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Antimicrobial efficacy of *Thymus vulgaris* extract against some *Staphylococcus* species isolated from subclinical mastitis in cattle in Basrah province, Iraq

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Abstract

Background: *Thymus vulgaris* extracts can play a significant role as alternatives for antimicrobial agents against bovine staphylococcus mastitis.

Aim: This research's goal was to evaluate the antibacterial properties of an extract from *T. vulgaris* as an alternative to antibiotics for bovine Staphylococcus mastitis. In addition, it is important to know the effect of the extraction methods (hot alcoholic, cold alcoholic, and hot water extract) on their effectiveness.

Methods: Two hundred ten cow milk samples from different areas of Basrah province had been suffering subclinical mastitis reported by using the California mastitis test (CMT). *Staphylococcus* species were identified by conventional microbiological technique, GP24 Kit, and *nuc* gene. Antimicrobial activity of various concentrations of *T. vulgaris* extracted (75, 50, 25) mg/ml with different methods of extraction (hot alcoholic, cold alcoholic, and hot water extract).

Results: Out of 210 samples, 99 (47.1%) were positive for the CMT, and the identification rate of *Staphylococci* spp. by conventional microbiological technique and GP24 kit was 78 (78.8%). Out of 78 isolates of *Staphylococcus* spp. 48 (61.5%) were identified as *Staphylococcus aureus,* by using both molecular techniques using PCR and miniaturized Kit GP24 and employing the miniature GP24, the remaining 30 (38.5%) were determined to be different species of Staphylococcus. Antibacterial activity of various concentrations of *T. vulgaris* extracted (75, 50, 25) mg/ml with different methods of extraction revealed that hot alcoholic extract (100%) was more effective than cold alcoholic extract (66.7%), whereas there is no effect on the bacteria species with the hot water extract.

Conclusion: *Thymus vulgaris* extracts can play a significant role as alternatives for antimicrobial agents against bovine staphylococcus mastitis.

Keywords: California, *Staphylococcu*s species, Subclinical mastitis, *Thymus vulgaris.*

Introduction

Bovine mastitis, which can result from physical harm or microbiological infections, is an inflammatory response of the mammary gland's tissues (Gomes and Henriques, 2016; Kukeeva *et al*., 2023). Because it reduces both the amount and quality of milk, it is regarded as the most common sickness that costs the dairy industry money (Sheet *et al*., 2023). On the other hand, milk with subclinical mastitis (SCM) could play a role in introducing the bacterium into the human food chain. The most typical bacteria to be identified from the milk of dairy cows is staphylococci (Taponen and Pyörälä, 2009; Mørk *et al*., 2012; Condas, 2017). The genus Staphylococcus belongs to the family Staphylococcaceae of the bacterial order Bacillales, which generates irregular clusters resembling bunches of grapes from spherical cells between 0.5 and 1.5 m in diameter (Lakhundi and Zhang, 2018). Staphylococci colonize the skin and mucous membranes, especially anterior nares. Staphylococci are characterized as non-spore-forming, non-motile, facultative anaerobes that are developed by way of aerobic respiration or by using fermentation, catalase-positive and oxidase-negative (Markey *et al*., 2013). Bacteria producing coagulase use it as a protective mechanism by coagulating the plasma areas around them, thereby avoiding phagocytosis (Taponen and Pyörälä, 2009).

Staphylococcus aureus is a major pathogen in both humans and a wide range of animals, in particular dairy cattle, which is of economic importance to the dairy industry (Heikki *et al*., 2018). Despite the fact that using antibiotics is still the primary technique for treating bovine mastitis, the advent of bacteria that are resistant to antibiotics is constantly growing (Cheng and Han, 2020). However, the pathogen's resistance to -lactam antibiotics, including methicillin, has shown that antibiotics are not an effective treatment (Rainard *et al*., 2018). Such strains are referred to as methicillin-

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resistant *S. aureus*, and the *mecA* gene that confers the resistance is present in these strains (Aboud, 2019).

In addition to phenolic chemicals, nitrogen compounds, vitamins, terpenoids (including carotenoids), and certain other endogenous metabolites, plants are also capable of synthesizing aromatic compounds. These compounds act as a plant's defensive mechanism against herbivores, insects, and microorganisms (Bharathi *et al*., 2011). Due to their pharmacological and biological characteristics, thymus species are regarded as therapeutic plants (Rota *et al*., 2008). Studies have shown that thyme oil (also known as thymus), which has a strong scent and therapeutic benefits, contains more than 44% phenols, mostly made up of 41% thymol and 3.6% karvacrol. Caffeic acid, triterpene, rosmarinic acid, and oleanic acid are polyphenolic acids found in the oil, while resins, gums, and tannins make up around 10% of this plant's total composition. It is the primary active ingredient in Listerine and toothpaste and is used as a disinfectant because of its antibacterial characteristics (Kakel, 2008; Mohsenipour and Hassanshahian, 2015). Therefore, the aim of the present study was to evaluate the antibacterial activity of *Thymus vulgaris* extract against some *Staphylococcus* spp. isolated from the SCM. In addition, compare the efficiency of different methods of extraction of *T. vulgaris* against these bacteria.

Materials and Methods

Samples collection

Two hundred and ten cow milk samples were collected from different areas of Basrah province that had been suffering from SCM reported by using the California mastitis test (CMT) during the period (February 2018 up to July 2019).

Identification of Staphylococcus spp.

Conventional microbiological identification

The positive milk samples to CMT were submitted to bacteriological examination by inoculation on both blood agar and mannitol salt agar and incubated overnight at 37°C under aerobic conditions. Hemolysis, Gram staining, and colony morphology were used to examine primary cultures. Catalase, oxidase, DNase, coagulase, and biochemical tests were performed on the suspicious colonies on mannitol salt agar (Macfaddin, 2000).

Miniaturize kit GP24

The GP24 (Slovak, Slovakia) test, which consists of 24 biochemical tests plus a homogeneous bacterial suspension in 100 ml of turbid solution at 3 McFarland turbidity, was used to analyze the suspicious isolates. The H1 and H2 wells' urea (URE) and arginine (ARG) wells were coated with a few drops of paraffin oil. Then, the plate was incubated for 24 hours at 37°C. By using an identification table and online program "DIAGNOSTICS s. r. o."

Molecular identification

Genomic DNA was extracted from probable *S. aureus* isolates using a DNA kit (Geneaid, USA) in accordance with the manufacturer's instructions. PCR identification of *S. aureus* isolates utilizing the *nuc gene* (423 bp) (Wongboot *et al*., 2013) The specific forward and reversed primers "5 – GCT TGC TAT GAT TGT GGT AGC C -3' and 5 – TCT CTA GCA AGT CCC TTT TCC A- 3'," respectively, were made by "Bioneer, Korea."

PCR amplification

PCR master mix reaction was prepared according to the company. The PCR procedure included "an initial denaturation at 94°C for 7 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, and extension at 72°C for 45 seconds. The last cycle was an extension at 72°C for 7 minutes." PCR products were run on agarose gel electrophoresis for 1 hour (100 V). The DNA bands were visualized by a gel documentation system and photographed.

Preparation of T. vulgaris extracts

Cold alcohol extract

Fifty grams of *T. vulgaris* leaves were mixed with 300 ml of 70% ethyl alcohol. The mixture was agitated at ambient temperature for 3 days before being filtered by Whattmann number 3. The filtrate was put into a Petri

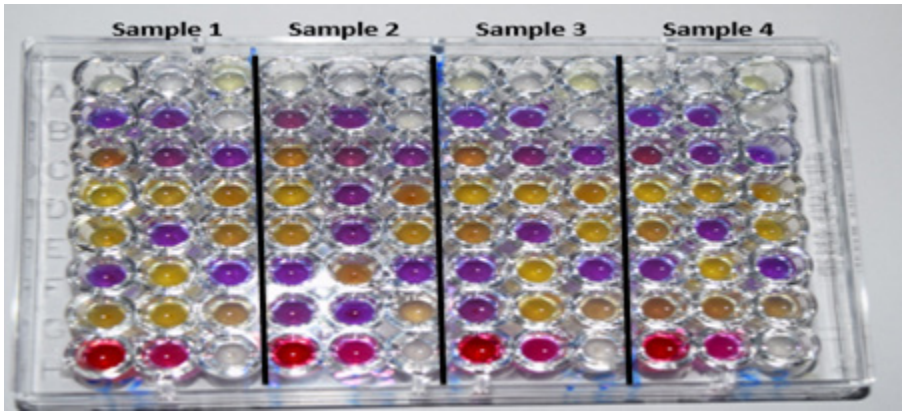

Fig. 1. Microtitration plate of GP24 (the plates designed for four isolates).

dish and allowed to dry at room temperature after being rotary evaporated at 80°C (Jonathan, 2009).

Hot alcohol extract

To make a hot alcohol extract, 300 ml of 70% ethanol were mixed with 50 g of *T. vulgaris* powder. The solution was refluxed for 3 days, then filtered through Whattmann No. 3 and evaporated at 60°C using a rotary evaporator (Hasan *et al*., 2009).

Hot water extract

To get hot water extraction, 300 ml of distilled water were added to 50 g of powdered *T. vulgaris* leaves. The solution was refluxed for 3 days, then filtered and evaporated at 60°C using a rotary evaporator (Hasan *et al*., 2009).

Antimicrobial efficacy

The antibacterial activity was evaluated using the disk diffusion method (NCCLS, 2013), which involves wetting absorbent sterilized paper discs (9 mm in diameter) with extracts to determine antimicrobial activity. Loopful isolated colonies from an overnight culture were selected from the agar plate culture and transferred into a tube containing 5 ml sterile normal saline until the turbidity was approximately equivalent to 0.5 McFarland standards (1.5 * 108 cells/ml). After 15 minutes. sterile cotton swab was dipped into the inoculum suspension, and it spread over the agar. The discs were placed on the agar's surface. The microorganisms thrived everywhere over the agar surface except where the substance that inhibited their growth was present. A definite circular inhibition zone was detected after incubation around the discs. The formation of clean zones surrounding the discs was used to determine the effect of the extracts on bacteria. All of the tests were carried out in duplicate.

Statistical analyses

Using IBM SPSS 22 software, the data were statistically processed.

Ethical approval

The ethical approval was provided by the scientific committee of the College of Veterinary Medicine at the University of Basrah.

Results

Detection of SCM

Ninety-nine (47.1%) cow milk samples out of 210 tested for SCM were positive for CMT.

Isolation and identification of Staphylococcus spp.

Using conventional microbiological methods, out of 99 samples investigated, 78 (78.8%) *Staphylococcus* spp. were isolated. All suspected isolates chosen by using the conventional bacteriological technique were subjected to identification by using a GP24 kit shown in Figure 1, and the results were identified by online software (Fig. 2); all isolates from conventional microbiological

Fig. 2. Result of online software for identification of *Staphylococcus* spp. by using GP24. (A) *S. aureus*. (B) *S. chromogenes*.

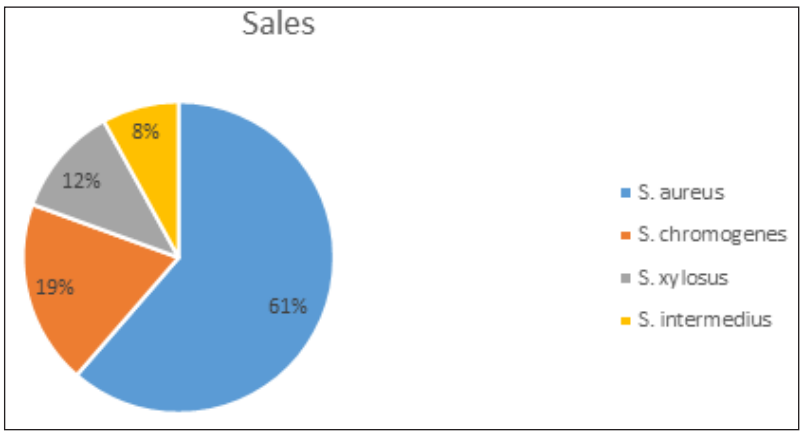

Fig. 3. The percentage of *Staphylococcal* spp. identified by using the GP24 kit (X^2 = 95.467; DF = 3; *p*-value = 0).

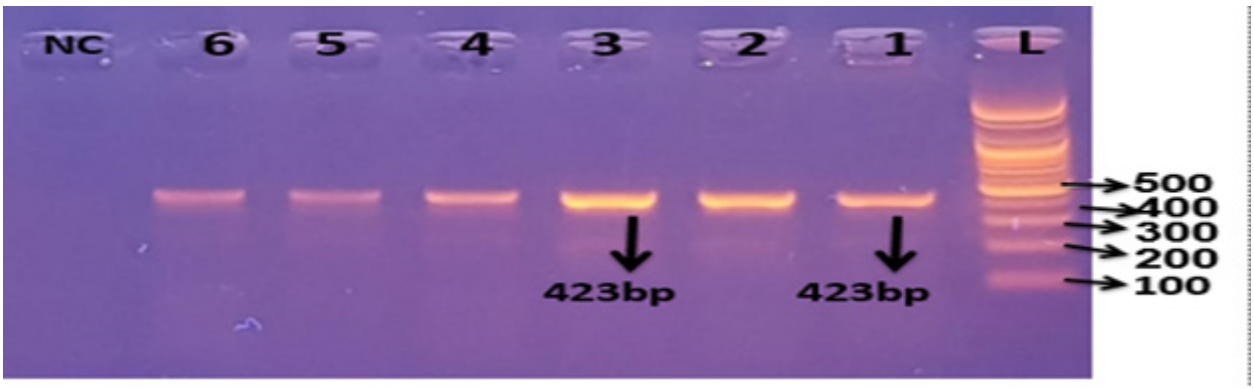

Fig. 4. PCR amplification of *nuc* gene from *S. aureus*. Using a 1.5% agarose gel and ethidium bromide as a dye, the 423 bp-sized pcr product was run. Negative control (NC) and ladder (L).

techniques were identified as *Staphylococcus* spp. 78 (78.8%) by using miniaturized GP24, the results showed that the high percentage of *S. aureus* isolates 48 (61.5%), followed by *Staphylococcus chromogenes* 15 (19%), *Staphylococcus xylosus* 9 (12%), *Staphylococcus intermedius* 6 (8%) (Fig. 3). There was a significant difference ($p < 0.05$) of isolation rates among the *Staphylococcus* spp.

Out of 78 isolates of *Staphylococcus* spp. 48 (61.5) were identified as *S. aureus* by using PCR analysis for the detection of *nuc* gene (Fig. 4).

The present study showed that the susceptibility of Staphylococcal isolates to *T. vulgaris* crude extracts is summarized in (Table 1). On the other hand, the hot alcoholic extracts are more effective at a concentration (75 mg/ml) on bacteria, followed by 50 mg/ml concentration (Fig. 5)

Discussion

Early detection of mastitis is critical for dairy farmers to avoid economic losses related to lower yields, treatment costs, and lost milk (Bhutto *et al*., 2012). In a dairy herd monitoring system, the CMT may be utilized as a screening tool for cows with intramammary infection (Sargeant *et al.*, 2001). In this study, the results of the CMT revealed the number of samples showing positive was 99 (47.1%). This result is in line with several studies such as (Kader *et al.*, 2002), which detected the prevalence as 44.61% SCM in Bangladesh. In Iraq studies, (Hussein, 2012; Mohammed, 2020) recorded that the incidence of SCM among the sampled cattle was 38.9% and 41.17%, respectively. According to additional research done in Basrah, the identification rate of SCM by CMT ranged from 38.5% to 57.6% (Al-Iedani, 2016; Al-Iedani and Ghazia, 2016).

In this study, 78.8% of mastitis bovine milk samples were positive for *Staphylococcus* spp. Additionally, these results were in line with (Shrestha and Bindari, 2012), who found the highest incidence of *Staphylococcus*, followed by *Escherichia coli*, *Streptococci*, and *Corynebacterium*. The opportunistic bacterium *Staphylococcus* can invade through the teat canal and can thrive on the skin of the udder (Pyorala and Taponen, 2009). On the other hand, results of culturing and identification of the causative agents revealed

Table 1. Antimicrobial activity of T. vulgaris crude extracts.

Staphylococcal spp.	No. of isolates	Inhibition zone diameter					
		Hot alcohol extract (125 mg/ml)		Cold alcohol extract (125 mg/ml)		Water extract (125 mg/ml)	
		No.	%	No.	%	No.	%
S. aureus	48	34	70.8	20	42.9	0	0
S. xylosus	9	9	100	6	66.6	0	0
S. chromogenes	15	15	100	8	50	0	0
S. intermedius	6	6	100	3	50	0	0
Total	78	64	82.05	37	47.43	0	0

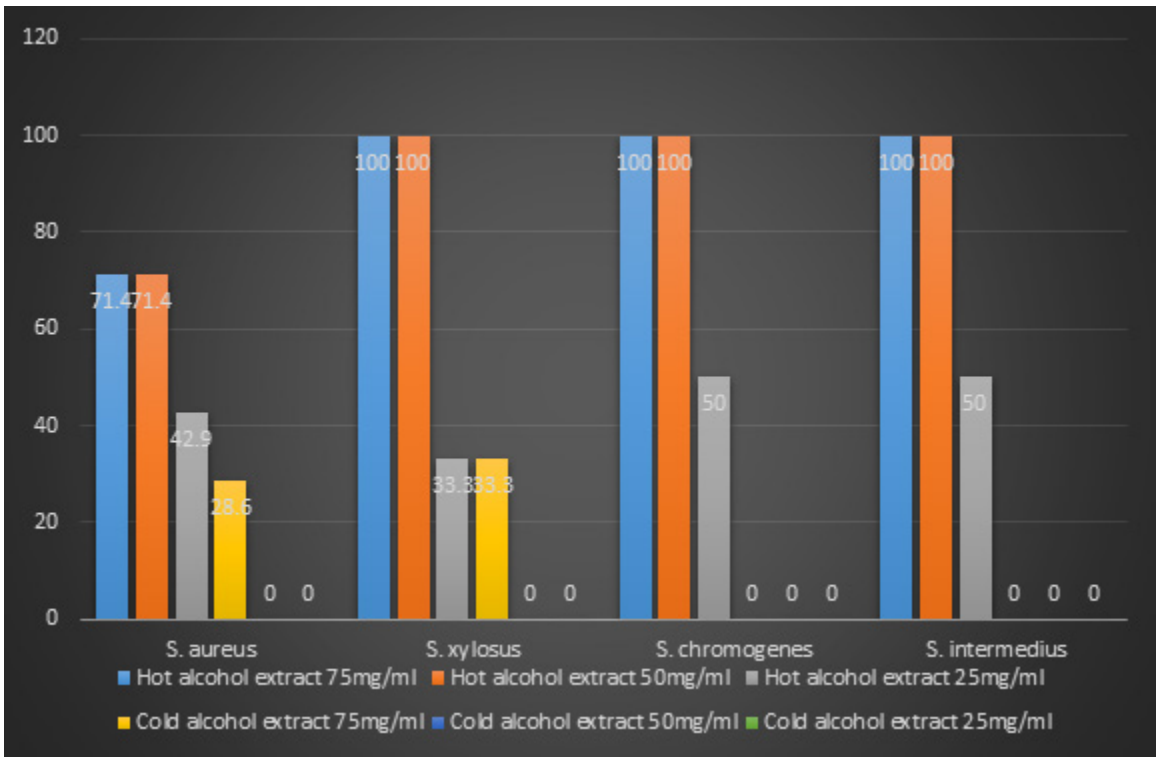

Fig. 5. Minimum inhibitory concentration of *T. vulgaris* extract to *Staphylococcal* spp. isolates.

that *S. aureus* was the most predominant bacteria as they were isolated 61.5% by *nuc* gene; this gene is an important pathogenic factor, and the thermostable nuclease (Sayhood *et al*., 2022). This result is in line with (Heikki *et al*., 2018). There are at least 43 species that were described in the *Staphylococcus* genus; four (*S. aureus, Staphylococcus epidermidis, Staphylococcus pseudintermedius,* and *Staphylococcus hyicus*) are significant in livestock.

In comparison with Iraqi studies, especially in Basrah province, the current results also were compatible with the study (Mohammed, 2020) which reported that *S. aureus* isolation rate (64.28%); on the other hand, lower results were obtained by Sheet (2022), Aboud (2019), Khudaier *et al*. (2013), and Hanon (2009) who recorded that *S. aureus* isolation rates 34.8%, 36.84%, 48.61%, and 48.57%, respectively.

The current study showed the highest bacterial activity of hot ethanolic extract of *T. vulgaris*, followed by cold ethanolic extract at 82.05% and 47.43%, respectively. The antibacterial activity of *T. vulgaris* extracts may be due to the presence of phenolic constituents (thymol and carvacrol), which make up a large percentage of the volatile oil (Nakatani, 2000). On the other hand, due to their hydrophobic nature, the two most researched monoterpenes from *T. vulgaris*, carvacrol, and thymol, can integrate into bacterial cell membranes, disrupting

normal membrane function and increasing ATP permeability (Kachu and Suntres, 2019).

The significant amount of active ingredient that precipitated during the extraction process as a result of the solvent may be used to explain the variations in the effects of the type of extraction on bacteria. Most phytoconstituents (alkaloids, saponins, carbohydrates, tannins, and flavonoids) were extracted from *Psidium guajava* L. leaves using ethanolic and hydroalcohol extracts (4:1 v/v), compared to other solvents like petroleum ether, chloroform, and water. Water extracts had equal efficiency to ethanol extracts, with the exception that no trace of alkaloids was found in the water extracts (Arya *et al*., 2012). There have been a number of reports validating the in vitro antibacterial and antifungal activities of this essential oil, including *S. aureus* (Azza *et al*., 2014; Nikolic *et al*., 2014; Lira Mota *et al*., 2012).

Conclusion

In conclusion, hot alcoholic extracts of *T. vulgaris* have more effective as antibacterial activity than both cold alcoholic and aqueous extracts. We recommended isolating and purifying the bioactive compounds from *T. vulgaris* extract, in addition to evaluating their extract *in vivo* as an alternative antibacterial activity.

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Authors contributions

This research was conceptualized by Abeer Laily Mohammed. The study was co-authored by all authors. The final manuscript was read and approved by all authors.

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Data availability

The data supporting the findings of this study are available within the manuscript. Any other data are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that there is no conflict of interest.

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