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Chemical analysis by GC-MS and *in vitro* antibacterial activity of *Alkanna tinctoria* extracts against skin infection causing bacteria.

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Abstract

Alkanna **is a traditional medicinal plant; Bedouin of Saudi Arabia use the roots of the plant for the treatment of many skin diseases. Water and ethanol extracts of** ***Alkanna*** **roots were evaluated for their antibacterial activity against four pathogenic bacteria responsible for skin infections i.e.** ***Pseudomonas areuginosa*** **ATCC 27853,** ***Bacillus subtilis*** **(clinical strain),** ***Escherichia coli*** **ATCC 25922 and** ***Staphylococcus aureus*** **ATCC 25928. The agar well diffusion method was employed for the antibacterial screening of crude extracts. Ethanol extract inhibited the growth of all bacteria, as depicted by the zone of inhibition of bacteria around the well filled with extract. The maximum zone of inhibition was observed against** ***B. subtilis*** **(28.0 mm) followed by** ***S. aureus*** **(24.7 mm),** ***P. areuginosa*** **(18.7 mm) and** ***E. coli*** **(15.3 mm). The water extract showed minor antibacterial activity against** ***B. subtilis*** **and** ***S. aureus*** **however, it was found ineffective against other two pathogens. The streak method was used to determine the Minimum Inhibitory Concentration (MIC). The MIC value of ethanol extract against** ***B. subtilis*** **and** ***S. aureus*** **was 2 µl/ml, whereas, against** ***E. coli*** **and** ***P. areuginosa*** **it was 4 µl/ml. The qualitative analysis of the ethanol extract was conducted by the GC-MS. The major compounds detected in the ethanolic extract of** ***Alkanna*** **were akanin, acetyl-alkanin, ethane, 1, 1-dichloro-, 1H-enzotriazole, 4-nitro, 2-chloroethyl (methylsulfonyl) methanesulfonate and 2, 5-cyclohexadien-1-one, 4-diazo-. Besides that several other napthoqinones and esters were detected in the extract. Results of present study manifest that extract of** ***Alkanna*** **has the potential to inhibit the growth of pathogens responsible for skin infections.**

Keywords: GC-MS analysis, *Alkanna tinctoria*, Antimicrobial activity, Pathogenic bacteria, Skin infection.

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Introduction

Natural products are a major source of discovery and development of new medicines due to the chemical diversity exhibited by plants. Hundreds of plant metabolites are reported to have many pharmacological activities [1]. Major population of the world still depends upon the herbal treatment of common ailments. With the increase in the dependency on the herbal medicines, scientists are paying more attention on exploring the medicinal plants for isolating bioactive compounds and developing new medicines. Members of genus *Alkanna* are known for medicinal and other properties from the ancient time. As evident, Hippocrates documented the use of alkanet root for the skin ulcers treatment. Similarly, Theophrastus and Dioscorides described the properties of *Alkanna* [2]. Traditionally, alkanet, are used for treating ulcers, inflammation and wounds. It is also used in dying cloth, colouring fats, cosmetics and food [2,3]. Naphthoquinone pigments, alkannin and its derivatives have been detected in the extracts of alkanet [4,5]. Several of these compounds are recorded for cytotoxic, antimicrobial, anti-leishmanial and anti-inflammatory activities [6-9]. The ointments, helixderm and histoplastin red contain alkannin as the active ingredient [5]. Bedouin of Saudi Arabia still use *Alkanna* roots for treating burns, skin wounds and other skin diseases [10]. *Streptococcus* and *Staphylococcus* are the common pathogens of skin infection. After burn injury diverse microorganisms such as gram positive and gram negative bacteria and yeast colonizes the burn wounds. Presently *S. aureus* and *P. areuginosa* are the most prevalent source of burn wound infection and are of great concern due to the development of resistant strains [11]. *B. subtilis* a ubiquitous bacterium is found on skin and also in epithelial wounds [12]. To find out the role of *Alkanna* in treating skin infections, the antibacterial activity of crude extract of *A. tinctoria* roots and the chemical composition of crude extract of *Alkanna* by GC-MS analysis was conducted.

Materials and Methods

Microorganisms

Four human pathogenic bacteria responsible for skin infection *viz. Pseudomonas areuginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25928, *Bacillus subtilis* (clinical strain) were procured from the King Khalid Hospital, King Saud University, Riyadh, Saudi Arabia. Stock

cultures of bacterial strains were maintained on the slants of nutrient agar, all stock cultures were stored at 4°C.

Collection of Alkanna roots and preparation of crude extracts

The roots of *Alkanna* were purchased from the local market of Riyadh, Saudi Arabia. For the preparation of water extract, dried powder of the sample (20 g) with 100 ml of distilled water was kept on water bath for 4 h at 45°C. After that solution was filtered by Whatman filter paper no.1 and the filtrate so obtained was used for the study. The ethanolic extract was prepared with help of Soxhlet extractor. Dried powder of the sample (20 g) and 96% ethanol (100 ml) were placed in the extraction tube and the apparatus was run for 24 h to get the extract. The liquid extract was evaporated to dryness in the oven at 50°C. The dried residue was scraped and dissolved in 96% ethanol to make the final volume of 2 ml. The so obtained crude extracts were used for further study.

Determination of antibacterial activity by agar well diffusion method

Antibacterial assay: The agar well diffusion method was employed for the antibacterial screening of crude extracts [13]. Fresh culture of bacteria to be tested were inoculated in 10 ml aliquots of nutrients broth and incubated at 37°C for 24 h. After required incubation the cultured broth was adjusted to a turbidity of 0.5 Mac Farland standards (10^8 CFU/ml). From the quantified broth, 10 µl bacterial culture was evenly spread over the entire surface of the nutrient agar plate. On the agar plate 4 wells/plate were made with the help sterilised cork borer and each well was filled with 50 µl crude extract, ethanol solvent was used as the negative control. Gentamycin (30 µg) was used for determination of susceptibility of tested pathogen to antibiotic. The plates were incubated at 37°C for 24 h. After 24 h, the antibacterial activity was determined from the size of the diameter (mm) of clear zone of surrounding the well.

Determination of minimum inhibitory concentration (MIC): The streak method was used to determine the Minimum Inhibitory Concentration (MIC) as described by Hancock [14]. Crude extracts were dissolved in nutrient broth and serially diluted in eppendorf tubes. 10 µl of overnight actively growing culture of the bacteria (1×10^6 CFU/ml) was transferred to the different eppendorf tubes. The tubes were incubated for 24 h at 37°C. The following morning, streaking was done from all samples on nutrient agar plates, agar plates were incubated overnight at 37°C. Next day plates were observed for the bacterial growth. MIC value was judged by the lowest concentration of the extract concentration that inhibited growth.

GC-MS analysis of crude extracts

To determine the chemical composition of crude extract, the gas chromatography coupled with mass spectrometer (GC-MS) analysis was conducted. The analysis was performed with Perkin-Elmer (Clarus 500, USA) gas chromatography coupled with (Clarus 500, USA) mass spectrometer (MS) equipped with RTx-5 column (30 × 0.32 nm). The oven temperature was set 50 to 150°C at 3°C/min, it was held isothermal for 10 min and raised to 250°C at the rate of 10°C/min. The carrier gas was Helium (1 ml/min). In this analysis neither internal nor external chemical standards were used. Interpretation of the resultant mass spectra was done by using NIST database and by studying the fragmentation pattern of such compound resulted from mass spectrometry analysis.

Statistical analysis

Experiments were performed in triplicate. The Standard Deviation of the mean (SD) of experimental results were calculated using SPSS statistics software.

Results

The water and ethanol extracts of *A. tinctoria* were evaluated for their antibacterial activity against four pathogenic bacteria responsible for skin infections (Table 1). Results depict that the ethanol extract had excellent potential in inhibiting the growth of all four pathogens tested, maximum zone of inhibition was observed against *B. subtilis* (28.0 mm) followed by *S. aureus* (24.7 mm), *P. areuginosa* (18.7 mm) and *E. coli* (15.3 mm). The water extract showed minor antibacterial activity against *B. subtilis* (8.3 mm) and *S. aureus* (8.3 mm) whereas, it was found ineffective against other two pathogens. In general, the pathogen, *B. subtilis* was observed to be most sensitive pathogen whereas, least was *E. coli*. The antibiotic, gentamycin (30 µg) was found much more effective than any of extract evaluated. Results of determination of Minimum Inhibition Concentration (MIC) of ethanol extract of *A. tinctoria* roots reported in Table 1 showed that the MIC values were depended on the pathogenic strain tested and the dilution used. The pathogens, *B. subtilis* and *S. aureus* were inhibited at a concentration of 0.2%. The MIC value of ethanol extract for *E. coli* and *P. areuginosa* was 0.4%.

The ethanol extract selected for the chemical analysis as it showed excellent antibacterial activity against all the tested pathogens. The qualitative analysis of the ethanol extract was conducted by the GC-MS (Table 2). The major compounds detected in the ethanolic extract of *Alkanna* were akanin, acetyl-alkanin, ethane, 1, 1-dichloro-, 1H-benzotriazole, 4-nitro, 2-chloroethyl (methylsulfonyl) methanesulfonate and 2, 5-cyclohexadien-1-one, 4-diazo-(napthoquinone). Besides that several other napthoqinones and esters were detected in the extract.

Table 1. *In vitro antibacterial activity and minimum inhibitory concentration (MIC) values of water and ethanol crude extract of A. tinctoria roots.*

Organisms	Extracts of *A. tinctoria* (50 µl/well)		Positive control*	MIC (v/v) ethanol extract
	Water	Ethanol		
	Zone of inhibition (mm)[a]			

B. subtilis	8.3 ± 0.58	28 ± 1.0	31.3 ± 1.15	0.20%
E. coli	0	15.3 ± 0.58	25.3 ± 0.58	0.40%
P. areuginosa	0	18.7 ± 1.15	23.7 ± 0.58	0.40%
S. aureus	8.3 ± 0.58	24.7 ± 0.58	28.0 ± 1.00	0.20%

[a]Mean ± SD for N=3; [*]Gentamycin (30 µg); negative control did not show any zone of inhibition.

Table 2. *The GC-MS analysis of ethanol extracts of A. tinctoria roots.*

Compound name	Molecular weight	Formula	Rev
1. 5, 8-dihydroxy-2-(1-hydroxy-4-methylpent-3-enyl) naphthalene-1, 4-dione alkanin	288	$C_{16}H_{16}O_5$	918
2. 2-(2-methoxyphenoxy) ethyl 2-acetyloxybenzoate (acetyl-alkanin)	330	$C_{18}H_{18}O_6$	917
3. Ethane, 1, 1-dichloro-	98	$C_2H_4Cl_2$	813
4. 2-chloroethyl methyl sulfone	142	$C_3H_7O_2ClS$	851
5. 2, 5-cyclohexadien-1-one, 4-diazo-napthoquinone	120	$C_6H_4ON_2$	903
6. 2-chloropropionyl chloride	126	$C_3H_4OCl_2$	889
7. 1H-benzotriazole, 4-nitro-azol dye	164	$C_6H_4O_2N_4$	808
8. 2-chloroethyl (methylsulfonyl) methanesulfonate clomesone	236	$C_4H_9O_5ClS_2$	751
9. Benzenediazonium, 4-hydroxy-, hydroxide, inner salt napthoqinone	120	$C_6H_4ON_2$	823

Discussion

The results of antibacterial activity of water and ethanol extracts of *A. tinctoria* showed that the ethanol extract had a potential to inhibit the growth of all bacterial pathogen tested. Whereas, the water extract showed almost negligible activity against these pathogens. The reasons for lesser antibacterial activity by water extract could be due to a lower concentration of antibacterial compounds in the extract or may be water was not a suitable solvent for the extraction of active ingredients of the *Alkanna* roots [15]. Cowan [16] documented that most of the active antimicrobial compounds of plants are obtained through the extraction with ethanol or methanol solvent. Moreover, the alkanet roots contain dyestuffs which are soluble in alcohol, ether and oils but insoluble in water. May be the bioactive ingredients are the part of non-water soluble constituents as it is also evident from the present results that ethanol extract gave much better results than water extract. In general, the pathogen, *B. subtilis* was found to be most sensitive pathogen whereas, least was *E. coli*. It is interesting to note that the ethanol extract was also effective against the gram-negative bacteria (*E. coli* and *P. areuginosa*) which are generally resistant to antibiotic. Khan et al. [17] reported that leaves extract of *A. tinctoria* prepared in ethanol, chloroform hexane and water had showed potential activity against *S. aureus* in addition these extracts were also effective to *A. baumannii*, *E. coli*, *P. aeruginosa*. Sengul et al. [18] reported that the methanolic extract of *A. tinctoria* had antimicrobial activity against 9 out of 32 microorganisms tested.

Results of determination of Minimum Inhibition Concentration (MIC) of ethanol extract of *A. tinctoria* showed that the pathogens tested in the current study with various concentration of ethanol extract exhibited different level of inhibition, which means that the crude extract alone was responsible for the inhibition of pathogenic species. This is the first report of determination of MIC value of ethanolic extract of *Alkanna* roots. In a study, the MIC of aqueous *Alkanna* leaves extract against *A. baumannii* and *S. aureus* was recorded to be 12.5 mg/ml whereas, against *E. coli* and *P. aeruginosa* it was 25 mg/ml [17]. The flavonoid sarothrin isolated from *A. orientalis* resulted in inhibiting the growth of *Mycobacterium smegmatis* (MIC 75 µM) and *S. aureus* (MIC>800 µM) [19]. Ogurtan et al. [20] reported the second-degree burn in rabbit model was healed by the root topical preparation in olive oil. Traditionally the *Alkanna* roots extract mixed with olive oil are used to treat burn wounds. Two bacterial species *S. aureus* and *P. areuginosa* are the most prevalent infective agents of the burn wounds. Results of present study manifest that extract of *Alkanna* has the potential to inhibit the growth of these pathogens. Hence the present study proves the role of *Alkanna* in treating burn wounds and skin infections.

After evaluating the antibacterial activity of water and ethanol extract of *A. tinctoria*, the ethanol extract of *Alkanna* was selected for the chemical analysis as the extract showed excellent antibacterial activity. The qualitative analysis of the ethanol extract was conducted by the GC-MS. The major compounds detected in the ethanolic extract of *Alkanna* were akanin, acetyl-alkanin, ethane, 1, 1-dichloro-, 1H-benzotriazole, 4-nitro, 2-chloroethyl (methylsulfonyl) methanesulfonate and 2, 5-cyclohexadien-1-one, 4-diazo-(napthoquinone). Besides that several other napthoqinones and esters were detected in the extract. The current GC-MS analysis of ethanol extract clearly showed the presence of important bioactive chemicals which were reported earlier by various workers, such as, alkanin, shikonin and naphthoquinone and their derivatives [21]. Through chromatographic purification Tung et al. [4] isolated two major naphthoquinones, alkannin and angelylalkannin from the roots

of *A. tinctoria*. Assimopoulou et al. [15] reported that dichloromethane extract of *A. tinctoria* roots contains mainly alkannin esters. In a review Ivancheva et al. [1] illustrated the presence of naphthoquinone, alkannin or shikonin, *Alkanna*n and alkane's esters in the roots of *A. tinctoria*. Some of these compounds are characterised as bioactive compounds [8,9]. Papageorgiou et al. [21] concluded that napthoquinone pigment with naphthezarin system is requisite for the activity. The present GC-MS analysis of ethanol extract reveals the presence of many napthoquinones, chemicals noticeable for their antibacterial activity.

Conclusion

The present study proves the role of *Alkanna* roots in treating skin infections. The ethanolic crude extract of *A. tinctoria* roots, can be a source new antibacterial compound which are yet to be explored.

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References

1. Ivancheva S, Nikolova M, Tsvetkova R. Pharmacological activities and biologically active compounds of Bulgarian medicinal plants. Phytochem Adv Res Filippo Imperato Res Signpost 2006; 87-103.
2. Papageorgiou VP, Assimopoulou AN, Ballis AC. Alkannins and shikonins: a new class of wound healing agents. Curr Med Chem 2008; 15: 3248-3267.
3. Bisset NG. Herbal drugs and phytopharmaceuticals: A handbook for practice on a scientific basis. Med Pharm Scientific Publishers, Stuttgart, Germany 1994.
4. Tung NH, Du GJ, Wang CZ, Yuan CS, Shoyama Y. Naphthoquinone components from Alkanna tinctoria (L.) Tausch show significant antiproliferative effects on human colorectal cancer cells. Phytother Res 2013; 27: 66-70.
5. Papageorgiou VP, Digenis GA. Isolation of two new alkannin esters from Alkanna tinctoria. Planta Med 1980; 39: 81-84.
6. Mohammed MS, Mohamed AL, Basudan O, El Tahir KEH, Osman B, Osman WJA. Evaluation of antipyretic, antinocieptive and sedative effects of Tribulus terrestris, Mimosa pigra and Alkanna tinctoria methanolic extracts. J Phytopharmacol 2016; 5: 105-107.
7. Alrheam A, Sheik GB, Al Mutairi NBK, Al Mutairi MBK. The efficacy of newly formula of Alkanna tinctoria oil for treatment of five bacterial species. Int J Adv Res 2015; 3: 646-654.
8. Assimopoulou AN, Papageorgiou VP. Radical scavenging activity of Alkanna tinctoria root extracts and their main constituents, hydroxynaphthoquinones. Phytother Res 2005; 19: 141-147.
9. Ali A, Assimopoulou AN, Papageorgiou VP, Kolodziej H. Structure/antileishmanial activity relationship study of naphthoquinones and dependency of the mode of action on the substitution patterns. Planta Med 2011; 77: 2003-2012.
10. Elsharkawy M, Elshathely GA, Jaleel HI, Al-Johar. Anti-inflammatory effects of medicinal plants mixture used by Bedouin people in Saudi Arabia. Herba polonica 2013; 59: 76-89.
11. Church D, Elsayed S, Reid O, Winston B, Lindsay R. Burn wound infections. Clin Microbiol Rev 2006; 19: 403-434.
12. Gonzalez DJ, Haste NM, Hollands A, Fleming TC, Hamby M, Pogliano K, Nizet V, Dorrestein PC. Microbial competition between Bacillus subtilis and Staphylococcus aureus monitored by imaging mass spectrometry. Microbiol 2011; 157: 2485-2492.
13. Khan MA, Inayat H, Khan H, Saeed M, Khan I, Rahman I. Antimicrobial activities of the whole plant of Cestrum nocturnum against pathogenic microorganisms. Afr J Microbiol Res 2011; 5: 612-616.
14. Hancock RE. Peptide antibiotics. Lancet 1997; 349: 418-422.
15. Assimopoulou AN, Boskou D, Papageorgiou VP. Antioxidant activities of alkannin, shikonin and Alkanna tinctoria root extracts in oil substrates. Food Chem 2004; 87: 433-438.
16. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999; 12: 564-582.
17. Khan UA, Rahman H, Qasim M, Hussain A, Azizllah A, Murad W, Khan K, Anees M, Adnan, M. Alkanna tinctoria leaves extracts: a prospective remedy against multidrug resistant human pathogenic bacteria. BMC Compl Alt Med 2015; 15: 127.
18. Sengul M, Yildiz H, Gungor N, Cetin B, Eser Z, Ercisli S. Total phenolic content, antioxidant and antimicrobial activities of some medicinal plants. Pak J Pharm Sci 2009; 22: 102-106.
19. Bame JR, Graf TN, Junio HA, Bussey RO, Jarmusch SA. Sarothrin from Alkanna orientalis is an antimicrobial agent and efflux pump inhibitor. Planta Med 2013; 79: 327-329.
20. Ogurtan Z, Hatipoglu F, Ceylan C. The effect of Alkanna tinctoria Tausch on burn wound healing in rabbits. Dtsch Tierarztl Wochenschr 2002; 109: 481-485.
21. Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KC. The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products. Angew Chem Int 1999; 38: 270-300.

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