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Antibacterial activity of camel whey in combination with various medicinal plant extracts

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Abstract

The aim of the present study was to evaluate the antibacterial potential of ethanol and water extracts from *Ballota undulata*, *Ruta chalepensis* and *Urtica urens* in combination with camel whey proteins. The bioactivity of all samples was tested against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Disk diffusion method was used to examine the antibacterial potential for all combinations. For further antibacterial investigation, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured by micro-broth dilution method. The obtained results showed that ethanol extracts of *B. undulata* and *R. chalepensis* were the most active. Synergistic antibacterial activity was observed for whey and ethanol extracts of *B. undulata* and *R. chalepensis*. The MIC and MBC values for these two combinations against *S. aureus* were equal to 1.56 and 12.5 mg/ml respectively. The obtained results form a platform for further studies on other combinations between camel whey and other plant extracts.

Keywords: Camel, Whey, Plant extracts, *Ballota undulata*, *Ruta chalepensis*, *Urtica urens*

1. Introduction

Animals have numerous antimicrobial systems that often evolved as part of their defense mechanisms [1]. Few studies have been reported on camel milk as it is a rich source of proteins with potential antimicrobial and protective activities [2, 3]. Studies showed that camel milk has antidiabetic, anti-hepatitis and bactericidal activity with stronger inhibitory system than that of cow's milk [4-6]. Camel milk contains a number of immunoglobulins that is compatible with human ones. These antibodies and their derived nanobodies were effectively used as antimicrobial agents [7-9].

Plants and their derivatives are used in traditional medicine as antimicrobial agents. They are a rich source of many potent and powerful drugs [10]. Moreover, the increasing use of plant extracts in the pharmaceutical industries suggests that in order to find active compounds, a systematic study of medicinal plants is very important [11].

Among these plants, *Ballota undulata* Fresen (Benth.) from Lamiaceae family was suggested to have anti-allergic, antispasmodic, antimicrobial and anti-inflammatory properties [12-14]. Moreover the aqueous extract of *B. undulata* was tested for antitumor and antimalarial activities [15]. The other plant species *Ruta chalepensis* L belongs to Rutaceae family [16]. Oil glands are principally present in *R. chalepensis* leaves, having strong deterrent smell [17, 18]. *R. chalepensis* has different pharmacological properties due to the presence of alkaloids, furoquinolone, flavonoids and coumarins [19, 20, 21]. Moreover, *R. Chalepensis* has anti-inflammatory properties in addition to its emmenagogue, abortifacient, anthelmintic, spasmolytic and antifungal activity against dermatophytes [22-24]. The last studied plant species is *Urtica urens* L. from Urticaceae family and it is commonly known as annual nettle [25]. *U. urens* leaves have a relatively high level of proteins [26]. The leaves of nettle are good sources of different significant minerals and vitamins in addition to flavonoids and terpenes [27-29]. Some studies were performed to evaluate the antimicrobial activity of *U. urens* [30-32].

In recent years, a large number of studies have been conducted searching the antimicrobial activity of natural products. However, the synergistic combined effect of antibacterial activity derived from camel whey proteins with plant extracts has not been addressed in literature. Therefore, this study was conducted to measure the antibacterial activity of water and ethanol extracts from three medicinal plants found in

Palestine that were utilized and combined with camel whey proteins. All combinations were tested against three bacterial isolates which are *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Materials and method

Bacterial Isolates

The *in vitro* antibacterial activities of all plant extracts and camel whey were evaluated against three reference bacterial isolates which are *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). All isolates were obtained from the American Type Culture Collection (ATCC).

Plant Materials

The three plant species *Ballota undulata*, *Ruta chalepensis* and *Urtica urens* were collected from different locations in West Bank, Palestine. The plant species were identified by Ghadeer Omar, Department of Biology & Biotechnology, An-Najah National University, Palestine. Representative plant specimens of the studied plant species were collected, pressed till drying, treated chemically, mounted on herbarium sheets and provided with voucher numbers, after that they were deposited at An-Najah National University herbarium. The plant materials for the antibacterial assay were washed, air dried, ground into powder using grinder and stored at room temperature until they were used.

Plant Extracts Preparation

Five grams of each dried plant leaves powder were soaked in 100 ml lukewarm distilled water, 100 ml of 99% ethanol for one week with continuous shaking at room temperature. Then the mixtures were centrifuged for 7 min at 4000 rpm. The supernatants were evaporated by freeze-drying for aqueous mixtures, while ethanol mixtures were evaporated by rotary evaporated. To adjust the final concentration at 100 mg/ml, the extracted powder from each plant species was dissolved in distilled water for aqueous powders, while ethanol powders were dissolved in 10% dimethyl sulfoxide (DSMO).

Milk Collection and Whey Preparation

Milk samples were collected from one female camel (Jenin, West Bank) by veterinary specialist. For whey protein preparation, the casein was precipitated from the skimmed milk sample [33]. In this technique milk renneting was done by commercially available rennin. The coagulated milk was heated to 56 °C for 10 minutes. Casein separation from lactoserum was carried out by filtration. For final clarification, the lactoserum was again centrifuged at 10,000 rpm for 30 min at 5 °C. The pellet was discarded and the supernatant was filtered using a millipore filter (0.45 µm), then the filtered supernatant was freeze-dried to produce whey powder. The prepared whey powder was dissolved in distilled water to a final concentration equal to 100 mg/ml and sterilized by microfiltration. Total protein content of camel milk whey sample was determined by Biuret method [34].

Identification of Camel whey Proteins Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Components of camel whey were fractionated by SDS-PAGE. This was performed in the discontinuous buffer system using 12% acrylamide-bisacrylamide separating gel (pH 8.8) and 4% acrylamide-bisacrylamide stacking gel (pH 6.8). Whey proteins were mixed in 3:1 ratio with sample buffer (pH 6.8). For band size determination, molecular weight protein

standards were used. The gel was stained with Coomassie brilliant blue R-250 and destained by 30% methanol until clear bands were seen.

Antibacterial Activity Assay:

The antibacterial activity of all plant extracts and whey proteins were determined by disk diffusion method [35]. The tested bacteria were grown overnight on nutrient agar plates. Broth turbidity was adjusted to 0.5 McFarland (1.5×10^8 CFU/ml). Then each bacterium was inoculated by streaking the swab over the entire sterile Mueller-Hinton agar surface. After that, 2 mg/ml of each plant extract under study and whey sample were loaded to 6 mm disk and added to the surface of inoculated agar plates. The plates were incubated at 37 °C for 18 h. After incubation all plates were examined for bacterial growth inhibition by measuring the inhibition zone diameter (IZD) to the nearest mm. The test was performed in triplicates. Antibiotic Gentamicin was used as positive control and sterilized distilled water and 10 % DMSO were used as negative controls. The same procedure was performed for all combinations. As 1 mg/ml whey proteins were mixed with 1 mg/ml plant extract and loaded to the disks.

Minimum inhibitory concentrations (MIC) for all plant extracts and camel whey in addition to all mixtures were determined by micro-broth dilution method [36]. The prepared samples were serially diluted two fold in Mueller-Hinton broth medium. Duplicates of each dilution (50.0, 25.0, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195 and 0.098 mg/ml) were inoculated with 1 µl of 5×10^7 CFU/ml. The last two duplicate wells were not inoculated and considered as negative controls. After that the inoculated microtiter plates were incubated at 37 °C for 18 h. The lowest extract concentration that inhibited the growth of tested microorganisms was considered as MIC. Minimum bactericidal concentrations (MBC) were determined. The contents of the wells resulting from MIC were streaked using a sterile cotton swabs on agar plate free of antibacterial agents and incubated at 37°C for 18 hours. The lowest concentration of the extract which showed no bacterial growth was considered as MBC. The same procedure was performed for camel whey and all mixtures that produce inhibitory effect.

Results

SDS-PAGE was done to determine the protein contents of camel whey sample (figure 1). Results showed that whey sample lack casein and contains immunoglobulins.

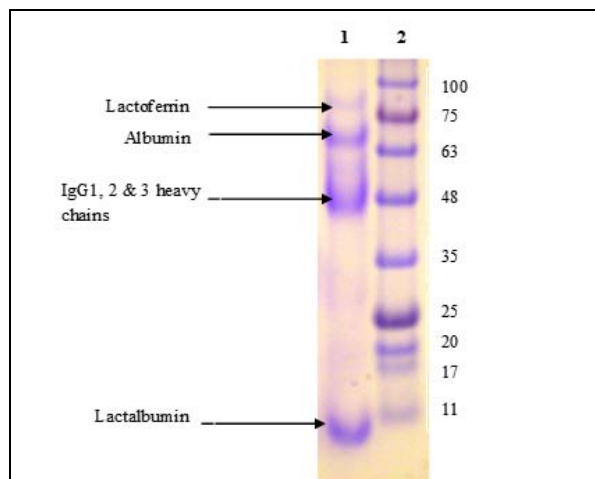


Fig 1: The SDS-PAGE pattern of camel whey (1) in addition to molecular weight standards (kDa) (2).

Agar disk diffusion method of aqueous and ethanol plant extracts under study exhibited antibacterial activity on at least one of the tested bacteria (Table 1 and 2). It was clearly noticed that *R. chalepensis* ethanol extract was the best. As it acted effectively against *S. aureus* with 15 mm inhibition

zone. After the combination of all prepared extracts with camel whey, a synergistic antibacterial activity was obtained from whey combination to both *R. chalepensis* and *B. undulata* ethanol extracts.

Table 1: Antibacterial activity of *B. undulata*, *R. chalepensis*, *U. urens* ethanol extracts with camel whey against three bacterial isolates.

	Camel Whey	<i>B. undulata</i>		<i>R. chalepensis</i>		<i>U. urens</i>		Positive Control GEN (10)
		Ethanol	Ethanol + Whey	Ethanol	Ethanol + Whey	Ethanol	Ethanol + Whey	
<i>S. aureus</i>	9	10	13	15	12	10	9	2.2
<i>E. coli</i>	9	8	11	10	12	10	11	2
<i>P. aeruginosa</i>	8	8	9	8	9	8	8	1.9

Table 2: Antibacterial activity of *B. undulata*, *R. chalepensis*, *U. urens* ethanol extracts with camel whey against bacterial isolates.

	Camel Whey	<i>B. undulata</i>		<i>R. chalepensis</i>		<i>U. urens</i>		Positive Control GEN (10)
		Water	Water + Whey	Water	Water + Whey	Water	Water + Whey	
<i>S. aureus</i>	9	9	9	8	9	8	no	2.2
<i>E. coli</i>	9	10	10	9	9	9	10	2
<i>P. aeruginosa</i>	8	10	10	8	8	9	8	1.9

The antibacterial activities of all extracts and camel whey were quantitatively assessed by determining their MIC concentrations against three bacterial isolates. Figure 2 and 3 clearly showed that ethanol extracts of *B. undulata* and *R. chalepensis* were more effective than ethanol extract of *U. urens*. *E. coli* was the most sensitive isolate to all aqueous extract for all plant in this conducted experiment.

Furthermore, a synergistic antibacterial activity was observed after combination between whey proteins and ethanol extracts of *B. undulata* and *R. chalepensis*. As the recorded MIC values against *S. aureus* were the lowest (1.56 mg/ml). In addition to that, whey combination to *U. urens* ethanol extract against *E. coli* showed inhibitory activity at 12.5 mg/ml.

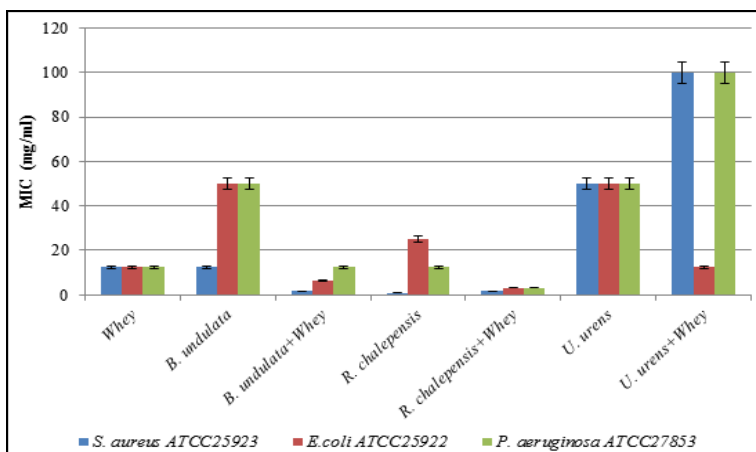


Fig 2: Antibacterial activity of *B. undulata*, *R. chalepensis*, *U. urens* ethanol extracts and camel whey against three bacterial isolates using micro-broth dilution method; (MIC) minimum inhibitory concentration (mg/ml).

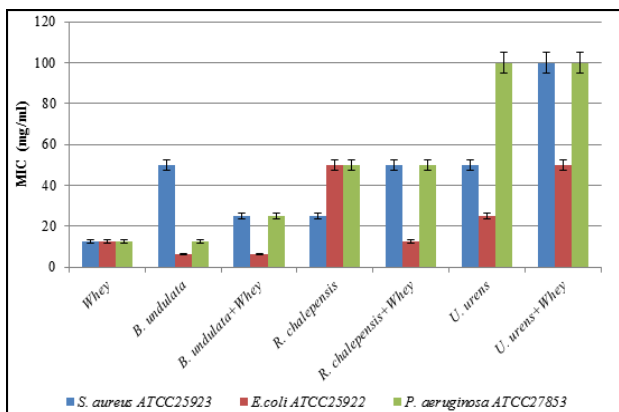


Fig 3: Antibacterial activity of *B. undulata*, *R. chalepensis*, *U. urens* water extracts and camel whey against three bacterial isolates using micro-broth dilution method; (MIC) minimum inhibitory concentration (mg/ml).

Minimum bactericidal concentrations (MBC) for all extracts that were given inhibitory effect in our research were determined. In addition to that, the MBC values after whey combination were also determined. Figure 4 and 5 showed that combination of whey proteins to either *B. undulata* or *R. chalepensis* ethanol extracts were the best, as these combinations killed *S. aureus* at (12.5 mg/ml). Moreover, *R. chalepensis* water extract combination to whey proteins showed bacteriostatic and bactericidal activity against *E. coli* at 12.5 and 25 mg/ml respectively. Furthermore, *E. coli* was killed by whey and *U. urens* ethanol extract combination at 25 mg/ml.

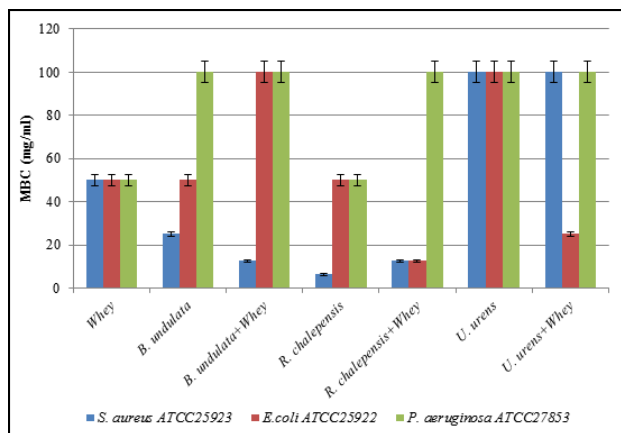


Fig 4: Antibacterial activity of *B. undulata*, *R. chalepensis*, *U. urens* ethanol extracts and camel whey against three bacterial isolates using micro-broth dilution method; (MBC) minimum bactericidal concentration (mg/ml).

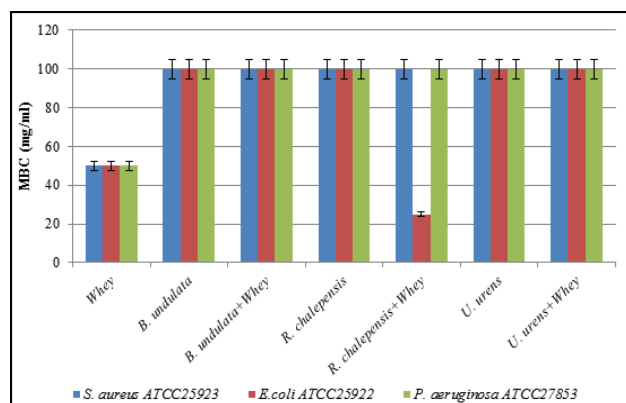


Fig 5: Antibacterial activity of *B. undulata*, *R. chalepensis*, *U. urens* water extracts and total and whey milk against three bacterial isolates using micro-broth dilution method; (MBC) minimum bactericidal concentration (mg/ml).

Discussion

Plants are important source of potentially useful compounds for the development of new chemotherapeutic agents. *In vitro* antibacterial activity of plant extracts was the first step towards this goal [37]. In the present study, the antibacterial assay results indicated that the highest antibacterial activity against *S. aureus* was obtained from *R. chalepensis* ethanol extract. In contrast to our finding, the antimicrobial activity of *R. chalepensis* essential oil showed that it was ineffective against bacteria including *E. coli*, *P. aeruginosa*, and *S. aureus* and it was significantly effective against fungi [38]. Moreover, solvent-free microwave extraction of the essential oils from fresh aerial parts of *R. chalepensis* revealed moderate antifungal activity [39].

Al-Bakri and Afifi were studied the antimicrobial activity of *B. undulata* by rapid XTT assay and viable count methods [14]. Their results showed moderate activity against *S. aureus*, weak activity against *E. coli* and no activity against *P. aeruginosa*. On the contrary to their results, *B. undulata* ethanol extract under study showed noticeable bioactivity mainly against *S. aureus* in addition to its effect against *E. coli* and *P. aeruginosa*.

The obtained results in this research provided that there was low antibacterial activity of *U. urens* extracts. Our results were partially similar to a study done by Mzid and his colleagues [32]. In their experiment water extract of *U. urens* are not effective against *S. aureus* and *E. coli*, while ethanol

extract exhibited bactericidal activity against *S. aureus* and no activity was observed against *E. coli*. In contrast to our results, *U. urens* extract could be considered a suitable alternative to topical antibiotic used to treat skin wound healing and to control bacterial contaminated the wound by *P. aeruginosa* [30].

The widespread usage of antibiotics has led to the increased resistance of pathogens and so they are more difficult to cure. Therefore, there is important need to find new antimicrobials such that obtained from food constituents like milk. The inhibitory effect of camel milk against several bacterial isolates has been reported [40, 41, 4]. Coliforms are more sensitive to the inhibitory system of camel's milk than any other groups of microorganisms [42]. On the other hand, It was shown that camel milk produce no antibacterial activity against *E. coli* and *S. aureus* while Miceller treatment of camel milk produced antimicrobial activity only against *S. aureus* [43]. Milk contain antimicrobial peptides such as lysozyme, lactoferrin, lactoperoxidase, immunoglobulins and short peptidoglycan recognition protein in addition to casein, lactalbumin and β -lactoglobulin [44]. Most of these proteins also found in whey including lactoglobulins, albumin and immunoglobulin [45]. It was proved that camel whey proteins were more heat resistant than cow and buffalo milk proteins. These proteins include the antimicrobial factors lactoferrin and immunoglobulin G [46]. Furthermore, whey sample under investigation was subjected to heat treatment, therefore, some of biofunctional peptides could lost their activity [47]. The main component suggested to be responsible for inhibitory activity was lysozyme [48]. Unfortunately, lysozyme was heat sensitive and this may explain the moderate activity of camel whey in our study. SDS-PAGE results demonstrated the presence of lactoferrin and immunoglobulins in the examined whey sample. It was reported that lactoferrin has bactericidal and bacteriostatic activity due to its iron binding ability. The presence of iron is necessary for bacterial growth and virulence [49]. In this conducted experiment, results indicated that camel whey has a synergistic action with some of the studied plant extracts. These observation coincide with other studies that carried out between camel milk and antibiotics. Camel milk have a synergistic action with antibiotic ciprofloxacin [50]. In addition to that, combination of lactoferrin with penicillin increased the inhibitory activity of penicillin and lactoferrin [51]. A study concerning camel milk antimicrobial activity showed that camel immunoglobulins had little effect against *E. coli* and *S. aureus* [4]. However, SDS-PAGE profile for whey sample that was used in our experiment showed that the main components that found at high concentrations were immunoglobulins. Whey proteins mainly immunoglobulins showed a synergistic effect when combined to *B. undulata* and *R. chalepensis* ethanol extracts against both gram negative and gram positive bacteria. However, *U. urens* ethanol extract combination to camel whey showed antibacterial activity only against *E. coli*.

Conclusion

In conclusion, our results showed that a synergistic antibacterial activity was obtained after the combination between camel whey proteins and different plant extracts. These combinations can be used for the infectious disease therapy and as preservatives in the food industry. Another possibility is to use them as products for the treatment of skin dermal infections. Further studies should be performed to analyze the components of both whey and plant extracts and their individual combination behavior against different microbes.

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