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Antibacterial Effect of *Thymus kotschyanus Boiss* Leaves Extract on Some Gram-Negative Bacteria

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ABSTRACT

Aims: The use of medications with plant origin covers a wide variety of maladies and constitutes an alternative way to antibiotic therapy, which seems to be no longer promising due to the widespread antibiotics resistance among the pathogenic microorganisms. Active principles having antimicrobial activity could be extracted and purified from plants and used in developing new medications. Among several diseases which have historically scourged man, some of the gram-negative bacteria are potentially epidemic and considered as one of the most outstanding causes of diarrhea. Therefore, this study aimed to evaluate the antibacterial activity of *Thymus kotschyanus* extracts.

Materials & Methods: The antimicrobial effect of *T. kotschyanus Boiss* leaves extract on some gram-negative bacteria strains was assayed *in vitro* by the disk diffusion technique. Dried and crushed plant materials were extracted from distilled water by evaporation and distillation. Finally, the antimicrobial assays were carried out for the plant, and the results were compared with an ampicillin disk results.

Findings: *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium, Entrobacter cloacae, Proteus mirabilis,* and *Shigella dysenteriae* were apparently killed by the extract, as judged by the presence of growth inhibition halos in the assays. The results of minimum inhibitory concentrations (MICs) showed that *E. coli* and *E. cloacae* strains were better inhibited by the extract.

Conclusion: The above results were similar to those from ampicillin disk, suggesting that *T. kotschyanus Boiss* could be used as a source of active principles against some gram-negative bacteria. Therefore, the tested *Thymus* extract could be considered as a valuable natural antibacterial source, which seems to be applicable in both medicine and food industry.

Keywords: Antibacterial effects, Plant extraction, Gram-negative bacteria.

CITATION LINKS

[1] In vitro antibacterial activity of Rheum ribes extract obtained from various plant parts against clinical isolates of gram-negative pathogens.[2] Effect of different ecological environments on... [3] The effects of essential oils and aqueous tea infusions of oregano (Origanum vulgare L. spp. hirtum), thyme (Thymus vulgaris L.) and wild thyme...[4] Effect of shiraz oregano(Zataria Multiflora Boiss) on growth probability of...[5] Antibacterial and antifungal activity of essential oils of...[6] Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and...[7] Evaluation of antimicrobial effects of four herbal essences on...[8] Extraction methods and... [9] Antibacterial properties of thymus pubescens. [10] Biologically active traditional medicinal herbs from Balochistan, Pakistan. [11] Flavonoids and further polyphenols in the genus Thymus. In: Stahl- Biskup...[12] RP-HPLC analysis of the phenolic compounds of plant extracts: Investigation of their antioxidant capacity and...[13] External leaf flavonoids of Thymus species from...[14] Antiviral and antimicrobial ... [15] Antibacterial activity of acylated flavonol glycoside from Waltheria indica.[16] Overview of antibacterial, antitoxin, antiviral, and...[17] Traditional medicinal plant extracts and natural products with activity against oral bacteria: Potential application in...[18] Antibacterial and antifungal activities and phytochemical profile of...[19] Essential oil composition of *Thymus kotschyanus* and T. pubescens from Iran... [20] Biological activities of flavonoid-rich fraction of Eryngium caucasicum...[21] Evaluation of the antioxidant and antibacterial capacity and phenolic content of three Thymus Species.[22] Isolation and identification of antibacterial compounds from... [23] Antibiotic susceptibility testing of the...[24] Antioxidant and antibacterial activities in the...[25] Study of the essential oils of Thymus ... [26] Correlation between phenolic compounds and ... [27] Developing the...[28] Antimicrobial activities of Eusteralis... [29] Anti-biofilm activity of Punica granatum... [30] In vitro antimicrobial activity of some medicinal plants against ...

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Introduction

Infectious diseases are the second leading cause of death worldwide. Treatment of infections continues to be problematic in modern time because of the severe side effects associated with some medications and the growing resistance to antimicrobial agents. Hence, the search for newer, safer, and more potent antimicrobial medications is an urgent need. Herbal medicines as a source of new antibacterial medications have received much attention because they are considered as time tested and comparatively safe for both human use and environment [1].

Knowledge about the medicinal properties of plants is extensively applied all over the world to cure or ameliorate suffering mankind. The use of medications with plant origin covers a wide variety of maladies and constitutes an alternative way to antibiotics therapy, which is no longer promising due to the widespread antibiotic resistance among the pathogenic microorganisms [2].

Theoretically, active principles having antimicrobial activity could be extracted and purified from plants to be used for developing new drugs. Scientific experiments on the antimicrobial properties of the plants compounds were first documented in the late 19th century. Plants are rich in a wide variety of secondary metabolites such as alkaloids and flavonoid, which have been found *in vitro* to have antimicrobial properties [3].

Nowadays, the extracts of many plants are known to exhibit antimicrobial activity. The use of herbal medicine predates the introduction of antibiotics and predates social, economic, and religious barriers [4-5]. Infectious diseases account for a large proportion of health problems in developing countries. Microorganisms have developed resistance to many antibiotics and as a result, a major clinical problem has been created in the treatment of infectious diseases [6].

Antimicrobial resistance in organisms has increased due to indiscriminate use of commercial antimicrobial medications commonly used to treat infectious diseases. This condition forced researchers to search for new antimicrobial substances from various sources including medicinal plants [7-9].

Iran is rich in ecological diversity and has a rich herbal flora which has not yet been well studied in terms of phytochemistry and bioactivity. Lamiaceae (formerly Labiatae) is one of the most important plant families in which *Thymus* with about 215 species is a significant genus [10]. *Thymus* species are commonly used as a tonic, carminative, digestive, antitussive, and expectorant for the treatment of cold in Iranian traditional medicine. Recent studies have implied that these species have strong antibacterial activities [11].

Extracts of *T. vulgaris* of Greek origin were examined as the potential sources of phenolic compounds and showed antimicrobial activity against the selected microbes [12]. In another research, aqueous and ethanolic extracts (10-200 mg/mL) of *T. capitatus* inhibited the growth of several bacteria and fungi [13]. It is believed that flavonoids are responsible for these activities [14-17]. They are known to be synthesized by plants in response to microbial infections [18]. In a study, the analysis of essential oil obtained from the aerial parts of *T. pubescens* revealed its main components, including carvacrol, thymol, α-terpineol, and p-cymene [19]. Abundantly distributed among the edible plants, flavonoids seem to contain some of the most potent next-generation drugs necessary for the treatment of infections as some of which possess unique antibacterial potency.

Objectives: In traditional medicine, medicinal herbs and plant products were

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used in the treatment of a wide spectrum of infections and other diseases. Therefore, in the present study, the extract of Iranian herb *T. kotschyanus Boiss*, which is rich in flavonoids [20], was evaluated for its antibacterial activities against some standard gram-negative bacteria.

Materials and Methods

Test Microorganisms: *Escherichia coli* (PTCC1763), *Pseudomonas aeruginosa* (PTCC1047), *Klebsiella pneumoniae* (PTCC1053), *Salmonella typhimurium* (PTCC1609), *Proteus mirabilis* (PTCC1776), *Entrobacter cloacae* (PTCC1003), and *Shigella dysenteriae* (PTCC1188) were used as standard strains. Test strains were obtained from Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology, Iran. The organisms were maintained on soybean casein digest agar (Merck, Germany) and regularly transferred onto fresh slants at 4°C [21].

Preparation of plant extracts: The aerial parts of *T. kotschyanus Boiss* were collected from the Paraw mountainside in the north east of Kermanshah city in the west of Iran. The plant parts were air-dried and finally crushed into a fine powder.

For aqueous extraction (evaporation and distillation), 10 g of dried and crushed plant material were weighted in 100 mL distilled water. The extract was then collected in a sterile vial and stored in a dark and cold place for further tests. But to avoid contamination and possible chemical alteration, the extract was ensured to be used within 3-4 days. Before using, the extract was sterilized by Whatman papers (pore size 0.2 μm) [22].

Antibacterial assay: Disk diffusion method was done to determine the zone of inhibition. The grown bacteria in Mueller Hinton broth culture medium (Merck, Germany) were centrifuged for 10 min at 3000g. By a spectrophotometer (230nm), the pellet was suspended in 5 mL of sterile peptone water to achieve a concentration of 1.5×10^8 CFU/mL (Shimadzo UV 120-1 spectrophotometer, Japan). Then 100 μL of this suspension were inoculated onto Mueller Hinton Agar (MHA) with a cotton swab (lawn culture), and 5 mm filter paper discs which were soaked with 20 μL of *T. kotschyanus Boiss* extract (100 mg/ml) were placed on MHA plates. After 24 hrs incubation at 37°C, the plates were examined for the presence of growth inhibition zones, and diameters were measured in millimeters, if there was any. Ampicillin disks (10 μg, Padtan Co., Iran) and sterile distilled water disk were used as positive [23] and negative controls, respectively. Relative inhibition zones (RIZ) was calculated as follows: RIZ (%) = $(IZD_{SAMPLE} / IZD_{AMPICILLIN}) \times 100$, where IZD was inhibition zones diameter (mm). Each experiment was duplicated along with controls [8, 24].

Minimum inhibitory concentrations (MICs): Broth dilution method was used to evaluate the minimum inhibitory concentrations (MICs) of *T. kotschyanus Boiss* extract by testing 8 concentrations (1-8 mg/mL) of the extract against gram-negative bacteria. Various concentrations of the extract were added to 10 mL Mueller Hinton broth, followed by inoculation with 1.5×10^8 colony forming units (CFU/mL) of bacteria suspensions and incubation in the incubator for 24 hrs at 37°C. After 5 min at room temperature, surviving CFUs were counted on Mueller Hinton agar medium incubated [8, 25]. The lowest concentration of the extract able to inhibit the bacterial growth was considered as MIC [11, 26].

Findings

Inhibition of bacteria growth: The results of some gram-negative bacteria growth

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inhibition clearly indicated that aqueous extract of *T. kotschyanus Boiss* leaves was effective against the bacteria (Table 1). In this case, 20 µL of aqueous extract was chosen for further assays. Indeed, a similar effect was observed when gram-negative bacteria cells were exposed to 20 µL of the antibiotic ampicillin (1 mg/mL) soaked in filter paper discs (Fig.1).

Determination of minimum inhibitory concentrations (MICs): The MIC results (Fig. 2) showed good antimicrobial effects against some gram-negative bacteria, including *E. coli, P. aeruginosa, K. pneumoniae, S. typhimurium, P. mirabilis, E. cloacae,* and *S. dysenteriae.* These results displayed that *E. coli* and *E. cloacae* strains were better inhibited by the extract.

Discussion

Therapeutic properties of herbs have had a prominent role in the evolution of most natural plant therapies. Because of low profitability of traditional medicine over a long period of time, traditional medicine knowledge started to be evanesced;

Table 1) Antimicrobial effect of *T. kotschyanus Boiss* leaves extract on some gram-negative bacteria

Name of microorganisms	RIZ (%)	MIC (mg/mL)
E. coli	100	1.0
E. cloacae	100	1.0
S. typhimurium	92	2.0
P. mirabilis	85	2.5
S. dysenteriae	70	3.5
K. pneumoniae	55	5.0
P. aeruginosa	32	7.5

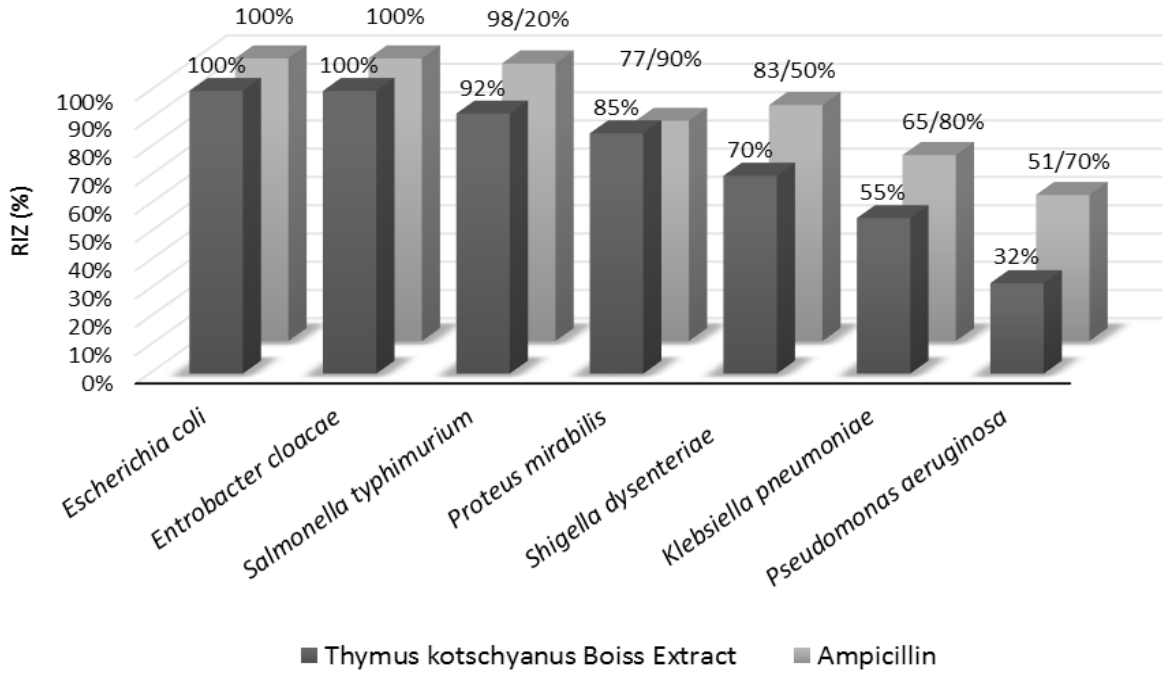

Figure 1: Relative inhibition zones (RIZ)

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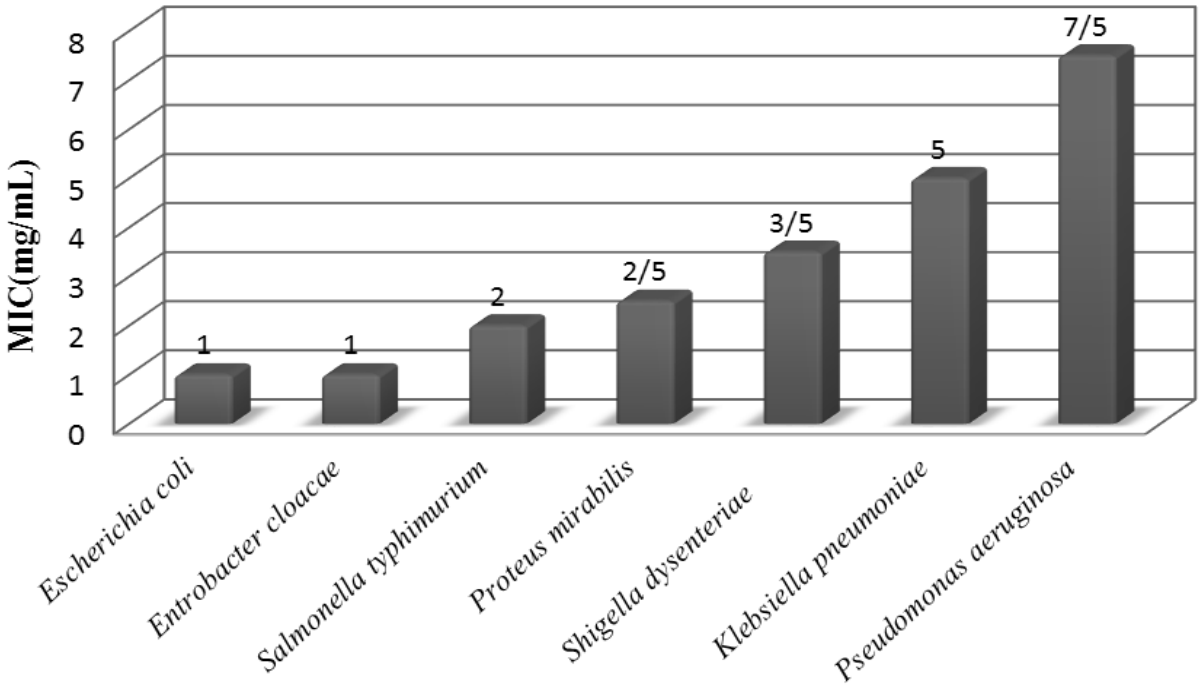

Figure 2) Minimal inhibitory concentrations (MIC)

although in recent years due to lesser side effects of plants, extensive studies have been conducted on their various effects on control and treatment of animal and human disease. Because of having lesser side effects than chemical drugs, natural medicines have been used for a long time to treat diseases, and in recent years, there has been a growing trend of such treatments [27].

The antibacterial activities of a large number of natural plants extracts have been known and tested. Due to their natural properties and various chemical components, they could be used as new medications against the microorganisms. Recent studies on antimicrobial effect of several species of *Thymus* have shown their effectiveness against some bacteria such as *Bacillus subtilis* and *Aeromonas hydrophila*. However, further studies are still needed to be conducted in order to explore their effectiveness in inhibiting the growth of parasites, viruses, and/or fungi. Another possibility for the limited antibacterial potency of some plants may be due to the use of soxhlet extraction method and crude extracts.

It is very difficult to compare the reported results about the antibacterial effects of different essences. The differences in the results could be attributed to such factors as the difference in methods used for evaluating the antibacterial effects of different extracts or essences, resources of extracts or essences, and genus of used bacteria [28-30]. In different studies, the powerful bacteriostatic effects of *T. revolatus* essence on both gram-positive and gram-negative bacteria was reported [5]. In a similar study, the bactericidal effects of *T. pubescus* essence on gram-negative bacteria was reported [9], like the previous studies. In another study, similar results were obtained about the effects of *Thymus* extract compounds on *S. sonei* and *S. flexeneri* [6].

In another research, disc diffusion analysis showed that *T. vulgaris* extracts had high ability to inhibit the growth of *P. aeruginosa* and *S. aureus* strains. However, these extracts showed low inhibitory effect on *E. coli* and *B. ceruse* strains and no inhibitory

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effect on other tested clinical bacterial pathogens. In a study, *T. vulgaris* extracts inhibited the growth of all tested bacteria in broth media with low concentration used in solid media [9].

In this research, *T. kotschyanus Boiss* leave extract showed a powerful bactericidal effect against some gram-negative bacteria. Although the exact nature of *T. kotschyanus Boiss* leave extract compounds having antimicrobial effect is unknown, these results might be useful for further assays concerning the purification of active principles. But the antibacterial activity of *T. kotschyanus Boiss* extracts might be due to the presence of phenolic constituents (thymol and carvacrol), making up a large percentage of the volatile oil.

The present study results supported the results of other advanced studies using *T. kotschyanus Boiss* extracts as antimicrobial agents due to the presence of both thyme essential oil and thymol. Also, these studies suggested the use of thyme as an antibiotic. Thymol is 25 times more effective than phenol but less toxic.

Since few popular applications of this plant have been suggested for intestinal disorders, *T. kotschyanus Boiss* could be considered as an alternative way to antibiotic therapy for diarrhea. The above results must be supported by further *in vivo* studies on experimental animal models involving pathogenic strains of some gram-negative bacteria.

In principle, phytotherapy could be assumed as highly effective, mostly in those cases where the malady becomes epidemic, and sanitary conditions are not promptly available. Additional research must be done in order to be able to elucidate the actual chemical composition of the compounds involved in the possible entero-bactericidal effect of this plant.

According to the obtained results in this research and due to the increasing restrictions in the use of chemical antimicrobial medications because of such issues as side effects and drug resistance, natural medicines and herbal essences could pave the way for studies about the replacement of mentioned substances to preserve alimentary substances and to control human diseases [4, 9, 28, 29].

Conclusion

In this study, the extracts of the aerial parts of *T. kotschyanus* were evaluated for antibacterial activity. The results indicated that all of the samples possessed high potent antibacterial activity. They are also expected to be rich in phenolic and flavonoid compounds. Water extract having the highest phenolic and flavonoid content showed an antibacterial activity and reducing power as compared with other extracts. Based on the obtained results from the antibacterial test, the extracts had an inhibitory effect (from moderate to good) against some gram-negative bacteria. Therefore, the tested *Thymus* extract could be considered as a valuable natural antibacterial source, which seems to be applicable in both medicine and food industry.

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