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Evaluation of antibacterial effects of *Withania coagulans* and *Cynara cardunculus* extracts on clinical isolates of *Brucella* strains

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ABSTRACT

Brucellosis is a common illness zoonotic and transmitted by eating infected food products. Medicinal plants are considered as new sources for the production of agents that can act as alternatives to antibiotics in the treatment of antibiotic-resistant bacteria. This study evaluated the effects of aqueous and ethanol plants of Cynara cardunculus and Withania coagulans extracts on Brucella strains. These plants are vegetarian rennet, which are used in cheese industry. Thirty Brucella strains provided by collection of microbial in research brucella center of Hamadan Iran. Strains identified by biochemical tests and then confirmed by polymerase chain reaction (PCR) method. Minimum inhibitory concentration (MIC) of plant extracts were determined by dilution method with several of bacterial concentrations. Sensitivity to antibiotics and herbal extracts were performed by Kirby-Bauer disc diffusion test. The results showed that among the tested antibiotics there was only 10% resistance to rifampin. Examination for plant extracts showed the mean zone of inhibition growth for C.cardunculus and W.coagulan were 28 and 17mm (in 40mg/ml) respectively by disk diffusion method and the highest Minimum inhibitory concentration (MIC) were 10.81µg/ml for C.cardunculus and 43.24µg/ml for W.œagulans. The present study showed C.cardunculus extracts possess compounds with antibacterial properties, therefore can be used as antimicrobial agents in new drugs for therapy of brucellosis .Also Results obtained the provide grounds for use this plant as a functional food in cheese-making industry.

Keywords: *Brucella, Withania coagulans, Cynara cardunculus,* antibacterial activity

INTRODUCTION

Brucellosis is a public-health problem in many developing countries, including Middle East, India, Iran, Central and South America [1].This is a disease caused by several of the genus *Brucella* and primarily infects animals, especially domestic livestock, but it can also be transmitted to humans [2]. *Brucella spp*. consists of four important species *B. melitensis, B.abortus, B. suis* and *B. canis*[3].The annual prevalence of brucellosis in the worldwide is more than500, 000 cases. The disease is endemic in Iran and its prevalence ranged from 1.5 to 107.5 cases per 100,000 people in 2003 [4]. Today, increased incidence of *Brucella* resistance to many antibacterial drugs has been reported, thus survey the antibacterial effects of medicinal plants on *Brucella* is important [5, 6]. Medicinal plants have

proven as an alternative source of antibacterial agents and have been traditionally used in treatment of disease [7]. According to WHO, medicinal plants would be the best source for obtaining a variety of drugs [8].*Cynara cardunculus*(*Cardoon*) and *Withania coagulans* are two medicinal plants which have reported their antimicrobial activities. *C. cardunculus* is composite family of a group of Mediterranea species, traditionally used in Southwest Europe such as globe artichoke, wild cardoon and cultivated cardoon [9].Several studies have been done on the properties of its pharmacological such as hepatoprotective, anticarcinogenic, antibacterial, antioxidative and anti-HIV effects. Flowers of *C. cardunculus* are rich in proteases, cardosins A and B, which aqueous extracts of its flowers have been used for years in the Iberian Peninsula for manufacturing of ovine and/or caprine milk cheeses [10].

Withania coagulans belong to *Solonaceae* family and native plants of indigenous South Asian countries, India, Pakistan, Afghanistan, and southern East of Iran. It is one of the important medicinal plants. Active compounds *W. coagulans*: Withanolides are a group of steroidal lactones found among members of *Solanaceae*. It was reported that Withanolides possess antimicrobial, anti- inflammatory, anti-tumor, anti-antigenic effects [11]. *W. Coagulans* is the source of coagulating enzyme for clotting the milk which is called "paneer" [12].

The aim of this study was evaluation the effects of *C. cardunculus* and *W.coagulans* extracts on clinical isolates of *Brucella* strains.

MATERIALS AND METHODS

Microorganisms and identification: Thirty *Brucella* strains provided by collection of microbial in research *brucalla* center of Hamadan Iran. The isolates were sub-cultured on *Brucella* agar (Merck, Germany) enriched with 0.05ml/L sheep blood and was incubated at 37 °C for 48 h with 5% CO2,then identified by conventional methods, colonial morphology ,consideration the gram stain characteristic, requirement of CO2 for growth, production of urease, oxidase, and H2S, ability to reduce nitrate to nitrite [13]. Finally isolates confirmed by PCR molecular method.

DNA Extraction: DNA Extracted by Modified boiling method was described by Queipo- Ortuño et al. [14].

PCR assay: The specific primers were used to amplification a target sequence of 223-bp within a gene code to produce a 31-kDa outer membrane protein specific to *B. abortus* which is common among all *brucella* species [15].Primers used were:

B4 (5'-TGG CTCGGTTGCCAATATCAA -3')
B5 (5'-CGC GCTTGCCTTTCAGGTCTG -3') [16].

PCR was performed in a final volume of 25 µl mixture containing12.5 µl mastermix (Fermentas), 1 µl of each primer and 2µl of total DNA extracted and distilled water was processed in a thermocycler (Eppendorf Co).The cycling conditions consisted of initial denaturation at 95 °Cfor5 minutes, followed by 35 cycles of 60 seconds of template denaturation at 95°C, 30s of primer annealing 60 °C and 1min primer extension at 72°C and final extension at 72°C for 7min.The products were analyzed by electrophoresis through 1.5% gel agarose .Then gels were stained and were visualized by UV transilluminator [15].

Preparation of the extracts:
The leaves of plants collected from South-east of Iran and approved, Research Center for Agricultural Hamadan Iran. Two ethanol and aqueous extracts were prepared from each plant. 100gr of each plant was beaten and soaked in 300mL of 90% ethanol (Merck, Germany) and distilled water, then incubated for 48 h in 37C°.Plants were condensed using rotary device and connected to a vacuum pump, then were sterilized by filtration 0.45 µm Millipore filters and were kept at 4 °C [17].

Tests on antibacterial activity
Disk diffusion assay for antibiotics: Antibiotic resistance of the isolated *bucella* strains was determined by Standard disc diffusion method(8)according to the clinical and laboratory Standards Institute (CLSI) protocols [18].

Different antibiotics (Hi-media, India) were used in the present work, Gentamicin ,Co-trimoxazol, Tetracycline, Streptomycin, Rifampin and Doxycycline.

Disk diffusion assay for extracts: The serial dilutions of 1/10 to 1/2560 from each extract prepared in sterile distilled water. Then the blank disks were floated in these dilutions and were dried. Then For each isolated *Brucella* strain was prepared 0.5 McFarland standard Kirby-Bauer)and inoculated to the culture medium and disks were put on each plate. A blank disk was floated in 80% ethanol was used as a negative control. Gentamicin disk was used as positive control. Finally, all of the plates were incubated at 37 °C for 48 h. Zone of inhibition bacteria growth was measured and compared with the CLSI fastidious bacteria [18].

Determination of minimal inhibitory concentration (MIC) for extracts:
In broth dilution method, duplicate serial dilutions of extracts were prepared in Mueller-Hinton broth medium (Merck, Germany).In this method serial dilution for each extract from1/10 to 1/2560 was determined. 1ml of bacterial suspension of 0.5 McFarland standard was added to each dilution. Negative and positive control was also done but without adding the bacteria and extract respectively. The final serial dilutions of extracts were 1/20 to 1/5120.Tubes incubated at 37 C ° and 5% Co2 for 48 h. The lowest concentrations without visible growth were defined as MIC [19].

Statistics
The results of this study were analyzed using SPSS and $P< 0.05$was considered statistically significant.

RESULTS

Brucella strains identified by biochemical tests and then confirmed by PCR (Figure1).Total isolates were *B. melitensis*.

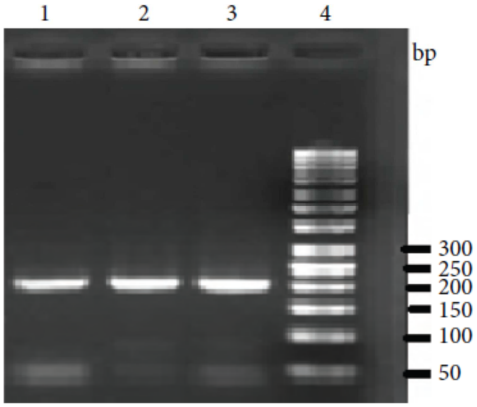

Figure1: lane1, 2: Bacteria that were separated with a 197 bp product,lane3: *Brucella melitensis*Rev-1 strain,lane4: DNA ladder 50 bp

In disc diffusion method rate of 30 *brucella* strains resistance to rifampin was 10% and there was no resistance to other antibiotics. Mean rate of antibacterial effects of each extract was studied on *Brucella* strains by disk diffusion method. The results of antibacterial activity showed *C.cardunculus* extracts were more effective than *W.coagulans* on *brucella* strains, p<0.05 (Table1).

Table1: The mean zone of growth inhibition for extracts on *Brucella* strains using the disk diffusion method

Concentration / Extract	disk contained 40mg/ml	disks contained 20mg/ml	Disks contained 10mg/ml
Extract ethanol *C. cardunculus*	28mm	20mm	15mm
Extract aqueous *C. cardunculus*	24mm	16mm	8mm
Extract ethanol *W. coagulans*	17mm	10mm	7mm
Extract aqueous *W.coagulans*	10mm	8mm	7mm

Maximal inhibition zones ethanol and aqueous *C.cardunculus* extracts by disc diffusion method were 28 and 24 mm and for *W.coagulans*were17and 10 mm (40mg/ml) respectively (Figure 2).

(A) (B)

Figure 2: Antibacterial effects of aqueous (A)and ethanol (B)*C.cardunculus* on *Brucella* strains by using disk diffusion method A:right to left :The concentration of 86.48 µg/mL(1/10) ,43.24 µg/mL(1/20) B:upto right and then to left : The concentration of 43.24 µg/mL(1/20), 43.24 µg/mL(1/40), 10.81 µg/mL(1/80)

These results compared with gentamicin disc (control positive) and with results of antibacterial susceptibility (Table2).

Table 2: Comparison of the statistical analysis mean inhibition zone of results of the antibacterial activity each extract n in 40mg/ml and antibiotic disks on *brucella* strains (P < 0.05)

Antibiotics / Extracts	Rifampin 20mm	Gentamicin 26mm	Tetracycline 39mm	Doxycycline 39 mm	Streptomycin 27mm	Co-trimoxazol 34mm
extract ethanolic *cynara cardunculus* 28mm	P <0.01	P=0.15	p< 0.001	P<0 .001	P = 0.14	P=0.04
extract aquatic *cynara cardunculus* 24mm	P=0.03	P = 0.17	P< .001	P< .001	P = 0.14	P<0.001
extract ethanolic *withania coagulans* 17mm	P=0.2	p<0.001	P<0.001	p<0.001	p<0.001	p<0.001
extract aquatic *Withania coagulans* 10mm	P<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

Also the MIC levels of each extract on the *Brucella* strains were evaluated. The mean MIC for ethanol and aqueous *C.cardunculus* extracts were10.80 µg/ml (in dilution of 1/80) and 21.62 µg/ml (in dilution of 1/40).The means MIC level for ethanol extract of *W.coagulans* was 43.24 µg/ml (in dilution of 1/20) but no observed any antibacterial effect to aqueous extract. Results showed that of the mean MIC level of *C.cardunculus* extracts were more than MIC level *W.coagulans* extracts (P < 0.001).

DISCUSSION

Treatment of human brucellosis needs antibacterial drugs that can act within the acidic intracellular environment. Despite a good treatment, many antibacterial drug-resistant strains may lead on to treatment failure [20].Recently, use of plant extracts have been developed in foods as natural antioxidants [21]or antimicrobials [22]and have been traditionally used worldwide in the treatment of many diseases from long time ago [3].Two plants that were used in this study traditionally are applied in regions of south and west of Iran in order to rennet to make cheese. In this study, we considered antibacterial resistant and effects of ethanol and aqueous *C.cardunculus* and *W.coagulans* on 30 species of *Brucella* isolated by disk diffusion method. The results of this research showed that the rate of resistance of *Brucella* strains to rifampin was 10% and antibacterial resistance was not observed to other antibiotics. Results of study by Baykam*et al*. in 2004 indicated that *Brucella* strains were sensitive to doxycycline, but less sensitive to rifampin [23].In Kasymbekov's study, during the years of 2009 to 2010, 17 species *B.mellitensis* was evaluated from aborted fetuses of sheep and other livestock. They showed these bacteria were sensitive to gentamicin, tetracycline, doxycycline, streptomycin, ofloxacine, rifampin and ciprofloxacin [24].In recent years, there are many reports the use of active compounds extracted from medicinal plants, which may benefit antibiotic development. Several studies including *Peganum harmalaL* [25] and aqueous *hops* extract [26] that possess good antibacterial effect against *B. melitensis*. Result of this study indicated that antibacterial effects *C.cardunculus* extracts were more than antibacterial effects of *W.coagulans* extracts by disc diffusion method and had not significant difference in anti-*brucella* activity Compared with the gentamicin disc. In biological assays demonstrated *C. cardunculus* extracts have antimicrobial activity comparable with some antibiotics [27].Our study showed

antibacterial activity *C.cardunculus* extracts more than the streptomycin and rifampin disc. On other hand our study determined MIC for ethanol and aqueous *C.cardunculus* extracts 10.80 & 21.62µg/ml respectively. MIC for ethanol *W.coagulans* extract was 43.24µg/ml and aqueous extraction had not antibacterial activity. These results confirmed previous studies reported that ethanol is a better solvent for more consistent extraction of antimicrobial substances from medical plants. The antimicrobial effect of *C.cardunculus* against pathogens such as *Salmonella typhimurium*, *Escherichia coli* and *Bacillus subtilis* was reported by Kukic and *et al* in 2008. The results of their experiment showed that all standard compounds, previously isolated from in volucral bracts of *C. cardunculus*, possess antimicrobial activity against all tested strains of bacteria and fungi (MICs, in a range of 0.03–0.10 mg/ml) [9].Similar results were also previously observed with compounds isolated from *C. Scolymus* leaves that were effective against fungi and bacteria with MICs ranging from 0.05 to 0.20 mg/ml [28].These kinds of differences in susceptibility among the microorganisms against antimicrobial agents in plant extracts may be explained by inheritance genes on plasmids that can be transferred among bacterial strains.

Cardoon leaves are used for their cholagogue, choleretic and choliokinetic actions, for treatment of dyspepsia and as anti-diabetics [29].Fernandez and Grammelis reported leaves of *C. cardunculus* have diuretic and hepatoprotective effects, improve gall bladder function, stimulate the secretion of digestible juices, especially bile and they can inhibit cholesterol synthesis [30, 31].Flowers of *C. cardunculus* are rich in proteases, cardosins A and B, which aqueous extracts of its flowers have been used for years in the Iberian Peninsula for manufacturing of ovine and/or caprine milk cheeses [12].About of *W. coagulans* many studies have been done.Choudhary et al. reported the volatile oil from the fruits of this plant showed antibacterial activity against *Vibrio cholera* and *Staphylococcus aureus* [32].The extract of *Withania coagulans* has hypotensive, respiratory stimulant and muscular relaxant activity in experimental animals [33].

CONCLUSION

The results of this study showed ethanol extract of *C.cardunculus* possess compounds with antibacterial properties even more than Rifampin antibiotic and *W.coagulans* extracts, therefore it can be used as antimicrobial agents in new drugs for therapy of infectious diseases .Also Results obtained support the traditional medicinal use of this plant provide grounds for further establishing its use as a functional food in chees industry.

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