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Research Article

ANTIBACTERIAL EFFICACY OF VARIETY PLANTS AGAINST THE RESISTANT STREPTOCOCCUS WHICH CAUSE CLINICAL MASTITIS IN COWS

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ABSTRACT:

Streptococcus is considered to be one of the most dangerous causes of Clinical mastitis in cows. The aim of this study was to investigate the effectiveness of extracts prepared from different parts of the flowing plants: *Oleaeuropea Linn(Oleaceae*), *Myrtuscommunis Linn (Liliaceae) ,thymus vulgaris Linn (Laminaceae)*, *Rosemery Linn(Laminaceae), Ficuscarica Linn (Moraceae),* and *Achilleafalcata Linn(Asteraceae)* against resistant *Streptococcus* in 1371 Samples of milk. This work was achieved in four stages: First of all, the presence of *Streptococcus* in 1371 Samples of milk, by using blood agar, Bile Esculin agar, and some bio-chemical tests were investigated .Secondly, the antibacterial activity of many antibiotics on these bacteria by using disc diffusion method were determined.Thirdly, the plants were extracted with water, absolute alcohol, and petroleum Ether by using soxhlet apparatus and rotary vacuum evaporator. Fourthly, the antibacterial activity of the extractions on resistant *Streptococcus* was determined by using disc diffusion method. This study has shown the presence of different antibacterial effectiveness of the extracts prepared from different parts of those plants. The extract of *thymus vulgaris* is more effective when compared to the extract of *Oleaeur opaea* against resistant *Streptococcus*.

KEY WORDS:

Oleaeuropea, *Myrtuscommunis ,thymus vulgaris*, *Rosemery*, *Ficuscarica ,Achilleafalcata, mastitis, Streptococcus.*

INTRODUCTION:

Therapeutic effects of medicinal plants have beenrecognized several years ago. Different studies have shown that medicinal plants can have a potential activity against drug resistant strains of bacteria, especially medicinal plants rich in various antimicrobial compounds such as tannins, flubatanins, trepenoides, saponin, steroids, alkaloids and flavonoids.

One of the important plants in this regard is the ***Ficuscarica(Fig)***, which recently is discussed due to high amounts of calcium as a major fruit for the survival of other plants and animals.Fruits, leaves and roots of *Ficuscarica*(fig) have been used in traditional medicine to treat various digestive disorders respiratory, inflammatory, cardiovascular diseases and cancer. Different studies indicated that figs have antimicrobial effects on various positive and gram-negative bacteria as well as drug resistant bacteria, yeasts and mold[(1)].

The ***Oleaeuropea Linn*** from the Oleaceae, is an evergreen long-lasting fruit tree, and is rooted in the Mediterranean region[(2)]. It has antimicrobial activity[(3)].***Myrtus communis Linn*** back to the Liliaceae, 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[(4)]. Many studies have shown its antibacterial

effectiveness[5].***Thymus vulgaris***is from the Laminaceae, the leaves are short-petioled, linear or oblong-round, acute, glandular-punctate with an involute margin and a tomentose under surface [4]. There are many studies that pointed to its anti-bacterialeffect[6]. As a member of the Laminaceae family, ***Rosemary Linn*** (*Rosmarinus officinalis*), grows wild in Mediterranean countries. The leaves are linear, coriaceous, entire-margined, light green and somewhat rugose above [4]. Many studies have shown its anti-bacterial effect [7]. Finally ***Achillea falcata Linn*** backs to the Asteraceae, is a herb perennial, ranging in height from 20 to 70 cm and sometimes higher. The leaves are light green, small, long, and gear.The flowers have yellow color, which grows in the Badia region of Syria[4]. Some studies have pointed to its anti-bacterial effect[8].

Bovine mastitis is one of the most important bacterial diseases in dairy cattle throughout the world, and it is responsible for great economic losses to milk producers as well as to the milk processing industries. These losses include reduced milk production, discarded milk, replacement cost, extra labor, treatment, and veterinary services. Many factors can influence the development of mastitis; however, 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*[9].Those strains that can cause infections, are becoming resistant to antibiotics. Prolonged usage of antibiotics has led to the emergence of drug resistance. That directed the researcher to medical plants. Therefore we tried in our research to investigate the possibility of owning those plants the capacity of confront these bacteria.We believe thatour researchisthe first report testing the activity of six Syrianplants against multidrug-resistant *Streptococcus*.

MATERIAL & METHODS:

Collection of plant materials :*Oleaeuropea*, *Myrtus communis, Thymus vulgaris* and *Ficu scarica* leaves ,and *Achillea falcata* flowers were Collected in the early morning hours during the periodfromJunetoAugust fromDamascus rural area, while the *Rosmarinuis officinalis* leaves were purchased from Damascus markets, which were identified by Prof. Dr.AnwarAl-Khatib from Damauscs university. The plant were washedwith cold water, distilled water, then dried with hot airat a temperaturenot exceeding60°C in shadow .Then were crushedproperly by metal mortaruntilafine homogeneous powder was obtained, kept inpaper bags with free humidity conditions [10].

Preparing plant extracts:

Plant parts were extracted separately by continuous extraction device(Soxhlet apparatus), adopted method described by Wang[11] for preparing plant extracts by organic solvents. 50 gof plant powder were placed byan electricmortar, inside the thimble-holder of Soxhlet apparatus, with 500 ml of each organic solvent (rate 1:10weight: volume). Threedifferentpolarsolventshavebeen selectedto extract the components of the plants, which are respectively: water, absoluteethanol, thenLight 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 driedusing rotary vacuum evaporatorat a temperaturenot exceeding40°C, while the aqueous extract was dried using lyophilizer (freeze dryer). The thicklayerof thebottom was stored insterilebottles at 4°C for further experiments.

All extracts were filter-sterilized using a 0.45μm membrane filters (whatman,UK)[10].

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 theCentral 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 *Streptococcus*.

Cultured Method of pathological sample:

Media cultures were Prepared according to the manufacturer's instructions. Thensterilized by autoclaving at 121˚Candunderthepressureof 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 3000cycles/minute, then the serum was pouredand 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 (Haimedia) plate, and incubated for 24 hours at 37°C. All the samples were planted within two hours from the time of sampling.

Selective cultures for bacterial growth:

-MacConky agar (Haimedia) which was used in order to distinguish *Streptococcus faecalis,* by using the sterilized platinumrod, then incubated for 40 to 48 hours at 35-37 °Cat an aerobic culture incubator.

-Bile Esculin Agar (Haimedia) was used for the growth and isolation of *Streptococcus* bacteria, and checked for Esculin and blood hydrolysis, by using the sterilized platinum rod, then incubated for 40 to 48 hours at 35-37 °Cat an aerobic culture incubator.

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-Mannitol,ramnose , Lactose,Sorbitol, Trihalose.

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 [12, 13] .

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[12, 13] .

4-5colonies ofbacteria were suspended (afterpure isolation andidentification) to the test tube in 2 ml of physiological solution, Mixed thoroughly until aturbidhomogeneous obtained. Sterile swab stick simmersed in suspension, and spread onto the surface of the Muller Hintonagar 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 24hours. 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.

A bacterial growth inhibition test of plant extracts by the disk diffusion method against Streptococcusthat 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 agarand incubatedfor 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 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:
Gram staining :round, Gram-positive, nonmotile, nonsporing bacteria that form winding chains, compatible with kayser and Ebeid[14].
Culture on blood agar: small, whitish-gray colonies surrounded by large hemolysis zones.These results were depended according to Kayser[14]. The results of Culture of *Streptococcus* type on selective media as shown in Table (1) according to Ibrahim[15]

Table (1): the results of Culture of *Streptococcus* type on culture medium

Streptococcus type	MaCconkey agar	Bile Esculin Agar
Streptococcus agalactiae	-	-
Streptococcus dysgalactiae	-	-
Streptococus uberis	-	+
Streptococcus faecalis	+	+

6-1-3- The results of biochemical tests:
The results were shown in Table 2, These results were depended according to(16).

Table(2):the results of biochemical tests of *Streptococcus* type

Test	***Streptococcus faecalis***	***Streptococusuberis***	***Streptococcus dysgalactiae***	***Streptococcus agalactiae***
Oxidase	+	+	+	+
Catalase	-	-	-	-
Indol	-	-	-	-
Hemolysis	α-hemolysis	α-hemolysis	α-hemolysis	β-hemolysis
Lactose	+	+	+	+
Mannitol	+	+	-	-
Raffinose	-	-	-	-
Sorbitol	+	+	-	-
Trehalose	+	+	+	+

Table (3): the percentage of affected samples out of the total number

Bacteria name	**percentage of affected samples %**
Streptococcus	14.01
Streptococcus agalactiae	1.92
Streptococcus dysgalactiae	2.09
Streptococusuberis	7.5
Streptococcus faecalis	2.5

Antimicrobial susceptibility results against *Streptococcus types*:

Testing was done according to manufacturer's instructions and according to guidelines developed by the National Committee for Clinical Laboratory Standards (NCCLS) [13]. The percentage of antimicrobial sensibility of *Streptococcus* isolates to the antibiotics are shown in Table (4).

Table(4):The percentage of antimicrobial sensibility to *streptococci*isolates

Antibiotic	Susceptible%	Intermediate sensitive%	Resistant %
Oxytetracycline(T)	3.2	9.3	**87.5**
Amoxicillin(AX)	3.1	6.2	**90.07**
Oxacillin(OX)	16.61	17.56	65.83
Cefadroxil(CER)	16.43	27.53	56.04
Pefloxacin(PEF)	10.54	22.56	66.9
Amikacin(AK)	3.8	11.53	84.67
Tetracyclin(TE)	5.7	9.6	84.7
Ciprofloxacin(CIP)	**19.23**	28.84	51.93
Norfloxacin(NOR)	9.19	18.57	72.24
Gentamycin(CN)	12.36	69.02	18.62
Chloramphenicol(C)	**22.12**	22.12	55.76
Enrofloxacin(ENR)	3.8	15.38	80.82
Doxycyclin(DO)	9.3	14.4	76.3
Cephalexin(CL)	9.74	19.62	70.64
Cephalotin(KF)	2.8	8.6	**88.6**

Table(5): Showsthe number of samples infected and their percentage

The total number of samples infected=1371	Number of samples infected	Percentage of samples infected
Infected samples by *Streptococcus* bacteria of the total number of samples	192	14.01%
Infected samples by resistant *Streptococcus* to all antibiotic of the total number of samples	98	7.14%

The results of Antibacterial Efficacy of plant extracts:

The ethanol extracts showed the antibacterial activity, on the contrarily, the water and ether petroleum extracts have no antibacterial effect (diameters of zone of inhibition were zero). Table (6) shows the results obtained of the antibacterial activity for the various extracts obtained from the studied plants, via determination of the diameter zones of inhibition .

Table (6): Antibacterial activity of different extracts of studied plants

Plant	Inhibition zones of plant extracts (mm)			Percentage of sensitive bacteria %
	Water extract	Ether petroleum extract	Ethanol extract mean ±S.D	
Control/5 µm	0	0	0	0
thymus vulgaris leaves	0	0	21.1±1.2	97.2
Ficus leaves	0	0	20.4±0.6	97.6
Rosemery leaves	0	0	19.2±0.7	96.9
Myrtuscommunis leaves	0	0	18.1±1.09	98.6
Achilleafalcata flowers	0	0	9.2±0.8	98.16
OleaEuropea leaves	0	0	7.5±0.8	98.08

DISCUSSION:

Mastitis is the most important disease in dairy milk production worldwide. *Staphylococcus* and *Streptococcus (S. aureus*, *Str. uberis*, *Str. dysgalactiae*and *Str. Agalactiae)*, are the major contagious pathogens of bovine mastitis, frequently combined with *E. coli*[17]. Out of 1371 milk samples were obtained from clinical cases of mastitis cows,192 (14.01%) infected samples by *Streptococcus*bacteria, and (7.4%) infectedsamples by resistant *Streptococcus* to all antibiotic ofthe totalnumber of samples. The same as Sumathi study has (16%) of *Streptococcus spp*[18]and Myllys found (11.3%) of *Streptococci* strains isolated from bovine mastitis in Finland[19]. The bacterial species isolated were *Streptococcusagalactiae*(22.11%), *Streptococcus dysgalactiae*(11.43%) in Atyabi study [20]. Other research reveald that *Str. uberis*(53%),*Str. dysgalactiae*(29%), and *Str. agalactiae*(27%)[17], while in our study were *Str. agalactiae*(1.29%), *Str. Dysgalactiae*(2.09%), *Str. Uberis*(7.5%),and *Streptococcus faecalis* (2.5%).

The in vitro antibiogram studies of the bacterial isolates from mastitis milk proved theChloramphenicol to be the drug of choice in this study (22.12%),and ciprofloxacin (19.23%),then Oxacillin (16.61%),Cefadroxil (16.43%), and Gentamycin(12.36%).Those results weren't in agreement with sumathi study, who revealed the gentamicin to be most effective drug (90%) followed by enrofloxacin (88%),ciprofloxacin (85%), and chloramphenicol (75%)[18].

On the contrary, the present study showed that streptococci isolated from the infected udder milk samples were resistance to 14 studied antibiotics out of 15.A higher resistance was recorded against Amoxicillin (90.07%), Cephalotin (88.06%), Oxytetracycline (87.5%),and Amikacin (84.67%), Tetracyclin (84.7%).While Martín study revealed a high resistance against lincomycin (61.9%) and enrofloxacin (38.1%)[9]. This difference may be due to the effects of antibiotics by antibiotic commonly used for treatment.That lead us that there is a need to develop alternative antimicrobial drugs for the treatmentof infectious diseases. So we have used six plants with different extracts. In the present study, the ethanol extracts of the plants exhibited disparate antibacterial activity (Table 6), while no effect for water, neitherpetroleum ether extracts was observed. The ethanolic extracts showed strong activity (inhibition zone 21-18 mm), and weak inhibition (zone 7-9 mm). According to this, the major effectiveness was achieved by the ethanolic extracts from *thymus vulgaris*,*Ficuscarica,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, ***Thymus Vulgaris*** extract had a significant antimicrobial activity against *Streptococci*[21].Essential oils derived from Thymus have been found to possess significant antifungal, insecticidal, and antimicrobial activities. These properties depend mainly to their phenolic compounds (thymol and carvacrol) [22]. According to Ivanovic research, there is no difference in antibacterial activity of thyme supercritical extract and thyme essential oil[23]. P-cymene (carvacrol precursor) is a very weak antibacterial, andswells bacterial cell membranes to a greater extent than carvacroldoes. By this mechanism p-cymene probably enables carvacrol to convey into the cell so

that asynergistic effect is achieved [24].The structure of thymol is similar to that of carvacrol; both substances seem to make the membrane penetrable. Their structure shatteredthe external membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP [25].

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, and Flavonoids acting as antioxidants [1].*Ficus capensis* revealed the presence of alkaloids, flavonoids, tanins, terpenes, resins, and sterols .The alkaloidsreveals its activity against pathogenic bacteria [26].The petroleum ether extract of *Ficus racemosa* leaves had effectiveness against *Escherichia coli*, and *Staphylococcus aureus*[27], whereas that was disagree with our results .Also water extract of methanol extracts of *Ficus carica* leaves had inhibited growth of *Staphylococcus aureus*, and *Streptococcus pyogenes*[28].

There is not much knowledge concerning anti-*streptococcal* activity of ***rosemary*** (*Rosmarinuso fficinalis*); the mechanism underlying this effect is not known.It was reported that both water and methanolrosemary extracts had different efficacy as antimicrobial agent, which linked to their different polyphenol compositions. 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 containing only rosmarinic acid revealed a narrow efficacy. Therefore, the antimicrobial rosemary extractsactivity was associated with their specific phenolic composition [29]. Ourextract has the same impact, where just the ethanol extract has the antibacterialefficacy. Another reported that aqueous and methanolic extracts of rosemary inhibited *S. sobrinus* growth and its glucosyl transferase activity[30], this result comply with my extracts, but only ethanol extract has the antibacterial efficacy.

Myrtuscommunis leaves contain different polyphenolic classes as flavonols and galloyl derivatives, which areresponsible to their antimicrobial action. Active phenolic compounds might have several targets which could lead to the inhibition of bacteria. Most of the studies on the mechanism of phenolic compounds have focused on their effects on cellular membranes. They attacked not only cell walls and cell membranes, but affecting their permeability and the release of intracellular constituents, and interfering with membrane functions such as electron transport, enzyme activity or nutrient uptake [31]. Moreover the inhibition activity may be due to presence of Tannin via producing hydrogen bonds with proteins, that converted its structure and lead to block the protein synthesis.Tannins considered as a phenolic compounds of plants which have anti oxidative effects [32]. Mansouri*et al* evaluated the antibacterial activity of methanol crude extract of *M. communis* against *Streptococcus pneumoniae, S. pyogenes, and S. agalactiae*[33]. The inhibition effect of *Myrtus communis* may refer to the polyphenolic and Tannin they contain.

*Achillea, and Olea*had the same impact,9-7 mm inhibition zonesdiameters.

Information about the antimicrobial activity of *Achillea* extracts is limited. The study of the 13 Turkish *Achillea* species demonstrated that not all of the *Achillea* species possess antibacterial activity. Achillea *falcata* showed mild to low antibacterial activity[8], which matched our results.

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 [34].The composition of the extract of *Achillea clavennae*are alkanes, fatty acids, monoterpenes, guaianesesquiterpenes, and flavonoids (apigenin and centaureidin) [35].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 had strong antimicrobial activity against both Gram-negative and Gram-positive bacteria, as well as mycoplasma[36].unfortunately, the ethanol extract we had 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[36].

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 [31].

CONCLUSION: The ethanol extracts of the studied plants revealed different antibacterial activity against *Streptococcus,* and had shown antibiotics resistance. While the water and ether petroleum extracts had no antibacterial effectiveness.

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