showing antibacterial, antimycotic, antioxidative, and natural food preservative[(25)] . According to Ivanovic research, there is no difference in antibacterial activity of thyme supercritical extract and thyme essential oil[(26)]. The Gram-negative bacteria generally have higher tolerance to the presence of essential oils due to hydrophilic outer membrane that blocks penetration of the hydrophobic essential oils into target cell membrane [(24)].

The structure of thymol is similar to that of carvacrol; however, they differ as to the location of the hydroxyl group in the phenolic ring. Both substances seem to make the membrane permeable. Their structure disintegrates the external membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP. The presence of magnesium chloride does not influence this

action, suggesting a chelating mechanism of different cations on the external membrane [27].

Ficus carica posse's large amounts of polyphenolic, benzaldehyde and coumarin compounds that has anti-cancer properties. Different studies indicated that figs have antimicrobial effects on various positive and gram-negative bacteria. Flavonoids of fig acting as antioxidants and neutralize free radicals of metabolism [13]. *Ficus capensis* revealed the presence of alkaloids, flavonoids, tanins, terpenes, resins, and sterols .The presence of alkaloids reveals its activity against pathogenic bacteria (28). The petroleum ether extract of *Ficus racemosa* Linn. leaves was the most effective against the tested organisms *Escherichia coli*,and *Staphylococcus aureus*[29], whereas that was disagree with our results.

The extracts of the four tested *Ficus* species had significant antibacterial activity [30].

There is not much knowledge concerning anti-*streptococcal* activity of *Rosmarinuis officinalis*; the mechanism underlying this effect is not known. Methanol rosemary extract containing carnosic acid, carnosol and rosmarinic acid was the most effective antimicrobial against gram positive bacteria , gram negative bacteria . By contrast, water extract contains only rosmarinic acid and shows narrow activity. Therefore, the antimicrobial rosemary extracts efficiency was associated with their specific phenolic composition. Carnosic acid and rosmarinic acid may be the main bioactive antimicrobial compounds present in rosemary extracts. It was reported that both water and Methanol rosemary extracts had different efficiency as an antimicrobial agent, which is linked to their different polyphenol compositions [31], this result comply with my extracts, but only ethanol extract has the antibacterial efficacy. The most susceptible species to ethanolic extracts of rosmarinus officinalis were *Staphylococcus aureus* (Penicillin G- and oxacillin-resistant),and *Streptococcus pyogenes* (Penicillin G, erythromycin- and clindamycin-resistant) [32].

Myrtus communis L. (Myrtaceae) has been reported to have antibacterial activity against *Staphylococcus aureus* [33]. *Myrtus communis L.* leaves are characterized by the presence of flavonols (myricetin and quercetin),glucosides, and galloyl derivatives, which include galloyl-glucosides, ellagitannins, and galloyl-quinic acids. Thus, myrtus Leaves contain different polyphenolic. It seems that their antimicrobial mode of action was related to the phenolic compounds present[34]. As in khder article these compounds were found in denature proteins and block enzymes and subsequently the bacteria losses its activity . Morever the inhibition activity 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 which have anti-oxidative effects [35].So the inhibition effect of *Myrtus* communis towards *Staphylococcus aureus* 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[12], which matched our results. In contrast, extracts of several species of *Achillea* possess a broad spectrum of antimicrobial activity against tested strains *Staphylococcus aureus*, and *Escherichia coli* [36]. As well as the essential oil of *Achillea millefolium* showed antimicrobial activity against *Streptococcus pneumonia*. However, water-insoluble parts of the methanolic extracts exhibited slight or no activity [37].

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 [12].

The composition of the extract of *Achillea clavennae* are alkanes, fatty acids, monoterpenes, guaiane sesquiterpenes, and flavonoids (apigenin and centaureidin) [36]. Eucalyptol (1,8-cineole) and camphor are well-known chemicals with their pronounced antimicrobial potentials. Antimicrobial activities of borneol have also been previously reported by other investigators[37].

The observed activity of the plants studied here in might be due to the presence of sesquiterpene lactones and flavonoids, and possibly due to synergistic interactions between the components of these extracts.

Researchers have published numerous studies concluding that **olive** leafs and its active ingredient oleuropein 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[38].unfortunately, the ethanol extract we had

Table 5: Antibacterial activity of different extracts of studied plants

Plant	Inhibition zones of plant extracts (mm) Water extract	Ether petroleum extract	Ethanol extract mean ±S.D	Percentage of sensitive bacteria %
Control/5 µm	0	0	0	0
thymus vulgaris leaves	0	0	20,1±1,4	98,4
Ficus carica leaves	0	0	20±0,9	97,6
Rosemery leaves	0	0	19±0,8	96
Myrtus communis leaves	0	0	19±1,09	95,7
Achillea falcata flowers	0	0	8±0,5	97,87
Olea Europea leaves	0	0	6±0,7	99,01

was less or no effectiveness than another researches and other stusdied plants; However, the exact mechanism of the antimicrobial activity of oleuropein is still not completely established[38].

Polyphenols or phenolic compounds are groups of secondary metabolites widely distributed in plants. Various publications have documented their antimicrobial activity. And 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.

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 [34].

CONCLUSION

The investigated *thymus vulgaris, Ficus carica, Rosemery, Myrtus communis, Achillea falcata,* and *Olea Europea* isolates showed strong antibacterial activity against *Coagulase-negative staphylococci* except by *Olea Europea*. The *thymus vulgaris, Ficus carica, Rosemery, and Myrtus communis leaves* were found to be the most effective in bacterial growth inhibition, followed by the *Achillea falcata* flowers. While *Olea europea* extract has no effect.

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