



Antibacterial Activity of Ethyl Acetate and Methanol Extracts of *Securigera securidaca*, *Withania sominefra*, *Rosmarinus officinalis* and *Aloe vera* Plants against Important Human Pathogens

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ARTICLE INFO	ABSTRACT
<p>Article type: Original Article</p> <p>Article history: Received: 05 Mar 2018 Revised: 17 Mar 2018 Accepted: 27 Mar 2018 Published: 15 May 2018</p> <p>Keywords: Antibacterial activity, Human pathogen, Inhibition, Plant extract.</p>	<p>Background: Considering the importance of new antibiotics, the potential antibacterial activity of ethyl acetate and Methanol extracts of <i>Securigera securidaca</i>, <i>Withania sominefra</i>, <i>Rosmarinus officinalis</i> and <i>Aloe vera</i> plants against important human pathogens was studied.</p> <p>Methods: <i>Streptococcus pneumoniae</i>, <i>Enterococcus faecalis</i>, <i>Proteus mirabilis</i>, <i>Staphylococcus aureus</i>, <i>E. coli</i>, <i>P. aeruginosa</i> were the pathogenic bacteria used to determine the antibacterial effect of ethyl acetate and methanol extracts of <i>Securigera securidaca</i>, <i>Withania sominefra</i>, <i>Rosmarinus officinalis</i> and <i>Aloe vera</i> in broth micro-dilution method.</p> <p>Results: The results of studying the methanol extract of <i>S. securidaca</i> with the lowest inhibitory concentration of 25, showed that all used bacterial pathogens were inhibited at this concentration, while the lowest inhibitory concentration of ethyl acetate extract of <i>Securigera securidaca</i> was 12.5, which were <i>P. mirabilis</i> and <i>S. aureus</i> is inhibited in this concentration. The highest bactericidal concentration (MBC) of <i>S. securidaca</i> ethyl acetate extract was 100, and <i>S. aureus</i>, <i>P. aeruginosa</i> were inhibitory at this concentration. The lowest inhibitory concentration of methanol extract of <i>W. sominefra</i> was 6.25, which <i>S. pneumoniae</i>, <i>P. mirabilis</i>, <i>S. aureus</i> and <i>E. coli</i> bacteria were inhibited at this concentration, while the highest inhibitory concentration was observed against <i>P. aeruginosa</i> (25 ppm), The lowest inhibitory concentration of the ethyl acetate <i>W. sominefra</i> extract was 3.1ppm in comparison with <i>P. mirabilis</i> and <i>E. coli</i>.</p> <p>Conclusion: In conclusion, it seems that <i>S. securidaca</i>, <i>W. sominefra</i>, <i>R. officinalis</i> and <i>Aloe vera</i> extracts could inhibit the growth of all of the mentioned bacteria.</p>

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Introduction

Medicinal herbs are a boon of nature to human and have been applied for centuries to cure a numeric of human diseases. In many regions of the world, medicinal herbs are applied in case of bacterial, fungal, and viral infections. Evaluation of herbs, bearing yield in healing various diseases is growing in last years. Innumerable biologically acting compounds of herbs are found to possess antimicrobial properties. According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs (1).

Withania somnifera (L.) Dual also known as "Aswagandha" belongs to the family Solanaceae and is widely applied in traditional medicine. It is an ingredient in many formulations prescribed for a diversity of musculoskeletal diseases (e.g. arthritis, rheumatism), and as a general tonic for improving the energy, better overall health and longevity, and prevent disease in athletes, and elderly (2, 3).

The total alkaloid content of Indian root varies from 0.13% to 0.31%. *W. somnifera* has been used as an anti-oxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, astringent and antibacterial agent (4, 5). The total alkaloid content of Indian root varies from 0.13% to 0.31%. *W. somnifera* has been used as an anti-oxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, astringent and antibacterial agent (4, 5).

Rosemary (*Rosmarinus officinalis* L.) originally grows in southern Europe. Its herb and oil are generally applied as a spice and flavoring factor in food processing for its desirable flavor, high antioxidant acting and lately because of its antimicrobial properties (6, 7). Moreno et al. (2006) reported that rosemary herbs are rich in phenolic compounds with high antimicrobial activities versus both of Gram-positive and Gram-negative bacteria (8).

Aloe vera belongs to the Liliaceae family, of which there are about 360 species. It is a cactus-like herb that amplify readily in hot, dry climates and currently, because of demand, is cultivated in large quantities (9). The gel of *A. vera* was applied to treat stomach ailments, gastrointestinal problems, skin disease, constipation, radiation injury, inflammatory outcome, healing wounds and burns, ulcer and diabetes. *A. vera* products are mainly for cosmetic, pharmaceutical, nutraceuticals and food industries. *A. vera* has been applied for many centuries for its curative and therapeutic properties. The inner gel have been identified, however, the therapeutic factors have not been correlated well with each individual component (10).

Securigera securidaca, an annual herb belonging to the Fabaceae family, is apply in Iranian folk medicine for treatment of diabetes. Experimental studies have disclose that administration of *S. securidaca* seeds decreases blood glucose in normal and diabetic subjects (11, 12, 13).

Material and methods

Bacterial strains and culture conditions

Bacterial strains were obtained from standard laboratory. Evaluate the antibacterial activity of the plant extracts were investigated using strain of bacteria *Streptococcus pneumoniae* ATCC 49619, *Enterococcus faecalis* ATCC 29212, *Proteus mirabilis* ATCC 35659, *Staphylococcus aureus* ATCC® 25923, *E. coli* ATCC25922, *P. aeruginosa* ATCC 27853. The type cultures of the above bacterial species was sub-cultured on Nutrient agar (Oxoid) and stored at 4 °C until required for further studies. The susceptibility of all antibiotics was carried out using disc diffusion method on Muller-Hinton agar as recommended by CLSI. The procedure followed is briefly described here. *S. pneumoniae*, *E. faecalis*, *P. mirabilis*, *S. aureus*, *E. coli*, *P. aeruginosa* plates were grown overnight on blood agar, Nutrient agar

and colony suspension was prepared using the sterile saline water equivalent to a 0.5 McFarland standard. Suspension (100 μ l) was spread over the media plate and antibiotic disc was transferred aseptically on the surface of inoculated media plate. Isolated plates were tested with different antibiotics and their concentration shown in parenthesis vise AN, OX- AMC- CZ-V- AM-GM-AZN (26).

Plant materials

The leaf of *Securigera securidaca*, *Withania somnifera*, *Rosmarinus officinalis* and *Aloe vera* was collection in the region of Iran (Zabol - south-eastern, Iran) and dried at room temperature. Samples were crashed and transferred into glass container and preserved until extraction procedure was performed in the laboratory. Each of 40 g grinded powders was soaked in 50 ml ethanol 95 % and 50 ml water, separately for 20 h (shaking occasionally with a shaker). After one day of dissolving process, materials were filtered (Whatman no. 1 filter paper). Then the filtrates were evaporated using rotary evaporator. At last, 0.97 g of dried extracts were obtained and then stored at 40 °C in air tight screw-cap tube.

Minimum Inhibitory Concentration (MIC) and MBC of plant extracts

The broth microdilution method was used to determine MIC and MBC. All tests were performed in Mueller Hinton broth supplemented with Tween 80 at a final concentration of 0.5% (v/v). Briefly, serial doubling dilutions of the extract were prepared in a 96-well microtiter plate ranged from 12.5ppm to 400 ppm. To each well, 10 μ l of indicator solution and 10 μ l of Mueller Hinton Broth were added. Finally, 10 μ l of bacterial suspension (10⁶ CFU/ml) was added to each well to achieve a concentration of 10⁴ CFU/ml. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The

plates were prepared in triplicates, and then they were placed in an incubator at 37 °C for 18–24 hours. The color change was then assessed visually. The lowest concentration at which the color change occurred was taken as the MIC value. The average of 3 values was calculated providing the MIC values for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate the visible growth. The microorganism growth was indicated by turbidity. The MBC was defined as the lowest concentration of the extracts at which does the incubated microorganism was completely killed.

Results

The results of this study showed that the lowest inhibitory concentration of methanol extract was 25 ppm which inhibited *P. mirabilis* while the lowest inhibitory concentration of *Aloe vera* ethyl acetate was 25, which was inhibited by *E. faecalis*, *S. pneumonia*, *P. aeruginosa* and The highest bactericidal concentration (MBC) of ethyl acetate extract was 100 ppm, which inhibited *P. mirabilis*, *S. aureus*, *E. coli* (Table 2).

The results of the study of methanol extract of *S. securidaca* with the lowest inhibitory concentration of 25, which all pathogens were inhibited at this concentration, while the lowest inhibitory concentration of ethyl acetate extract of lentils was 12.5, which were *P. mirabilis*- *S. aureus* is inhibited in this concentration. The highest bactericidal concentration (MBC) of *S. securidaca* ethyl ester acetate extract was 100, and *S. aureus*, *P. aeruginosa* bacteria were eliminated at this concentration.

Table 1. Results of Minimum Inhibitory Concentration and Minimum Concentration of *S. securidaca* and *W. somenetra* against bacterial pathogens.

Bacterial Code	<i>Methanol S. securidaca</i>	<i>Ethyl acetat S. securidaca</i>	<i>Methanol W. somenefra</i>	<i>Ethyl W. somenefra</i>
<i>S. pneumoniae</i>	25/50	25/50	6.25/12.5	6.25/12.5
<i>E. faecalis</i>	50/100	25/50	12.5/25	6.25/12.5
<i>P. mirabils</i>	12.5/25	25/50	6.25/12.5	3.1/6.25
<i>S. aureus</i>	50/100	25/50	6.25/12.5	12.5/25
<i>E. coli</i>	12.5/25	25/50	6.25/12.5	3.1/6.25
<i>P. aeruginosa</i>	50/100	25/50	25/50	6.25/12.5

Table 2. Results of Minimum Inhibitory Concentration and Minimum Concentration of *R. officinalis* and *A. vera* against Bacterial pathogens.

Bacterial Code	<i>Methanol R. officinalis</i>	<i>Ethyl acetat R. officinalis</i>	<i>Methanol A. vera</i>	<i>Ethyl acetat A. vera</i>
<i>S. pneumoniae</i>	6.25/12.5	3.1/6.25	50/100	25/50
<i>E. faecalis</i>	6.25/12.5	3.1/6.25	50/100	25/50
<i>P. mirabils</i>	6.25/12.5	3.1/6.25	25/50	50/100
<i>S. aureus</i>	6.25/12.5	6.25/12.5	50/100	50/100
<i>E. coli</i>	12.5/25	25/50	100/200	50/100
<i>P. aeruginosa</i>	12.5/25	6.25/12.5	50/100	25/50

Table 3. Antibiotic resistance pattern of bacteria.

Bacterial	AN	OX	AMC	CZ	V	AM	GM	AZN
<i>S. pneumoniae</i>	S	R	S	R	I	I	S	S
<i>E. faecalis</i>	S	R	I	R	S	R	S	R
<i>P. mirabilis</i>	R	R	S	I	S	S	S	R
<i>S. aureus</i>	R	R	S	S	I	S	I	R
<i>E. coli</i>	S	R	S	S	S	S	S	R
<i>P. aeruginosa</i>	R	R	R	R	R	R	R	R

The lowest inhibitory concentration of methanol extract of *W. sominefra* was 6.25, which *S. pneumoniae*, *P. mirabilis*, *S. aureus*, *E. coli* were inhibited at this concentration, while the highest inhibitory concentration was observed against *P. aeruginosa* (25 ppm), The lowest inhibitory concentration of the ethyl acetate *W. sominefra* extract was 3.1ppm in comparison with *P. mirabilis* and *E. coli*, while the highest inhibitory concentration was observed at 12.5 ppm *Pseudomonas aeruginosa*.

The lowest inhibitory concentration of Rosemary ethyl acetate extract was 3.1ppm in *S. pneumoniae*, *E. faecalis*, *P. mirabilis* and has the highest inhibitory concentration of 25, which was observed against *E.coli* bacteria.

Discussion

Since multidrug resistance of microorganisms is a main medical concern, screening of natural Products in a search for new antibacterial factors that would be active versus these microorganisms is the need of the hour. The finding disclose that the maximum inhibition of bacterial growth was seen at 1:8 dilution of WSR extract. The highest dilution of the juice that inhibited the increase of

the test organism when compared with control was 1:16. Therefore, the minimum inhibitory concentration of aqueous juice of WSR is 1:16(14).

The study of Rizwana was conducted to evaluate the antimicrobial acting of chloroform, acetone, methanolic, and ethanolic crude juice of stem, leaves, and roots of *Withania somnifera*. The region of blockage was maximum with acetone juice ranging between 38 and 10 mm, followed by methanolic juice ranging between 28 and 10 mm and ethanolic juice ranging between 25 and 8 mm, respectively. However, *K. pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA) did not respond to root juice of both methanolic and ethanolic juice. The chloroform juice of stem and leaves showed significant acting versus all pathogens with inhibition region between 20 and 8 mm (15).

The study of Sepehri et al (2016) evaluated the antimicrobial acting of *Withania somnifera* L. *Dunal* (Solanaceae; root and leaves)], an Indian traditional medicinal herb versus pathogenic bacteria. The methanolic juice was further sub-fractionated apply different solvents and the butanolic sub-fraction was found to possess maximum inhibitory acting versus a spectrum of

bacteria including *Salmonella typhimurium*. Moreover, in contrast to the synthetic antibiotic (viz. chloramphenicol), these juice did not induce lysis on incubation with human erythrocytes, advocating their safety to the living cells. Finally, the antibacterial efficacy of the juice isolated from herb (both root and leaves) was determined versus experimental salmonellosis in Balb/C mice (16).

Rosmarinus officinalis L. essential oil and three of its main components 1, 8-cineole (27.23%), α -pinene (19.43%) and β -pinene (6.71%) were measure for their in vitro antimicrobial activities and toxicology properties. *R. officinalis* L. essential oil possessed similar antimicrobial activities to α -pinene, and a little bit better than β -pinene, while 1,8-cineole possessed the lowest antibacterial activities. *R. officinalis* L. essential oil exhibited the strongest cytotoxicity towards three human cancer cells. Its inhibition concentration 50% (IC₅₀) values on SK-OV-3, HO-8910 and Bel-7402 were 0.025%, 0.076% and 0.13% (v/v), respectively. The cytotoxicity of all the experiment samples on SK-OV-3 was significantly stronger than on HO-8910 and Bel-7402. In general, *R. officinalis* L. essential oil demonstrate greater acting than its components in both antimicrobial and anticancer test systems, and the activities were mostly related to their concentrations (17).

The study of Mashhadi et al (2016), the most important antimicrobial acting of both essential oils was expressed on *Escherichia coli*, *Salmonella typhi*, *S. enteritidis*, and *Shigella sonnei*. A significant rate of antifungal acting, especially of essential oil of rosemary, was also exhibited. Antioxidant acting was evaluated as a free radical scavenging capacity (RSC), together with the effect on lipid peroxidation (LP). RSC was assessed by measuring the scavenging acting of essential oils on 2,2-diphenyl-1-picrylhydrazil (DPPH) and hydroxyl radicals(18).

The study of Masoumipour the main components of these fractions were alpha-pinene, 1, 8-cineole, camphor, verbenone, and borneol, constituting ca. 80% of the total oil. The antibacterial acting was investigated by the disc diffusion and broth dilution methods versus six microbial species,

including gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram-negative bacteria (*E. coli* and *P. aeruginosa*), a yeast (*Candida albicans*), and a fungus (*Aspergillus niger*). All of the essential oil-rich fractions obtained demonstrated antimicrobial acting versus all of the microorganisms experimental, with inhibition range and minimal bactericidal and fungicidal concentration values in the range of 17 to 33 mm and 2.25 to 0.25 mg/ml, respectively. The most acting fraction was the one obtained in experiment 4 (4% ethanol as modifier; extraction pressure, 25 MPa; extraction temperature, 60 degrees C). *S. aureus* was found to be the most sensitive bacteria to the rosemary juice, whereas the least susceptible was *A. niger*. alpha-Pinene, 1,8-cineole, camphor, verbenone, and borneol standards also showed antimicrobial acting versus all the microorganisms experimental, borneol being the most effective followed by camphor and verbenone (19).

The study of Jahani et al (2016) antimicrobial activity of the essential oils and methanolic juice of *R. officinalis* collected of three different regions at four different time intervals of the year versus *S. aureus*, *Proteus vulgaris*, *P. aeruginosa*, *K. pneumonia*, *E. feacalis*, *E. coli*, *S. epidermidis*, *B. subtilis* and *C. albicans*. Essential oils were obtained from the aerial parts of the herb by apply a Clevenger apparatus, for 4 h. After distillation, the distillates were filtered, air-dried and then extracted by using a Soxhlet apparatus for 9 h to obtain the methanolic juice. The antimicrobial activities of the essential oils obtained from *R. officinalis* were determined by minimum inhibitory concentration (MIC). The results indicated that the tested bacteria were sensitive to the essential oils and partially to the methanolic juice. The antimicrobial activities of the essential oils against the experimental bacteria differed, depending on location and seasonal variations (20). The study of Hydari et al (2016) was to evaluate the antimicrobial effects of Rosemary (*R. officinalis* L.) essential oils against *Staphylococcus* spp. fourteen clinical isolates of *Staphylococcus* were cultured from patients. The disc diffusion

method was used for determination of antimicrobial activity of essential oil. Results showed that this inhibitory effect is dose-dependent, to wit, by increasing the concentration of the extract in the culture media, reduction in growth was obviously revealed (21).

The study of Mohsenipour and Hassanshahian (2014) was to determine the antimicrobial activity of three selected plants (*R. officinalis*, *O. majorana*, and *Trigonella foenum-graecum*) against Extended Spectrum Beta Lactamase (ESBL)—producing *Escherichia coli* and *K. pneumoniae* and to identify the specific plant fraction responsible for the antimicrobial activity. The Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) were determined using broth microdilution. The MICs ranged between 1.25 and 80 µg/ml. The majority of these microorganisms were inhibited by 80 and 40 of the crude extracts. The petroleum ether fraction of *Origanum majorana* significantly inhibited 94% of the tested strains. Ethyl acetate extracts of all selected plants exhibited relatively low MICs and could be therefore described as strong antibacterial (22).

The study of Mohsenipour and Hassanshahian (2015) was to evaluate the antimicrobial efficacy of *Aloe vera* and to determine its effectiveness in decontaminating gutta percha cones. The zones of inhibition on the agar plate were measured as 24 mm, 21 mm and 24mm respectively. The broth remained clear even after 48 hours of incubation (23).

The study Mohsenipour et al (2015) was to determine the anti-microbial and inhibitory activities of various concentration of *Aloe vera* Gel (AVG) against oral pathogenic bacteria. Various staining and biochemical tests confirmed that the sample contained *A. actinomycetemcomitans*, *Clostridium bacilli*, *Streptococcus mutans* and *S. aureus*. AVG showed anti-bacterial property at 100% and 50% concentration ('t' value = 7.504, p-value <0.001). At lower concentration there was no effect against the bacteria. At 100% AVG concentration, zone of inhibition measured was 6.9 mm in *A. actinomycetemcomitans*, 6.3 mm in *C.*

bacilli, 6.8mm in *S. mutans* and 6.6mm in *S. aureus*. The standard drugs were also used to compare anti-bacterial property of AVG. Result showed that higher concentration (100%, 50%) of AVG has comparable zone of inhibition with Ofloxacin (5mcg) and Ciprofloxacin (30 mcg)(24).

The antibacterial property of *Aloe vera* gel extracted using different solvents showed varying degree of response towards the selected pathogens. Using ethanol extracts the zones of inhibition ranged from 12.66 – 23.33 mm being maximum for *B. cereus* and minimum for *E. coli* (p<0.05). Methanol extract exhibited maximum antibacterial activity against *B. cereus* (22.33 mm) followed by *S. pyogenes* (15 mm) and least for *S. typhi* (9.66 mm); the differences being statistically significant (p<0.05). Acetone extract gave lower values of zones of inhibition ranging from 6.00 mm for *E. coli* to 7.33 mm for *S. pyogenes*, while no response was observed for *P. aeruginosa* and *S. typhi* (p<0.05) (25).

Conclusion

The resistant of bacteria to some antibiotics is increased in these recent years. Then, selection of an alternative method for overcome to these antibiotic resistance is very important. The results of this study confirm that the extract from *S. securidaca*, *W. sominefra*, *R. officinalis* and *Aloe vera* have a good potential for decrease antibiotic resistance in some pathogenic bacteria.

Conflict of interest

The authors declare that they have no conflicts of interest.

Financial disclosure

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