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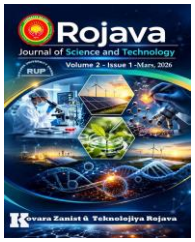

Thymus Vulgaris Extract as a Natural Antimicrobial to Prevent North and East Syrian Nosocomial Microbes

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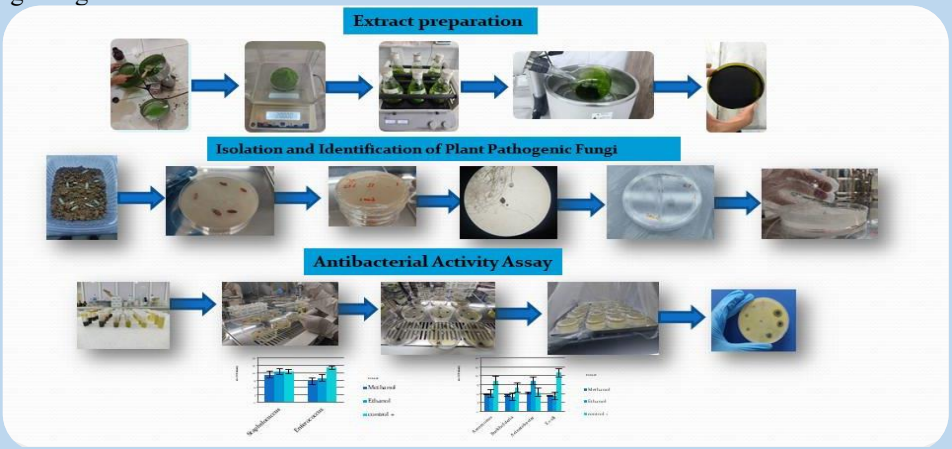
Abstract

Local herbal plants represent an important source of the traditional medicine and are a good source of nutrients. Healthcare-associated infections are a major issue in healthcare systems worldwide especially in low- and middle-income countries. Nosocomial infections following antibiotic resistance are an emerging problem in Middle Eastern countries, leading to significant morbidity and mortality. The objective of the present research was to verify the antibacterial activity of the methanol and ethanol extracts of *Thymus vulgaris* against seven nosocomial microbes, *Staphylococcus*, *Enterococci*, *Aeromonas*, *Burkholderia*, *Acinetobacter*, *E. coli* and *Candida* spp., isolated from hospitals of North and East Syria. For this, the analyses of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by the well agar diffusion technique using and also disc diffusion method. Gas chromatography-mass spectrometry (GC-MS) technique was used for characterization of bioactive compounds in the extracts. Ethanol extract of *Thymus vulgaris* showed a strong inhibitory activity against *Staphelococcus*, *Acinetobacter* and *Enterococcus* with the MIC values ranging from 20 to 50 mg/mL while no differences between the ethanolic and methanol extracts were recorded for *Burkholderia*, *E. coli*, *Aeromonas*, with the MIC values ranging from 100 to 200 mg/mL and bactericidal effect for most strains, with MBC values ranging from 50 to 200 mg/mL. Moreover, the ethanol extract demonstrated a higher inhibition of *Candida* spp. compared to the methanol extract. Spathulenol (6.86%), thymol (3.41%) and p-cymene (3.4%) were reported as a major ethanol extract components of *T. vulgaris*. Therefore, Thyme ethanol extract presents itself as an antibacterial and antifungal agent against nosocomial microbes.



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1. Introduction

Plant extracts have been utilized in cosmetics for their aromatic and therapeutic properties since antiquity, serving as a valuable source of bioactive compounds that present an alternative to synthetic chemicals in personal care formulations. Developing of antibiotic resistance microbes because of overuse and also unnecessarily using of antibiotics is one of the main problems in North and East Syria (Rojava). With the promotion of this problem, herbal plants have considered as an undertaking source for the discovery of new antibacterial drugs. *Thymus vulgaris* is a species of flowering plant from *Lamiaceae* family, native to southern Europe from the western Mediterranean to southern Italy. *Thymus vulgaris* Syrian native is a bushy, woody-based evergreen sub shrub with linear, highly aromatic, grey-green leaves and clusters of purple or pink flowers in last spring or early summer (Hosseinzadeh *et al*., 2015). The extract and essential oil of *Thymus vulgaris* exhibit significant biological activity and was used in herbal medicine for centuries. *T. vulgaris* is described by chemical polymorphism according to the major volatile compounds and its geographical region. Its primary constituents include thymol, p-cymene, 1,8-cineole, γ-terpinene, and carvacrol. Due to the antimicrobial properties of these compounds in extract and essential oil, *T. vulgaris* has identified with high antimicrobial and antioxidant activities (Thompson *et al*., 2003). Its antimicrobial activity ranges from moderate to very strong.

Nosocomial infections, healthcare-associated infections (HAI), are infection(s) that occur in different areas of healthcare delivery, such as in hospitals, long-term care facilities, and ambulatory settings, and may also appear after discharge. The responsible pathogen for nosocomial infection, bacteria, virus or fungi can spread to a susceptible patient host via invasive procedures and surgery, indwelling medical devices, and prosthetic devices and cause infection. The incidence of nosocomial infections (NIs), as reported in most studies, ranges from 3.6% to 12% in high-income countries and from 5.7% to 19.1% in low- and middle-income countries (Wang *et al*., 2019).

Antibiotics are powerful drugs that are used to fight fatal diseases. As with any powerful medication and high beneficiary effect, an antibiotic carry a wide range of adverse effects and out weights the risks. The inappropriate use of antibiotics in one patient might interrupt the composition of the infectious agent, causing bacterial adaptation or mutations, and in turn, leading to new strains that are resistant to the current antibiotic regimen. The mechanism of antibiotic resistance can be occurred on the basis of Intrinsic Resistance, Acquired Resistance, Genetic Change or DNA Transfer. In 2015, 30% of the outpatient antibiotics prescribed were unnecessary, with acute respiratory infections holding the highest unnecessary use of antibiotics at 50%. Moreover, microbes can to evade the effect of antibiotics through multiple different mechanisms that it causes to antibiotic resistance microbe (Habboush and Guzman, 2023). Nosocomial infections (healthcare-associated infections) following antibiotic resistance are an emerging problem in Middle Eastern countries, leading to significant morbidity and mortality (Nimer, 2022). Previous studies showed that increasing of antibiotic resistance microbes in hospitals is related to nosocomial infections (Hormozi *et al*., 2018).

Staphylococci and *Enterococci* are important Gram-positive bacilli caused nosocomial infections (Sizar *et al*., 2023). *Staphylococci* can cause many types of infection such as superficial skin lesions, osteomyelitis, endocarditis, furunculosis and hospital acquired (nosocomial) infection of surgical wounds and causes infections associated with indwelling medical devices. They express many potential virulence factors such as surface proteins for promotion colonization of

host tissues, phagocytosis inhibition factors and toxins to damage host tissues. *S epidermidis* is one of the species that can readily colonize on implanted devices (Foster, 1996). *Enterococci* are a common cause of Urinary Tract Infections (UTIs), bacteremia, and infective endocarditis and rarely intra-abdominal infections and meningitis, which cause difficult-to-treat infections in the nosocomial setting. They have intrinsic resistance to some antibiotics and they can transfer this ability to other bacteria via mobile genetic elements like plasmids, conjugation, and transposons (Said *et al*. 2024).

Aeromonas, Burkholderia, Acinetobacter and *E. coli*, gram-negative bacilli, are commonly associated with nosocomial infections such as pneumonia, surgical site infection (SSI), urinary tract infection (UTI), and bloodstream infections in ICUs (Gaynes *et al*., 2005). The proportion of Acinetobacter species related to ICU pneumonia increased from 4% in 1986 to 7.0% in 2003, which main increases in resistance rates were seen for selected antimicrobial-pathogen combinations (Gaynes *et al*., 2005).

Candida species are other potential nosocomial pathogens. The infections caused by *Candida* are usually endogenous, although, the exogenous infections may also occur. There are some important factors leading to nosocomial candidiasis such as overusing of antibiotics, immunosuppression, malignancy, surgical intervention, diabetes and prolonged hospitalization. Nosocomial infections caused by *Candida* are difficult to diagnose and to therapy (Jahagirdar *et al*., 2018).

The most commonly used techniques for studying the component profile of plant extracts are maceration methods and GC-MS technique. Gas chromatography-mass spectroscopy (GC-MS) is one of the best, fast and accurate techniques to determine and identify various compounds, including alcohols, alkaloids, nitro compounds, long chain hydrocarbons, organic acids, steroids, esters and amino acids (Razack *et al*., 2015). This technique needs a small volume of plant extract for determining the components of extract. It combines two analytical techniques to a single method for analyzing mixtures of chemical compounds and separates the components of the mixture (Shi *et al*., 2005).

This study's purpose is to analyze the biological activity of the ethanol and methanol extracts of *T. vulgaris* as well as determine the chemical composition, of the Thyme extracts using GC-MS. This study focused on antifungal and antibacterial effects of North and East Syria local Thyme extracts on nasocamial fungi and bacteria isolated from local hospitals of North and East Syria (Rojava).

2. Materials and methods

The completely dried and powdered plant materials were allowed to soxhlet extraction with two different solvents, methanol (80%) and ethanol (96%) by shaking on 150 rpm for 48 h at room temperature. Then, the methanolic and ethanolic extracts were filtered through Whatman No-1filter paper. The extracted liquids were subjected to Rotary evaporator and then concentrated under reduced pressure in vacuum at 40 °C. The residue obtained were nominated as crude extracts, were labeled and stored in a sterile screw cap bottle at 5 °C for further study (Nofouzi *et al.*, 2016). The dried plant extract residues were redissolved in 0.1% Dimethyl Sulfoxide (DMSO) to get different concentrations (6.25 mg/ml- 400 mg/ml) of crude extracts and filtration through a 0.45µm membrane filter and stored in a refrigerator at 5 °C until using for bioassays.

2.1 Collection and maintenance pathogenic microorganisms

Totally, six nosocomial bacteria (*Staphylococcus aureus, Enterobacter spp, Aeromonas hydrophila, Burkholderia gladioli, Acinetobacter baumannii and Escherichia coli*) and one nosocomial fungi

cultures were procured from Institute of Science and Modern Technology, Rojava university, North and East Syria. Bacterial cultures were sub-cultured on Nutrient agar medium and stored in refrigerator at 4℃. *Candida* spp. culture was sub-cultured on Glucose-methylene blue Mueller Hinton Agar (GM-MHA) and stored in refrigerator at 4℃.

2.2 Preparation of microbial Inoculum for antimicrobial assay

The bacterial inoculums were prepared by transferring 3–4 fresh colonies into separate tubes containing normal saline. The cultures were incubated on a shaker at 37°C until homogeneity was achieved. The turbidity of each sample was adjusted to an absorbance of 0.08–0.1 at 600 nm using a spectrophotometer (Phylo, UV-920). This turbidity level visually corresponds to the McFarland standard of 0.5. The MH agar was inoculated by streaking the swab against the plate (Kowalska-Krochmal and Ruth, 2021). Fungal inoculum was prepared by transferring 3–4 fresh colonies into tube containing normal saline. The cultures were incubated on a shaker at 35°C until homogeneity was achieved. The turbidity of each sample was adjusted to an absorbance of 0.08–0.1 at 600 nm using a spectrophotometer (Phylo, UV-920). This turbidity level visually corresponds to 0.5.Mc Farland standards and then applied for the evaluation of antifungal activity.

2.3 Antimicrobia assay

The antibacterial activity of *T. vulgaris* plant ethanol and methanol extracts were determined by Agar well diffusion method proposed by Valgas *et al*. (2007). For this purpose, 50 µl of the prepared inoculum was evenly spread on a solid Mueller-Hinton agar (MHA) plate containing wells, 6 mm in diameter made using a sterile borer. The test materials, consisting of 50 µL of plant extract at a concentration of 200 mg per 1 ml dimethylsulfoxide (DMSO 10%), were applied into each well. Amikacin and imipenem (30 µg/disc and 10 µg/disc respectively) were used as positive controls and DMSO was used as negative control for antibacterial assays. For antifungal assay, nystatin (200 mg/ ml) and DMSO were used as control positive and control negative, respectively. Bacteria plates were incubated at 37°C for 24 hours and fungi plates were incubated at 35°C for 48 hours.

2.4 Chemical Characterization of T. vulgaris Ethanol Extract by Gas hromatography/Mass Spectrometry (GC/MS)

In order to identify the bioactive components, plant extracts was analysed using a GC-MS instrument (Perkin Elmer Clarus 500). The GC-MS system was equipped with a capillary column elite-5 (30m × 0.25mm), which consists of 5% phenyl and 95% dimethylpolysiloxane. Helium (99.999%) served as the carrier gas, flowing at a rate of 1 ml per minute with a split ratio of 1:10. A 2µl acetone extract from the sample was injected into the column at an injector temperature of 280°C. The oven temperature began at 60°C without a hold and increased at a rate of 6.0 until it reached 150°C, where it held for 2 minutes. Subsequently, the temperature ramped up to 280°C, holding for 5 minutes at a rate of 4.0. The injector temperature was maintained at 280°C while the detector temperature was kept at 160°C. The mass spectrum of the compounds in the sample was acquired by operating the mass spectrometer in positive Electron Ionization (EI) mode at 70 eV. The detector functioned in scan mode with an interval of 0-5 seconds, capturing fragments from 40-600 Da atomic units.

2.5 Statistical analysis

Data were analysed as a completely randomized design with three replicates by analysis of variance (ANOVA) using the SPSS software (SPSS 26, 64 bit). For antibacterial test, mean of inhibition zone was compared among treatments and two controls. Also for antifungal analysis, fungal growth inhibition was calculated by Mori *et al*. (1997) method and then the mean of growth

inhibition was compared between treatments and two controls. All analyses, was performed at a significance level of %1 (or P<0.01).

3. Results

Plants are a main source of potentially useful materials for the development of new bioactive issues. The knowledge of secondary metabolites and their biological activities is acceptable, not only for discovery of novel bioactive principles, but also for disclosing new sources of already known biologically active compounds. *Thymus vulgaris*, a member of *Lamiaceae* family, contains wide-range of chemical constituents. The stems, leaves, and flowers of *Thymus vulgaris* are used in traditional medicine to treat of variety of diseases such as gastroenteric and bronchopulmonary disorders. The nosocomial infection leads to serious problems and affects humans throughout their life. Often, nosocomial microbes are multidrug- resistant pathogenes acquired by invasive precedures, overuse or improper use of antibiotics. *Thymus vulgaris* have been reported to have a huge variety of compounds which have various bioactivities such as antibacterial, antifungal, antioxidant, anthelmintic, anti-cancer etc (Hosseinzadeh *et al*., 2015).

3.1 Antimicrobial Activity and Minimal Inhibition Concentration (MIC)

The antimicrobial activity was investigated by the well agar diffusion technique evaluating the effect against gram-positive bacteria (*Staphylococcus aureus, Enterobacter spp*), gram-negative bacteria (*Aeromonas hydrophila, Burkholderia gladioli, Acinetobacter baumannii and Escherichia coli*), yeast (*Candida* spp.). In all tested microorganisms, we observed very strong and moderate inhibitory activity of Thyme methanol and ethanol extracts. The results showed that the ethanol extract was effective than methanol in *Staphylococcus aureus, Enterobacter spp and, Acinetobacter baumannii*, which the highest effect was recorded for *S. aureus* (18.66±1.15 methanol and 20.66±0.57 ethanol extract), while no differences between the ethanol and methanol extracts were recorded for *Burkholderia gladioli*, E. coli and *Aeromonas hydrophila* (8.66±0.57 methanol and 8.66±0.57 ethanol extract) (Table 3.1, Fig 3.1 and 3.2). Since the ethanol extract was effective than the methanol extract, only the ethanol extract was examined for the MIC and MBC experiments. The results demonstrate *Staphylococcus aureus* showed highest sensitivity (25 mg/ml for MIC and 50 mg/ml for MBC) to *thymus vulgaris* extract. The MBCs for other bacteria were slightly higher: *Enterococcus* and *Acinetobacter baumannii* 100 mg/ml, *Aeromonas hydrophila, Burkholderia gladioli,* and *E. coli* 200, mg/ml. These results indicate that *Staphylococcus aureus* is more sensitive to the *T. vulgaris* extract while higher concentrations of extract were required to inactivate *Aeromonas hydrophila, Burkholderia gladioli,* and *E. coli* Mohsenipour and Hassanshahian (2015) evaluated the effect of T. vulgaris against three Gram-positive (*S. aureus*, *B. cereusin* active form,and *S. pneumoniae*), and three Gram-negative (*P. aeruginosa*, *E. coli*, and *K. pneumoniae*) bacteria by disk diffusion method and recorded a very strong inhibition in all tested microorganisms. Borugă *et al*. (2014) examined the antibacterial activity of Thyme essential oil against *S. aureus, E. faecalis, C. albicans, S. typhimurium*, and *P. aeruginosa* and recorded a very strong effect of this plant EO on these organisms. Rota *et al*. (2008) recorded zones of inhibition more than 20 mm in the tested microorganisms and showed the Thyme EO as very effective. Our findings on the strong inhibitory activity of *T. vulgaris* extracts are consistent with the authors work above.

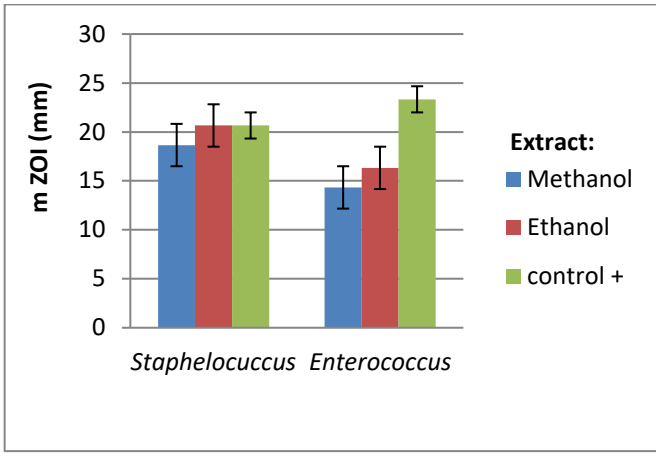

Fig 3.1 Comparison of mean of inhibition zone of methanol and ethanol extracts of *T.s vulgaris* against two strains of gram positive bacteria. Bars within a bacterial treatment, indicate significant difference at α=0.01.

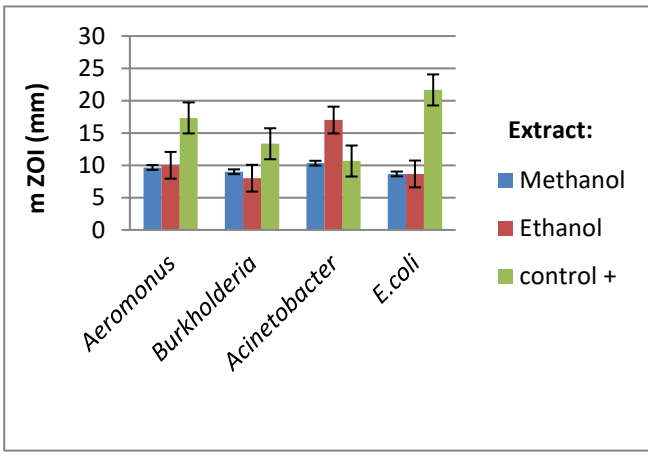

Fig 3.2 Comparison of mean of inhibition zone of methanol and ethanol extracts of *T. vulgaris* against four strains of gram negative bacteria. Bars within a bacterial treatment, indicate significant difference at α=0.01.

Moreover, Thyme ethanol extract (200 mg/ml) demonstrated a higher inhibition zone (19 ± 1.00 mm) against *Candida* spp. compared to the methanol extract (17 ± 1.00 mm), suggesting greater antifungal efficacy (Table 3.1, fig 3.3). These results were agreed with the results by Baj *et al* (2020) and they indicate that *Candida* was affected by *Thymus vulgaris* essential oil. Moreover, Jafri and Ahmad study (2020) revealed the effect of by *Thymus vulgaris* essential oil and its synergistical interact with antifungal drugs against on drug resistant strains of *Candida albicans* and *Candida tropicalis*.

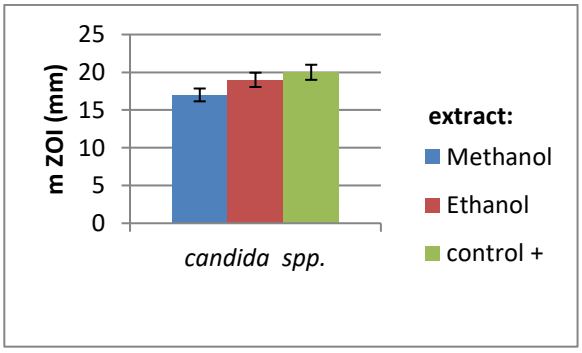

Fig 3.3 Comparison of mean of inhibition zone of methanol and ethanol extracts of *T. vulgaris* against *Candida* spp.. Bars within a fungi treatment indicate significant difference at α=0.01.

Table 3.2 Antimicrobial activity of *Thymus vulgaris* extracts against nosocomial infection microorganisms

microorganisms	**Zone Inhibition**		**Activity of Extract**	**MIC**	**MBC**	**ATB**
	Ethanol extract	Methanol extract				
S. aureus	20.66±0.57	18.66±1.15*	***	25 mg/ml	50 mg/ml	20.66±0.57 Amikasin
Enterococcus spp.	16.33±0.57	14.33±0.57	***	50 mg/ml	100 mg/ml	23.33±0.57 Ipm
A. hydrophila	10.00±1.00	9.66±0.57	*	200 mg/ml	200 mg/ml	17.33±0.57 Ipm
B.gladioli	8.00±0.00	9.00±1.00	*	200 mg/ml	200 mg/ml	13.33±0.57 Amikasin
A. baumannii	17.00±0.00	10.33±0.57	***	50 mg/ml	100 mg/ml	10.66±0.57 Amikasin
E.coli	8.66±0.57	8.66±0.57	*	200 mg/ml	200 mg/ml	21.66±0.57 Amikasin
Candida spp.	19± 1.00	17± 1.00	***	100 mg/ml	---	20.66±0.57 Nystatin

* Weak antimicrobial activity (zone 5–10 mm). ** Moderate inhibitory activity (zone 10-15 mm). *** Very strong inhibitory activity (zone > 15 mm), ATB—antibiotics, positive control (amikacin and imipenem for bacteria and nystatin for *Candida* spp).

3.2 Chemical Composition of *T. vulgaris* Ethanol Extract

The GC-MS analysis of ethanolic extract of *T. vulgaris* identified 24 chemical compounds in different ranges. A total of 24 compounds were identified comprised 39.2% of the extract. The ethanol extract was dominated by the presence of alcohol and phenol derivatives (17.01%) followed by fatty acides (7.38%), alkyl benzens (4.62%), esters (4.35%) and monoterpenes which represent 0.55% of the total extract (Table 3.2). Spathulenol was the most prominent compound found in highest concentration (6.86%) followed by p-Cymene-2,5-idol (as an alkyl benzene: 3.4%) and thymol (3.41%), 9-Octadecyne (2.5%) and trans-13-Octadecenoic acid, methyl ester (2.39%) respectively. Extract preparation and identification of chemical compounds were done as described in the material and methods section. Results were expressed as percentage relative to total chemical compounds extracted from *T. vulgaris*. Our results are in agreement with some other studies (Saleem *et al*. 2022), where spathulenol, thymol and p-cymene were reported as a major methanol extract components of *T. vulgaris*. In a recent study, spathulenol was reported as major compound of ethanol extract from *T. vulgaris*. Spathulenol, a tricyclic sesquiterpene with 5,10-cycloaromadendrane skeleton, has important bioactivity such as anticholinesterase (Karakaya *et al*., 2020), anti-nociceptive, anti-hyperalgesic (Dos Santos *et al*., 2022), anti-mycobacterial (de Jesús Dzul-Beh *et al*., 2019), antioxidant, anti-proliferative, anti-oedematogenic (do Nascimento *et al*., 2018) and cytotoxicity (Mirzaei *et al*., 2017). Since the extract of *T. vulgaris* contains high levels of spathulenol, therefore, the above mentioned biological activities are expected for this compound, yet needs to be examined in the future.

Table 3.2 Chemical composition of Ethanol extract from *Thymus vulgaris.*

S. no.	% area	R. time	Compound name	Biological activity
1	0.81	4.109	Phenol, 2-methoxy	Expectorant and anti-oxidant properties
2	1.22	7.02	Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)	A valuable intermediate in organic synthesis and various chemical transformations such as Demethylation Reactions
3	0.36	9.03	a-Thujene	Anti-inflammatory, antimicrobial and anti-arthritic
4	0.19	10.00	Myrcene	Reducing inflammation and pain, relaxation and sedation, and potential anti-ageing
5	0.07	10.241	Phenol, 5-ethenyl-2-methoxy	Antimicrobial, apoptotic, antimetastatic, anti-inflammatory and antibiotic properties
6	2.16	10.88	Eugenol	Analgesic and Anesthetic Properties, antimicrobial, anti-Inflammatory and antioxidant activity
7	0.61	11.92	Mequinol	Antioxidant properties
8	1.71	12.36	Aromandendrene	Anti-inflammatory, anti-cancer and antibacterial properties
9	3.40	12.89	p-Cymene-2,5-diol	Hepatoprotective, anti-inflammatory, antioxidant, cytotoxic and anti-cancer properties
10	3.41	13.43	Thymol	Anti-inflammatory and antibacterial properties
11	6.86	14.45	(-)-Spathulenol	Anti-Inflammatory, antimicrobial, antioxidant antitumor potential
12	1.02	16.08	Methyl-(2-hydoxy-3-ethoxy-benzyl)ether	Antioxidant, Anti-Inflammatory and Antimicrobial activity against bacteria and fungi.
13	1.42	17.92	Tetradecanoic acid	Skin Care and Anti-Microbial properties
14	0.72	18.01	Isopropyl myristate	Pediculicide activity
15	2.5	18.87	9-Octadecyne	Antibacterial and antioxidant properties
16	0.8	19.43	2-Pentadecylfuran	Antifungal, anti-inflammatory, antibacterial, antiviral, antioxidants, anticonvulsants, antihypertensive and anti-leprosy activities
17	1.01	19.81	Pentadecanal	Inhibits biofilm formation by interfering with the quorum sensing system of certain bacteria.
18	1.96	20.21	Pentadecanoic acid, 14-methyl-, methyl ester	Antifungal and antimicrobial activity
19	1.34	21.91	Methyl 9-cis,11-trans-octadecadienoate	Antimicrobial activity
20	2.39	22.517	trans-13-Octadecenoic acid, methyl ester	Anti-inflammatory and cancer preventive properties
21	1.35	23.00	Phytol	Anti-hyperalgesic, anti-inflammatory, and antiarthritic effects
22	1.11	23.96	Octadecanaoic acid	Antiviral, antibacterial and antioxidant activities
23	0.77	31.51	dl-.alpha.-Tocopherol	Protect cells against the effects of free radicals
24	2.01	45.11	Gamma-sitosterol	Antihyperlipidemic activity

4. Conclusion

The most prominent components of T. vulgaris ethanol extract were Spathulenol found in highest concentration (6.86%) followed by p-Cymene-2,5-idol (as an alkyl benzene: 3.4%) and thymol (3.41%), 9-Octadecyne (2.5%) and trans-13-Octadecenoic acid, methyl ester (2.39%) respectively. *T. vulgaris* essential oil had strong antibacterial and antifungal activity against multi-drug resistance nosocomial microorganisms.

From the obtained results, *T. vulgaris* ethanol extracts have a greater potential to be used as antimicrobial compounds against human pathogens in the treatment of nosocomial infectious caused by pathogenic microbes.

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