In Vitro Study to Evaluate Antibacterial and Non-haemolytic Activities of Four Iranian Medicinal Plants

S Sepahi[1], A Ghorani-Azam[1], S Sepahi[2], A Asoodeh[1], S Rostami[3]

ABSTRACT

Objective: *Aqueous extracts of four medicinal plants including* Ferula gummosa, Echinophora orientalis, Nasturtium microphyllum *and* Verbascum thapsus *were used to determine their antibacterial activities and minimum inhibitory concentration (MIC). The aim of this study was to assess antibacterial activity of extracts of four medicinal plants against a Gram-positive and a Gram-negative bacteria* (Staphylococcus aureus *PTCC1431, and Escherichia coli HP101BA 7601c).*
Methods: *Radial diffusion assay was used to assess the antibacterial activity of extracted samples. Haemolysis assay was also used to examine their nontoxic effects on human red blood cells.*
Results: *This study showed that all the mentioned plants have satisfactory antibacterial effects against both Gram-positive and Gram-negative bacteria. Minimum inhibitory concentration values of these samples were less than 750 µg/mL. In addition, no significant haemolytic activity was observed at their MIC values.*
Conclusion: *The results of this study showed that all these studied plants have good potential for further studies for drug discovery.*

Keywords: Antimicrobial activity, medicinal plants, minimum inhibitory concentration, traditional medicine

Estudio *In Vitro* para Evaluar Actividades Antibacterianas y No Hemolíticas de Cuatro Plantas Medicinales Iraníes

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RESUMEN

Objetivo: *Extractos acuosos de cuatro plantas medicinales – incluyendo Ferula gummosa, Echinophora orientalis, Nasturtium microphyllum y Verbascum thapsus – fueron utilizados para determinar sus actividades antibacterianas y la concentración inhibitoria mínima (CIM). El objetivo de este estudio fue evaluar la actividad antibacteriana de los extractos de cuatro plantas medicinales frente a bacterias Gram-positivas y Gram-negativas (Staphylococcus aureus PTCC1431 y Escherichia coli HP101BA 7601 c).*
Métodos: *Se usó un ensayo de difusión radial para evaluar la actividad antibacteriana de las muestras extraídas. También fue utilizado un ensayo de hemólisis para examinar los efectos no tóxicos de las muestras en los glóbulos rojos de los seres humanos.*
Resultados: *El estudio demostró que todas las plantas mencionadas tienen efectos antibacterianos satisfactorios contra las bacterias Gram-positivas y Gram-negativas. Los valores CIM de estas muestras fueron menos de 750 µg/mL. Además, no se observó actividad hemolítica significativa en sus valores CIM.*
Conclusión: *Los resultados de este estudio mostraron que todas las plantas estudiadas tienen buen potencial para continuar su estudio con el fin de hallar nuevos fármacos.*

Palabras claves: Actividad antimicrobiana, medicina tradicional, plantas medicinales, CIM

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INTRODUCTION

Therapeutic purposes of medicinal plants are known by people in different countries. Medicinal herbs are used for pain relief in some countries (1–3). Plants, specifically medicinal plants, are widely used in medicine and pharmacological sciences. These medicinal plants contain some organic compounds such as polyphenols, aliphatic compounds and peptides which have antimicrobial properties (4–10). Medicinal herbs have antibacterial compounds which are effective in the treatment of diseases (11–17). Several recent studies showed that some plants have promising anti-inflammatory effects (18–20). In some countries, plants and herbs are widely used as anti-cancer agents and antioxidants in traditional medicine (21–24). Medicinal herbs generally contain ingredients that have been shown to have relatively low side effects on the human body in comparison with other chemically synthetic antibiotics (21). Different parts of the plant including root, leaf, seed, fruit and stem could be used in pharmacology for drug discovery. *Ferula gummosa* belongs to the family *Umbelliferae*. In traditional medicine, *Ferula gummosa* is used in the treatment of physiological disorders such as backache and haemorrhoid. This plant grows in the north-east of Iran. *Echinophora orientalis* from *Apiaceae* mostly grows in north-west Iran. It also grows in Armenia and Turkey, especially eastern Anatolia. It grows on fallowing lands, sandy steppes, gravel and dry hills. *Echinophora orientalis* is used as an ingredient in food and dairy. *Nasturtium microphyllum* (watercress) belongs to the family *Brassicaceae*. Some members of this family have antiviral, antibronchitic, antipyretic and tonic properties and are suitable for the treatment of respiratory illnesses (25–26). *Verbascum thapsus* has antioxidant and antiviral properties and is generally used to treat wounds (27–28). It belongs to the family *Scrophulariaceae*. All these plants are traditionally used in Iran for treating various diseases. Due to the lower frequency of side effects associated with the use of plants on the human body in comparison with other synthetic antibiotics, traditional medicine and the use of medicinal plants is growing worldwide. In this study, we travelled from the north-east to the north-west of Iran to collect these four medicinal plants and some basic information about their uses. These four medicinal plants are widely used in Iran for different therapeutic purposes including the treatment of intestinal illnesses caused by microbial contamination, although their role in the treatment of such diseases has not been proven. For this reason, they were chosen for antimicrobial study to evaluate their antibacterial properties.

MATERIAL AND METHODS

Sample collection and preparation of plant extracts

The plant samples were collected in spring 2012 from the north-east and north-west of Iran and their genus and species were confirmed taxonomically. To prevent mildew, avoid chemical changes and thus maintain its chemical composition as well as the ease of powdering plant for the extraction of the constituent materials, the plant was dried in an oven at 50 °C after collection. The dried plants were washed with deionized water and were then powdered with a grinding machine. Fifty grams of each dried sample was mixed in 50 mL of deionized water and centrifuged at 5000 rpm for five minutes. The supernatant was filtered through Whatman Filters Grade 1 Filter Paper. All these processes were performed at 4 °C. Extracted solutions of plants were then concentrated by ultrafiltration membrane, cut off of 1 kDa. Finally, samples were lyophilized by freeze drier to enable easier preparation of the samples in subsequent processes of antibacterial assay. Ten milligrams of each sample of dried powder was dissolved in 5 mL of phosphate buffered saline (PBS) and used as a 2 mg/mL stock for preparation of serial dilution (2 mg/mL, 1 mg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL and 125 µg/mL) for antimicrobial tests and minimum inhibitory concentration (MIC) assay.

Antimicrobial test

Antimicrobial activity of extracted samples was assayed on two Gram-positive and Gram-negative bacteria according to the radial diffusion assay (RDA). For this purpose, specific amount of bacteria (4×10^6 CFU) were poured into 5 mL of 10 mM cold phosphate buffer and were mixed with 1% agarose (Sigma-Aldrich) in 0.03% Tripticase soy broth (TSB) as an underlay culture and were poured into plates. Then, extracted samples were poured into the punched well of the plate, and then they were overlaid with media cultures containing pre-autoclaved 6% TSB and 1% agarose, and were kept at 37 °C for 12 hours. After two to three hours, a light-halo was obvious around the punched wells. The antibacterial activity of the test agents was determined by measuring the mean radius of zone of inhibitions in millimetre. All results were compared with the standard antibacterial antibiotic (Pattan Teb Company, Tehran, Iran). Each experiment was repeated three times.

Minimum inhibitory concentration determination

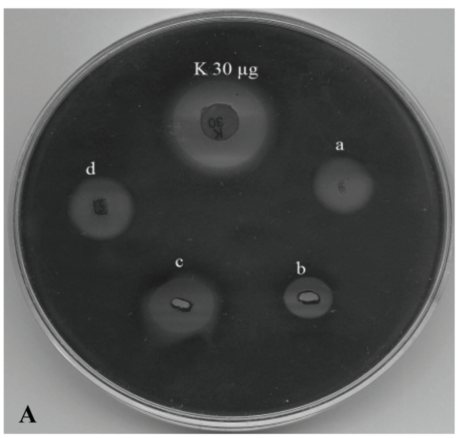
Almost all antimicrobial components are subjected to the MIC test. In microbiology, MIC is the lowest concentration of an antimicrobial compound that will inhibit the growth of a microorganism after overnight incubation (29, 30). Minimum inhibitory concentration is important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. Enzyme-linked immunosorbent assay (ELISA)-reader was used to measure MIC values at 630 nm. To determine MIC values, two types of bacteria, including *E coli* HP101BA 7601c (Gram-negative) and *S aureus* PTCC1431 (Gram-positive) were cultured. A bacterial suspension (1×10^6 CFU/mL) and 80 µl of TSB medium were poured into a 96-well microplate, after which serial dilutions of the extracted antimicrobial components were added. The 96-well plates were incubated at 37 ºC for 12 hours.

Haemolysis assay

Haemolytic activity of the samples was determined according to the method by Minn *et al* (31). Two millilitres of human red blood cells were washed several times with 5 mLof PBS by centrifugation at 4000 rpm for 10 minutes. Washed cells were diluted to a final volume of 40 ml of PBS; 20 µl of extracted sample was added to 180 µl of 5% diluted erythrocytes and the treated cells were kept at 37 ºC for 30 minutes. Triton X-100, at 0.1%, was used as a positive control with 100% haemolytic activity. After 30 minutes, the solution was centrifuged at 4000 rpm for five minutes and the supernatant was gently diluted to 1 ml of PBS. Absorbance of the solution was measured at 567 nm. Experiments were performed in triplicate.

RESULTS

Samples were collected and prepared with initial concentrations of 2 mg/mL. Bacteria were cultured and the antimicrobial components were poured into the punched wells. After 12 hours incubation in 37 ºC, the growth inhibitory zone around the wells was obvious (Fig. 1).



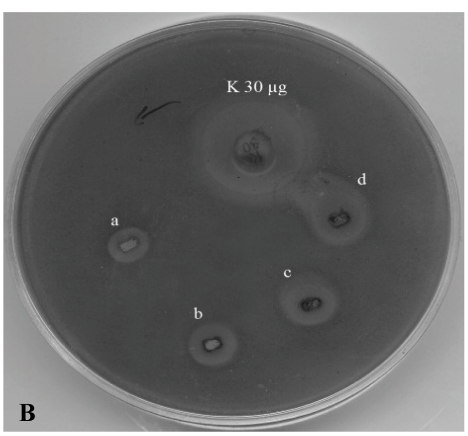


Fig. 1: Antimicrobial assay of four medicinal herbs against *E coli* (A) and *S aureus* (B). K shows kanamycin (30 µg) and the letters a, b, c and d show antimicrobial activity of (20 µg), *Verbascum thapsus*, *Nasturtium microphyllum*, *Echinophora orientalis* and *Ferula gummosa*, respectively.

Several independent experiments confirmed that these plants have great antimicrobial activity on both Gram-positive and Gram-negative bacteria. Kanamycin (30 µg) was used as positive control; it had great antibacterial properties and was suitable for a comparative antimicrobial assay. The overall MIC values for these samples were between 350 and 750 µg/mL (Table).

Table: Minimum inhibitory concentration (MIC) values of extracted samples

Species and family	**Folk uses**	**Micro-organism/MICs (µg/ml)**	
		E coli	*S aureus*
Ferula gummosa	Backache and haemorrhoid	350	500
Echinophora orientalis	In food and dairy	400	750
Nasturtium microphyllum	Antibronchitic and antipyretic	700	600
Verbascum thapsus	Tranquilize	550	750

Among the studied plants, the aqueous extracts of *Ferula gummosa* had more antimicrobial properties; however, almost all of the samples in this study had satisfactory antimicrobial activity. Figure 2 represents the relative anti-

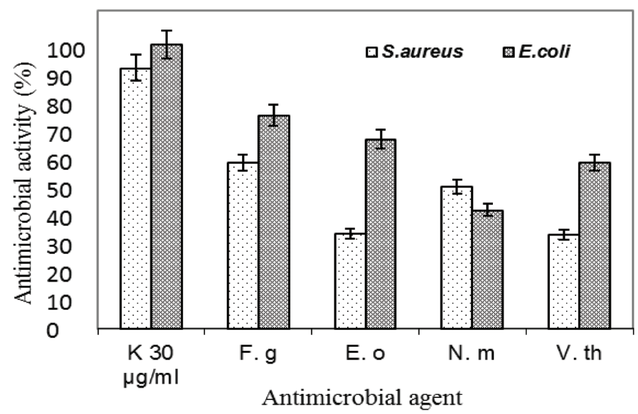


Fig. 2: Antibacterial activity (%) of samples against *S aureus* PTCC1431 and *E coli* PTCC1431. K shows control (kanamycin 30 µg) and F.g, E.o, N.m and V.th are abbreviations for *Ferula gummosa, Echinophora orientalis, Nasturtium microphyllum and Verbascum thapsus* (20 µl of 2 mg/mL stock for each), respectively.

bacterial properties of studied plants in comparison with kanamycin. Their antimicrobial activity was similar to kanamycin as a usual antibiotic but with more concentrations of aqueous samples (2 mg/mL).

Antibacterial activity of kanamycin was defined as 100%, and antimicrobial activities of aqueous extracts were compared with kanamycin. Only a very small amount of haemolysis was observed for all tested samples, and it was only less than 1.2% in comparison with Triton X-100 as positive control with 100% haemolysis (Fig. 3). But, other toxicological studies are required to confirm their non-toxic effects on human cells.

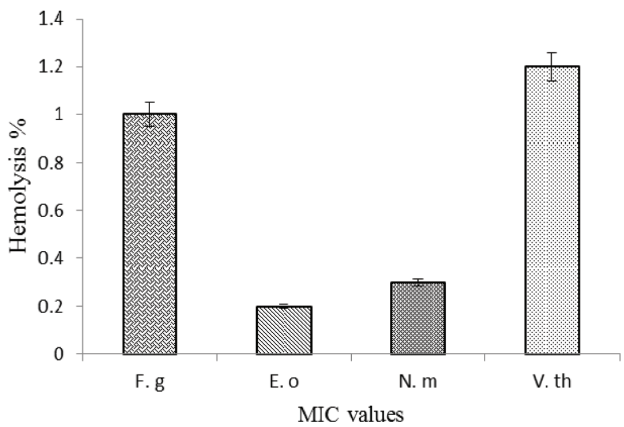


Fig. 3: Haemolysis assay at the minimum inhibitory concentration (MIC) values. For all extracts, no significant haemolytic effects were observed at their MIC values. F.g, E.o, N.m and V.th are abbreviations for *Ferula gummosa, Echinophora orientalis, Nasturtium microphyllum* and *Verbascum Thapsus*, respectively.

DISCUSSION

All the plants investigated in this study have satisfactory antimicrobial properties. However, all of them have more antimicrobial effect on Gram-negative bacteria than the Gram-positive ones. None of these plants has significant haemolytic activity, which makes them potentially good candidate for further studies in drug discovery and the search for new antibiotics from natural sources.

In many other reports, the antibacterial, antiviral and other medicinal properties of different medicinal plants have been studied (32, 33). This is due to the importance of plants and medicinal herbs in herbal therapy and drug discovery. Madubunyi showed that the constituents of *Garcinia kola* seeds have antimicrobial activities (34). In 2011, Hu *et al* conducted a study using light microscopy, transmission electron microscopy and scanning electron microscopy methods and reported that *Magnolia officinalis* extract has antimicrobial effects on *Staphylococcus aureus* (5). Lazarević *et al*, in 2011, showed that *Allium sphaerocephalon* has strong antioxidant and antimicrobial properties (18). Some other reports suggested that antibacterial and antiviral activities of plants could make them a potential reservoir of antibiotics in the near future (21, 22, 35). In this study, the authors introduced four medicinal plants which are widely used in Iran for different purposes. They all have great antibacterial and non-haemolytic activities. Our results provided evidence that the studied plant extracts might be potential sources of new antibacterial drugs in the near future.

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