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EXPERIMENTAL PAPER

Antibacterial activity of *Tribulus terrestris* methanol extract against clinical isolates of *Escherichia coli*

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Summary

Introduction: *Tribulus terrestris* L. is traditionally used for treatment of urinary tract infections. *Escherichia coli*, as the most prominent agent of urinary tract infections, can be sensitive to *T. terrestris* extract. **Objectives:** The aim of this study was to evaluate the antibacterial activity of *T. terrestris* methanol extract against clinical isolates of *E. coli* from urinary tract infections. Saponins were determined as main constituents of *T. terrestris* methanol extract. **Methods:** The antibacterial activities of *T. terrestris* methanol extract were evaluated by micro-broth dilution assay. The synergistic effects of *T. terrestris* methanol extract were screened with gentamicin by micro titer plate and disc diffusion assays. The isobologram curve was figured and the Fractional Inhibitory Concentration Index (FICI) was determined. **Results:** The saponin content of *T. terrestris* methanol extract was 54% (w/w). The means of MIC and MBC values for *E. coli* clinical isolates (n=51) were 3.5±0.27 and 7.4±0.5 mg/ml while these amounts were 3.9±1.3 and 6.4±1.8 μg/ml for gentamicin. *T. terrestris* methanol extract and gentamicin had synergistic effect with FICI equal to 0.1375. **Conclusion:** Therefore, *T. terrestris* can be applicable as alternative treatment in management of urinary tract infections.

Key words: *Tribulus terrestris, methanol extract, synergy, gentamicin, saponins*

INTRODUCTION

Tribulus terrestris L. as a plant from *Zygophyllaceae* family has been traditionally used as analgesic, anti-hypertensive, and diuretic agents. Also, it is famous in traditional medicinal system because of its importance in the treatment of urinary tract infections [1]. In Chinese traditional medicine, *T. terrestris* has been used for treatment of respiratory tract infections, mastitis and different kinds of eye infections [2]. As the traditional systems are shown, *T. terrestris* has been used from the ancient times as antimicrobial agents. In recent years, the antibacterial activities of *T. terrestris* extracts were confirmed against a large number of bacteria [1, 3-5], although the role of *T. terrestris* in management of urinary tract infections has not been studied yet.

Escherichia coli is the major cause of many different infectious diseases in humans and animals [6-10] and the most common bacteria isolated from patients with urinary tract infections [11, 12]. It is also the major cause of nosocomial blood [13], nosocomial neonatal [14] and genital tract [13] infections, and also many other lethal infections in humans and animals.

Regardless of the critical role of *E. coli* in lethal infections, the appearance of multidrug resistant *E. coli* occurs in different parts of the world [8, 15, 16]. Furthermore, these treatments are associated with undesired adverse effects in humans and animals [17]. Therefore, finding new sources of antimicrobial agents is valuable and essential.

Although the antibacterial activities of *T. terrestris* extracts against bacteria were evaluated, the antibacterial activity of *T. terrestris* methanol extract against clinical isolates of *E. coli* in patients with urinary tract infections was evaluated for the first time.

In this study, according to the traditional usage of *T. terrestris* for treatment of urinary tract infections, the antibacterial activity of *T. terrestris* methanol extract against clinical isolates of *E. coli* (n=51) from the patients with urinary tract infections was evaluated.

MATERIAL AND METHODS

Plant material

Tribulus terrestris methanol extract of whole aerial parts (Barij Essence Pharmaceutical Company) was prepared. *T. terrestris* methanol extract was a yellowish brown to brown hygroscopic dried powder with no excipients.

Determination of saponin contents of *T. terrestris*

100 mg of *T. terrestris* was mixed with 40 ml of methanol and refluxed for 2 hours at 60°C. 2 ml of this solution and 2 ml of PDBA (para-di-methyl-amino

benzaldehyde) reagent (0.5 g of PDBA in 17 ml of HCl and 50 ml methanol) (tube 1), 2 ml of *T. terrestris* solution and 2 ml of methanol (tube 2), 2 ml of PDBA reagent and 2 ml of methanol (tube 3) in three separate tubes were mixed completely and put in the water bath (60°C) for 2 h. The OD for tube 1 was determined at 515 nm on the basis of the tube 2 and 3. The amount of saponins in *T. terrestris* extract was determined on the basis of the OD for cobalt chloride at 510 nm by this equation:

$$C=([(A_{tube1}- A_{tube2})\times W_s\times 2]/[A_{cobalt\ chloride}\times W\times 5])\times 100,$$

where C = saponins in the sample (%), W_s is the weight of cobalt chloride (300 mg), W is the weight of *T. terrestris* extract.

Microbial isolates

In this study, we used 50 clinical isolates of *E. coli* isolated from patients with urinary tract infections. *Escherichia coli* ATCC 8739 was used as a standard isolate. The bacterial isolates were cultured on nutrient agar a night before the experiment and they were incubated at 37°C. 1-2 colonies of bacteria were suspended in normal saline and their turbidities were adjusted to 0.5 McFarland (1×10^8 CFU/ml).

Antimicrobial evaluation of *T. terrestris* methanol extract by micro-broth dilution assay

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of extract were determined by micro broth dilution assay. The extract was twofold serially diluted (51.2–0.1 mg/ml). Cation adjusted Muller Hinton broth was used as a broth media. After shaking, 100 μl of extract was added to each well. The above mentioned microbial suspensions were diluted to 1×10^6 and then 100 μl were added to each well and incubated at 35±2°C. MICs were defined as the lowest concentration of extract that inhibits bacteria after 24 h. MBC values were the first tube that showed no growth on Eosin Methylene Blue agar (EMB) [18].

Synergistic evaluation of gentamicin and *T. terrestris* methanol extract by check board micro-titer assay

Eight serial, twofold dilutions of extract (12.8–0.4 mg/ml) and gentamicin (8–0.25 μg/ml) were prepared. 50 μl of each dilution extract and 10 μl of gentamicin dilutions were added in horizontal and vertical orientations of 96-microtiter plates.

50 μl of *E. coli* ATCC 8739 (10^6 CFU/ml) was added to each well and was incubated at 35ºC for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the division of MIC for combination of extract and gentamicin to the MIC of extract or gentamicin alone. The sum of FIC for extract and gentamicin was FIC index (FICI) and interpreted as synergistic effect: FICI <0.5, as indifferent: FICI $\geq 0.5–2$ and as antagonistic: FICI >2.0. The synergistic effect is shown graphically by applying published Isobole methods [19].

Synergistic evaluation of gentamicin and *T. terrestris* methanol extract by disc diffusion assay

The MIC values for 51 clinical isolates of *E. coli*, and finally the MIC_{90} (MIC value for 90% of isolates) were determined. The sub-inhibitory concentration of extract (one quarter and half of MIC_{90}) was added to Muller Hinton agar. Muller Hinton agar without extract was used as control. Plates were inoculated with the microbial suspension (1×10^6 CFU/ml) and gentamicin discs (10 μg) were put on the inoculated plates and were incubated at 35°C, nightly. Then, the diameter of inhibition zone was measured by Kulis-Vernieh method and compared [20].

Statistical analysis

The results of experiments were compared in Graphpad prism and the results were reported as means $\pm$SD. The differences between the results were determined at level of 0.05 by ANOVA analysis.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

T. terrestris methanol extract was containing 54% (w/w) of saponins. Antibacterial activity evaluation of *T. terrestris* methanol extract by micro broth dilution assay showed that the means of MIC and MBC values for these clinical isolates were 3.5 ± 0.27 and 7.4 ± 0.5 mg/ml, respectively. These amounts were 3.9 ± 1.3 and 6.4 ± 1.8 μg/ml for gentamicin, respectively (fig. 1).

The synergistic evaluations were determined by two different methods. In disc diffusion assay, the addition of *T. terrestris* extract to media at sub-inhibitory concentrations increased the inhibition zone diameters of gentamicin from 17.6 ± 3.5 (control) to 19.8 ± 3.7 mm (1/2 MIC value). There was significant difference between 1/2 MIC_{90} and 1/4 MIC_{90} of *T. terrestris* methanol extract and control (Fig 2A). Therefore, *T. terrestris* methanol extract increased the sensitivity of clinical isolates of *E. coli* to gentamicin.

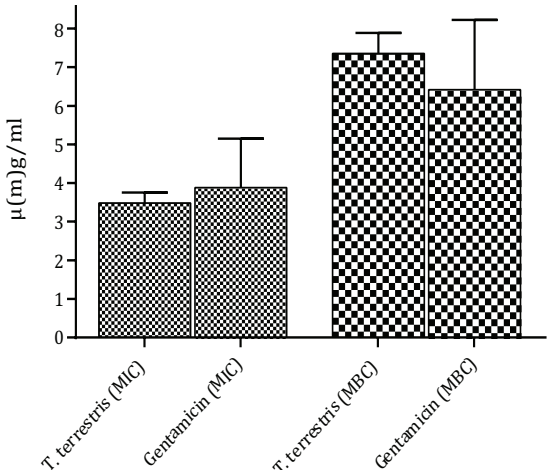

Figure 1.

The means MIC and MBC values for *T. terrestris* extract (mg/ml) and gentamicin (μg/ml) against clinical isolates of *E. coli*

The synergistic evaluation between *T. terrestris* methanol extract and gentamicin by check board micro-titer assay showed gentamicin decreased the MIC value of *T. terrestris* methanol extract from 3.2 mg/ml to 0.4 mg/ml. The *T. terrestris* methanol extract decreased the MIC value of gentamicin from 1 to 0.0125 μg/ml. The FIC values for *T. terrestris* methanol extract and gentamicin were 0.125 and 0.0125, respectively. The FICI was 0.1375. Therefore, the FICI of combination gentamicin and *T. terrestris* methanol extract was lower than that of 0.5 and exhibited the synergistic effects (fig. 2B).

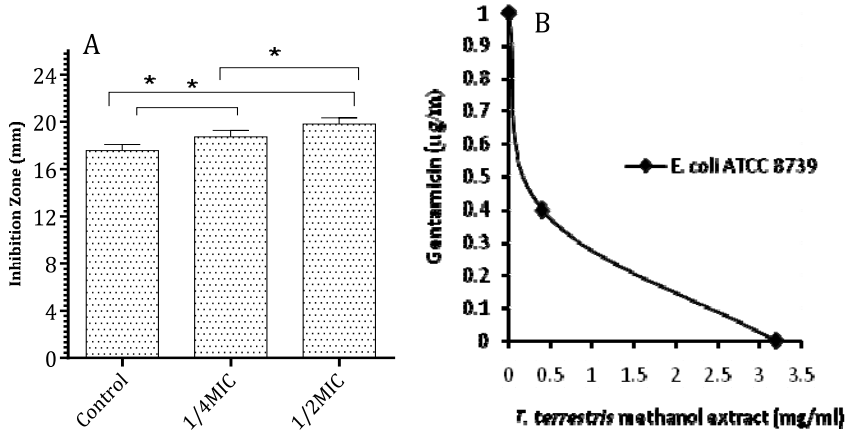

Figure 2.

Synergistic evaluation of gentamicin and *T. terrestris* methanol extract by disc diffusion assay (A) and check-board microtiter assay (B)

According to the traditional uses of *T. terrestris* for treatment of urinary tract infections, the results of our study showed that *T. terrestris* methanol extract had acceptable antimicrobial activity against clinical isolates of *E. coli*. In recent years, the antibacterial activity of *T. terrestris* methanol extract was confirmed against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Proteus vulgaris* [21]. *T. terrestris* aqueous extract had been antimicrobial effects against *E. coli*, *Pseudomonas aeruginosa* and *Candida albicans* [1], overhand, others [4] refuted the antibacterial activity of *T. terrestris* and they reported no antibacterial activity against *E. coli*, *P. aeruginosa* and *C. albicans* [5].

The antibacterial activity of ethanol extract from Turkish and Iranian *T. terrestris* has been confirmed against *E. coli*, *P. aeruginosa*, *S. aureus* and *Enterococcus faecalis* [1, 3] while the Yemeni ones have showed no activity against four above mentioned bacteria [22]. Therefore, there are confusing reports about the antibacterial activities of *T. terrestris*.

The main components of *T. terrestris* are saponins and these compounds play critical role in antimicrobial activity of this plant. The antimicrobial activities of saponins were confirmed against different microorganisms [23-25]. Saponins are detergent like substance and with surface active properties may disturb the bacterial membrane cells of bacteria [26]. Although, our result showed that *T. terrestris* containing a lot of saponins exhibited the high antibacterial activity against clinical isolates of *E. coli*, but saponins from *Quillaja saponaria* enhanced the growth of *E. coli* strains via increasing the influx of nutrients from medium into *E. coli* cells [27]. Indeed, the biological activity of saponins depended on the nature of their aglycone structure and the number of their sugars [28]. Furostanol and spirostanol saponins of tigogenin, gitogenin, neotigogenin, hecogenin, neogitogenin, neohecogenin, chlorogenin, diosgenin, ruscogenin and sarsasapogenin types were found frequently in *T. terrestris* extract [29] and have many biological activities. Hence it could be a good choice for treatment of impotence and sexual deficiency as well as cardiac diseases. They have cytotoxic effects, antihelmintic and antimicrobial activities [29]. Furthermore, a reason for different reports for antimicrobial activity of *T. terrestris* against microorganisms is the screening method used. In the evaluation of the antimicrobial potency of *T. terrestris* extract against clinical isolates of *E. coli*, discs containing different concentrations of *T. terrestris* extract showed no inhibition zone diameters (data has not been shown), while in broth dilution, the high antibacterial activity was observed for *T. terrestris* extract. Therefore, weak diffusion of extract in agar media decreased the antimicrobial activities. For this reason, disc diffusion method is not a gold method for the evaluation of antimicrobial activity [30].

Other issue of this manuscript was the evaluation of the synergistic effects of gentamicin and *T. terrestris* methanol extract by two different methods. The results of our experiment showed that gentamicin decreased the dose of *T. terrestris*, so it had a synergistic effects with gentamicin. The synergistic effects of saponins with amphotericin B, itraconazole [31], mancozeb [32], and vancomycin [33] were confirmed, while saponins had no synergistic effects with ampicillin, streptomycin

and ciprofloxacin against clinical isolates [27]. The synergy between *T. terrestris* methanol extract and gentamicin were confirmed by two methods in this study and are reported for the first time. Indeed, *T. terrestris* methanol extract increased the sensitivity of clinical isolates of *E. coli* to gentamicin. Therefore, it can be used as alternative treatment for decreasing the dose of gentamicin in patients or for the reduction of the time of treatment.

CONCLUSIONS

The results of our study have confirmed the traditional uses of *T. terrestris* for treatment of urinary tract infections *in vitro* and therefore it can be a suitable candidate in patients with urinary infections or other *E. coli* related infections. For achieving this aim, *T. terrestris* should be formulated and more toxicological, pharmacological and clinical studies should be done for clarify the suitability of *T. terrestris* extract in treatment of *E. coli* induced infections.

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Conflict of interest: Authors declare no conflict of interest.

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DZIAŁANIE ANTYBAKTERYJNE WYCIĄGU ETANOLOWEGO Z *TRIBULUS TERRESTRIS* NA KLINICZNE IZOLATY *ESCHERICHIA COLI*

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Streszczenie

Wstęp: *Tribulus terrestris* L. jest tradycyjnie stosowany w leczeniu zakażeń układu moczowego. *Escherichia coli*, jako główny czynnik zakażeń tego układu, może wykazywać wrażliwość na działanie ekstraktu z *T. terrestris*. **Cel:** Celem badań było określenie aktywności przeciwbakteryjnej ekstraktu metanolowego uzyskanego z ziela *T. terrestris* w stosunku do szczepów *E. coli* izolowanych od osób z zakażeniami układu moczowego. **Metody:** Działanie przeciwbakteryjne ekstraktu metanolowego z *T. terrestris* określano metodą rozcieńczeń w pożywce płynnej. Działanie synergistyczne ekstraktu metanolowego z *T.*

terrestris z gentamycyną badano metodą krążków bibułowych w podłożu agarowym. Na tej podstawie wyznaczono krzywą izobolograficzną oraz ułamkowe stężenie hamujace dla obu substancji (Fractional Inhibitory Concentration Index – FICI). **Wyniki:** Zawartość saponin w ekstrakcie metanolowym z *T. terrestris* wynosiła 54% (w/w). Średnie wartości MIC i MBC dla szczepów klinicznych *E. coli* (n=51) wynosiły odpowiednio 3,5±0,27, 7,4±0,5 mg/ml, a dla gentamycyny odpowiednio 3,9±1,3 i 6,4±1,8 μg/ml. Ekstrakt metanolowy i gentamycyna wykazywały działanie synergistyczne na poziomie FICI równego 0,1375. **Wniosek:** Wykazano, że ekstrakt metanolowy z ziela *T. terrestris,* może być stosowany jako alternatywny środek do leczenia zakażeń układu moczowego.

Słowa kluczowe: *Tribulus terrestris, ekstrakt metanolowy, działanie synergistyczne, gentamycyna, saponiny*