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A Comparative Investigation on Growth of Three Food Born Pathogenic Bacteria Inoculated with Withania somnifera: an Invitro Experimental Study

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ARTICLE INFO

Article history
Received: 23 June 2019
Accepted: 13 July 2020
Published Online: 16 October 2020

Keywords:
E. coli
Pathogen
Salmonella typhimurium
Shigella dysenteriae
Withania somnifera

ABSTRACT

Background: Withania somnifera (WS) is proposed as one of the alternatives instead of the antibiotic. This study is aimed to evaluate the inhibitory potency of enzymatic extract of the fruits of the WS. Methods: As an invitro experimental study, the growth rate of Shigella dysenteriae, Salmonella typhimurium, and Escherichia coli inoculated in different concentrations (25%, 12.5%, 6.25% and 3.125%) of the extract were assessed. A microtitre plate method was conducted. ANOVA was applied to identify statistical differences with $p$-value <0.05. Results: Different concentrations of extract, in comparison with control, declined the growth rate of all tested bacteria. All concentrations inhibited the growth of $S. typhimurium$ ($p<0.05$). Compared to the microorganism control, effective concentration of the extract inhibiting the growth of $E. coli$ was 12.5%, and 6.25%, while it was 12.5%, and 6.25% for $Sh. dysenteriae$ ($p<0.05$). A dose-dependent response of $E. coli$ was observed. The antibacterial activity of the extract tested was found mainly against $E. coli$ and $Sh. dysenteriae$. The most resistant microorganism compared to $E. coli$ and $Sh. dysenteriae$ was $S. typhimurium$ ($p<0.05$). 25% of the concentration of the extract showed the different inhibitory effect among three tested bacteria ($p<0.05$). Conclusions: The extract was labeled as an antibacterial agent against the representative of three food-borne bacteria, Invitro. The common effective concentrations of the extract (12.5, and 6.25%) is recommended for further research, as food additive, to remedy digestive ailments related to $E. coli$, $S. typhimurium$ and $Sh. dysenteriae$.

1. Key Messages

The results of this study highlight the antibacterial effect of Withania somnifera (WS), a medicinal plant in Sistan and Baluchistan province, Iran, against $Shigella dysenteriae$, $Salmonella typhimurium$, and $Escherichia coli$ (E. coli), as food born human/animal pathogens. As a continuous work to our previous relevant study, the present study figures out the effect of different concentrations of crude enzymatic extract of WS berries against $E. coli$, $S. typhimurium$ and $Sh. dysenteriae$ growth, and also, register the local understanding of traditional medicines’ use by residents in Sistan and Baluchistan.

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Source(s) of support: University of Zabol (grant No. UOZ/GR/9618/32)
2. Introduction

Today, around 60% of anti-infective and antitumor medicines, found in the market, composed of natural origin. [1] Medicinal herbs have fewer side effects, and are cheaper than chemical drugs, and also, easily available. [2] Statistics on the use of herbal medicines are significant in recent years. The World Health Organization revealed that about 4 billion people, now, use herbal medicine to treat illnesses. [3] Maybe, it is an unlikely explanation for many people that a variety of the chemical drugs in colorful packaging is the result of scrutiny on the effective elements of medicinal plants. This has led to form a widespread investigation by pharmaceutical companies on the healing properties of plants in different parts of the world. [3]

Designation of a national pharmacopeia, monographs of medicinal materials, standards and guidelines regarding medicinal herbs is suggested. [2] WS is considered as a medicinal plant in Sistan and Baluchestan province, Iran. [4-5] This plant is one of the vegetation of many cities of South of the province, including Saravan, Iranshahr, Sarbaz, Sib -o- Soran, Mehrestan, and Khaash (Figure 1). [6] It is widely distributed and different parts of WS are prescribed, empirically, in traditional medicine, in above locations, for treatment of diarrhea, vomiting and hypertension. [6-8] Aqueous, ethanol, methanol, petrol, and chloroform extracts of leaves and roots of WS have been examined on \( S.\ typhimurium \), \( S.\ dysenteriae \) and \( E.\ coli \). Nevertheless, the effect of berries of WS is ambiguous. [9-11]

Intestinal bacteria are divided into opportunistic pathogens like \( Escherichia coli \), and primary pathogens including \( Salmonella \) and \( Shigella \) spp. They infect the digestive system of human and animal, lead to diarrhea and digestive system disorders. [12] In addition, they could act as food borne pathogen via consumption of contaminated water or meat. [13] Antibiotics are used to inhibit the growth of these bacteria and it results in the emergence and development of the resistance isolates. Hence, to discover new agents with distinct origin, as substitution for antibiotics, is logical, nowadays [2].

The present study is carried out to investigate the antimicrobial activity of different dilutions of extract of fruits of WS, using a microtitre plate method, against three commercially available bacterial strains, including \( E.\ coli \), \( S.\ typhimurium \), and \( Sh.\ dysenteriae \), as representative of intestinal food borne gram-negative pathogens in Iran.

3. Materials and Methods

3.1 Bacterial Strains and Culture Media

\( E.\ coli \) (ATCC® 25922™; PTCC® 1399™), \( S.\ enterica\ subsp.\ enterica\ serovar\ typhimurium\) (ATCC® 14028™; PTCC® 1709™), and \( Sh.\ dysenteriae\) (PTCC® 1188™), were delivered from archive of Laboratory of Microbiology, Faculty of Veterinary Medicine (LMFVM), University of Zabol, Zabol, Sistan and Baluchistan, Iran, and used for the current study. Bacterial cultures were grown in Peptone Water Broth (PWB). Two hours culture of tested bacteria, amplified in 5 mL PWB, were adjusted to 0.5 McFarland standard (about \( 10^8\) CFU, confirmed by plate colony count). [2]

3.2 Plant Material

WS were collected from cities of Sistan and Baluchistan province (Figure 1), Iran, at the markets, and at the homes of traditional healers during April - July 2017, and transferred to LMFVM. The identification of the plant was conducted entirely by Department of Plant Pathology, Faculty of Agriculture, University of Zabol, Iran.

3.3 Extract Preparation

Briefly, enzymatic extract of WS fruits was obtained by homogenization of 10 g of WS berries in 60mL of 85% NaCl for 24 hours via mild shaking, and then, centrifugation at 20,800 × g for 30 min at 4°C. The supernatant was filtered and applied for in vitro study or stored at 4°C for next steps. [6, 14] Identification and quantification was performed, considering total protein concentration of enzymatic extract via Bradford method. [15]
3.4 Formulation of Different Concentration of Extract

Five dilutions of extract (0%, 25%, 12.5%, 6.25%, and 3.125%) were prepared in this study.

3.5 Antimicrobial Assay

100 μL of adjusted culture to 0.5 McFarland standard was distributed into flat-bottomed 96-well microtiter plates and mixed with 100 μL of different concentration of WS berries extract. As microorganism control, 100 μL of PWB was mixed with 100 μL of adjusted culture to 0.5 McFarland standard. In addition, culture medium control consist of 100 μL of PWB mixed with 100 μL of PWB was included. Plates were incubated at 37 °C, and the growth, as Optical Density (OD), was evaluated using a micro-plate reader (Stat fax-2100, UK), set at 490 nm, at time 0 and 24 hours after incubation. All bioassays were carried out in triplicates. The antimicrobial assay was carried out for E. coli, S. typhimurium and Sh. dysenteriae, individually.  

3.6 Statistical Analysis

The growth curve of tested bacterium, based on OD, for different concentration of WS, and control, was computed and the slope value was calculated via regression coefficient (B). In better words, the increases and decreases in growth rate were calculated by slope of trend lines during tow measurements (0 and 24) using Microsoft Excel [16]. More negative slopes value demonstrated more inhibition. Statistical Package for the Social Sciences (SPSS) was applied to analyze statistics via ANOVA and to identify differences with p-value <0.05 [16].

4. Results and Discussion

High interest in traditional medicine has been declared by WHO. [3] In Sistan and Baluchistan, one of the biggest provinces of Iran, primary health care have been mediated via traditional medicines. [5] Bacterial infections and inflammation are among the sicknesses treated by traditional healers in study area. Few investigations regarding traditional medicine have been accomplished in South-East of Iran, Sistan and Baluchistan, one of the old provinces of Iran. [2]

Regional healers for treatment of diarrhea administrate WS in Sistan and Baluchistan. Based on our literature review no study about WS was performed in the study area [4].

There are literally many published scientific papers from around the globe describing the antimicrobial activities of WS extracts and its chemical constituents, but none of them deals with fruit extract of WS. [17-19]

As a continuous work to our previous relevant study, [2] the present study is performed to figure out the effect of different concentrations of crude enzymatic extract of WS berries against E. coli, S. typhimurium and Sh. dysenteriae growth, and also, to register the local understanding of traditional medicines’ use by residents in Sistan and Baluchistan.

The growth rate of E. coli, S. typhimurium and Sh. dysenteriae were assessed via the calculation of slope line trend. All microorganisms tested were found to be susceptible to the extract and their growth was inhibited compared to their control (Figures 2). The lowest growth rate, with significant difference (p<0.05), was observed at 25%, 12.5% and 6.25% of the concentrations of the extract for E. coli (Figures 2a). Moreover, based on figure 2b, all concentrations of the extract showed inhibitory effect, with statistical significant difference (p<0.05), on the growth of S. typhimurium compared to the microorganism control. Finally, two concentrations of the extract, including 12.5% and 6.25%, decreased the growth rate of Sh. dysenteriae, statistically significant (p<0.05), compared to its control (Figure 2c). In addition, as can be seen in figure 2, notably (p<0.05), 12.5% and 6.25% of concentrations of the crude enzymatic extract of WS berries showed the best inhibitory effect for three tested bacteria.
antibiotic resistant *Staphylococcus aureus* was assessed and concluded that ethanol extract of *WS* leaf might be exploited as natural drug for the treatment of several infectious diseases caused by this pathogen. A study evaluated the antibacterial activity of aqueous and alcoholic extracts of root and leaves of *WS* against pathogenic bacteria including *S. typhimurium* ATCC 23564, *E. coli* K-12 DSM 4060 and *Staphylococcus aureus* ATCC 9144 by in vitro agar gel diffusion method and it was found to possess strong antibacterial activity against mentioned bacteria. According to the antimicrobial properties of the plant, our findings in the current study was not different from those of Bokaeian and Saeidi and Owais et al.

A study investigated the antibacterial activity of *WS* root (*WSR*) against *E. coli* O78. The turbidity optical density was measured. The results revealed that the maximum inhibition of bacterial growth was observed at 1:8 dilution of *WSR* extract. The authors concluded that *WSR* possessed good antibacterial activity. It is consistent with our findings which was observed the lowest growth rate of *E. coli*, at 25%, 12.5% and 6.25% of concentrations of the extract ,with significant difference (p<0.05).

As shown in Figure 2a, the slope values, for the extract with the E.coli showed a dose-dependent decrease with the increase in the concentrations of extract, while, it was not seen for both *S. typhimurium* and *Sh. dysenteriae* (Figures 2b and 2c). One study found that *WS* caused dose-dependent suppression of α2-macroglobulin (an indicator for anti-inflammatory drugs) in the serum of rats inflamed by injection of carrageenan suspension. Furthermore, a study revealed that the aqueous extract of *WSR* inhibited the growth of bacteria in dose-dependent manner. Also, in present study, as detailed information, the dose-dependent response of the extract of a *WS* component against *E. coli* has been shown.

Due to the antibacterial activity of the extract, the results presented in this paper documented that tested plant used by the healers in Sistan and Baluchistan for treatment of diarrhea, may act toward diarrheal diseases believed to be of bacterial origin. These facts support the medicinal value of *WS* and suggest that it could be the new sources of antibacterial therapies.

Again, it is worthwhile to note that according to our literature review, the present study is the first report related to the effect of different concentrations of enzymatic extract of *WS* fruits on three important intestinal food borne gram-negative pathogens including *E. coli*, *S. typhimurium*, and *Sh. dysenteriae*. Among tested bacteria, different concentrations of *WS*, in term of inhibitory effect, are compared, statistically. All microorganisms tested were found to be of bacterial origin. These facts support the medicinal value of *WS* and suggest that it could be the new sources of antibacterial therapies.

As shown in Figure 2a, the slope values, for the extract with the E.coli showed a dose-dependent decrease with the increase in the concentrations of extract, while, it was not seen for both *S. typhimurium* and *Sh. dysenteriae* (Figures 2b and 2c). One study found that *WS* caused dose-dependent suppression of α2-macroglobulin (an indicator for anti-inflammatory drugs) in the serum of rats inflamed by injection of carrageenan suspension. Furthermore, a study revealed that the aqueous extract of *WSR* inhibited the growth of bacteria in dose-dependent manner. Also, in present study, as detailed information, the dose-dependent response of the extract of a *WS* component against *E. coli* has been shown.
to be susceptible to the extract. Different concentration of the extract, showed various extent of growth inhibition among tested bacteria, individually (Fig 3). The power of the inhibition of the extract among three bacteria, based on the concentration is compared in table 1. As can be seen in table 1, notably (p<0.05), 25% of concentration of the extract showed the different inhibitory effect among three tested bacteria (table 1, Fig 3), indicating that the inhibitory effect of the 25% of concentration of the extract could be species-specific among bacterial population. The decreasing order of the growth rate, with significant difference, in confronting with the extract were as E. coli > Sh. dysenteriae > S. typhimurium (Fig 3). The results clearly indicated that E. coli and Sh. dysenteriae are the bacteria with the highest sensitivity to the extract compared to S. typhimurium (Fig 3).

There are studies that report selective antibacterial activity of extract of WS inhibiting the growth of bacteria, which is consistent with our results. [24]

Table1. Comparison of the inhibitory effect of different concentration of the extract on the growth of tested bacteria

| tested bacteria           | Concentration (%) |
|---------------------------|-------------------|
|                           | 25                |
|                           | 12.5              |
|                           | 6.25              |
|                           | 3.125             |
| E. coli vs S. typhimurium | ■                 |
| E. coli vs Sh. dysenteriae| ■                 |
| S. typhimurium vs Sh. dysenteriae | ■ |
| E. coli vs Sh. dysenteriae | ■                 |

The comparison that are significantly different (p<0.05) from each other at the same concentration point indicated by ■.

A study screened the crude extracts of different parts of WS including unripen fruit; ripen fruit, and calyx, for their antimicrobial activity invitro against Bacillus subtilis, Pseudomonas aeruginosa, and Enterobacter aerogens by disc diffusion assay. Chloroform extract of calyx of WS showed highest activity against Bacillus subtilis. [25] Our study was the first report to evaluate the effect of crude fruit extract on E. coli, S. typhimurium and Sh. dysenteriae and the results obtained in the present study may be due to the genera of tested bacteria and methodology compared to the study conducted by Singariya et al. [25]

Antimicrobial activity of crude acetone extract from the aerial part of WS was tested in vitro against six pathogenic bacteria, using disk diffusion method, in comparison with gastrointestinal microbiota, and the results suggested that WS could act as an effective antibacterial agent against human pathogenic bacteria with lowered harmful effect on bifidobacteria [9]. The examination of different part of WS with different methodology in the present work may justify the difference of our observed data compared to the findings of Halamova et al [9].

Identification, extraction, and purification of more than 35 chemical constituents of WS has been widely studied. Alkaloids, steroidal lactones, saponins, and withanolides have been introduced as the biologically active chemical compounds of WS. [19] The structural and non-structural proteins of examined bacteria, in comparison with other tested bacteria, may play a role to super-induce/prevent the inhibitory effect of the extract [26].

The results of the present study portray the prospect of using WS as a substitution for antibiotics in the bacteriology. It is important to demonstrate scientifically that the remedies employed in folk medicine are indeed therapeutically active and therefore, potentially active compounds must be isolated from tested plant and according to our outcomes, WS would be interesting candidates for future research regarding to E. coli, S. typhimurium and Sh. dysenteriae. Bear in mind that the mutagenic and/or toxic effects of WS is still ill-defined and could act as growth depressor. Further studies on the mutagenesis/toxicity of the plant must be employed, as well as its application in often complex traditional mixtures. It would allow to elucidate possible candidates for future development of antimicrobial agents.

It should not be out of mind that minimum inhibitory concentration, double disc diffusion test, standard susceptibility breakpoints and resistance cut-off breakpoints for this plant fruit extracts need further investigation.

Studies showed that fruits extract of WS possesses good radical scavenging activity. [27] It is, also, reported that synthetic oxidants presenting in both food and drugs

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**Figure 3.** The effect of different dilutions of extract on three tested bacteria; Dots show slope value of trend lines and 95% confidence of interval of Mean are presented by error bars. More negative slopes value demonstrated more inhibition.
can lead to undesirable health effects. With the latest trend, crude fruit extracts of WS as antioxidants have been potentially proposed to add by many food technologists to increase the nutrient values. The use of natural antibacterials for treating diseases, and as food additives, have better consumer acceptability and a trend over the use of the available synthetic products. [27]. Our result, as a research of quantification of the antibacterial activities of the WS, indicated that two concentration of the extract, including 12.5% and 6.25%, play significant role to decrease growth rate of three important food borne pathogens, including E. coli, S. typhimurium and Sh. dysenteriae. Our result is important since the information on the antibacterial properties of WS against E. coli, S. typhimurium and Sh. dysenteriae is available prior to incorporating them into food products. The findings may be used as a fundament for further experiment in food technologies to control diseases. It is notable that harmful adverse effect on beneficial human microbiota, regarding to plant extracts and compounds, need more investigation. On the other hand, vegetarianism can also lead to increase the demand for substitution of plant material instead of chemical drugs, especially antibiotics. Through this investigation, we have shown that all dilutions of extract exhibit antibacterial activities against three tested bacteria, proposed a good potential to be used in therapeutics. The results presented in this report will also provide a suitable guide in choosing dilutions of extracts by the medical practitioners as natural antibacterial treating and controlling diseases.

In sum, this paper reports and establishes a scientific basis for the therapeutic use of WS as an antibacterial agent against three food borne pathogens. This experiment reveals and proposes the effective dilutions of crude enzymatic extract of WS berrie against the growth of E. coli (25%, 12.5%), S. typhimurium (25%, 12.5%, 6.25%, 3.125%) and Sh. dysenteriae (12.5% and 6.25%), and also, it increases the local understanding of traditional medicines’ use by residents in Sistan and Baluchistan. 12.5% and 6.25% dilutions of crude enzymatic extract of fruit of WS can be proposed for further research, as food additive to remedy ailments related to examined bacteria.

Acknowledgement

This study was funded by University of Zabol (grant No. UOZ/GR/9618/32). This study was performed in partial fulfillment of the requirements for a BSc in Laboratory Sciences student’s project (Abdoljamal Azar). We acknowledge the assistance provided by the staff of the Laboratory of Microbiology, especially, Mr. Saeed Shahriari.

Conflict of Interest

The authors declare no conflict of interest for all potential sources of bias, including affiliations, funding sources, and financial or management relationships.

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