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Original article

Evaluation of antimicrobial effect of hops extracts on intramacrophages *Brucella abortus* and *B. melitensis*

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How to cite this article:
Shapouri R, Rahnema M. Evaluation of antimicrobial effect of hops extracts on intramacrophages *Brucella abortus* and *B. melitensis.* Jundishapur J Microbiol. 2011; 4(Supplement 1): S51-S8.

Received: August 2010 **Accepted:** November 2010

Abstract

Introduction and objective: Brucellosis is a zoonosis disease among animal and human, and has been endemic in Iran. The most important virulence factors of *Brucella* are related to their capability of intraphagocytic survival. Because of the side effects of brucellosis treatment regime, it is necessary to find new antimicrobial agents. The hop plant (*Humulus lupulus*) extract reported as having antimicrobial effects. The present study evaluated the antimicrobial activity of hops extracts against *Brucella abortus* 544 and *B. melitensis* 16M.
Materials and methods: Hops extracts were prepared in water, acetone and ethanol. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for two strains of *Brucella* determined by broth macrodilution and agar well diffusion methods. Effect of extracts on intramacrophge surviving of *Brucella* strains studied on cell culture of mouse peritoneal macrophages.
Results: Results indicated that MIC for aqueous extract was 1:80 (0.625mg/ml) and for acetonic and ethanolic extracts was 1:160 (0.05mg/ml). MBC for aquatic extract was 1:40 (1.2mg/ml) and for two other extracts was 1:80 (0.1mg/ml). In cell culture results indicated that all of extracts were effective and eradicated intramacrophage *Brucella* strains in 1:40 (1.2mg/ml for aqueous and 0.2mg/ml for ethanolic and acetonic extracts), 1:80 (0.625mg/ml for aqueous and 0.1mg/ml for ethanolic and acetonic extracts) and 1:160 (0.312mg/ml for aqueous and 0.05mg/ml for ethanolic and acetonic extracts) of extracts dilutions after 24h.
Conclusion: Overall this study indicated that aquatic, acetonic and ethanolic extracts of hops showed antimicrobial effect against *B. abortus* and *B. melitensis*, therefore, they are useful in the treatment of brucellosis.

Keywords: *Brucella abortus*; *B. melitensis*; Hops extract; Antibacterial activity; Cell culture

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Introduction

Brucella is the causative agent of brucellosis, one of the most important and widespread zoonoses world's [1]. All of the main species of farm animals are susceptible to brucellosis. As a zoonotic disease virtually human infections derive from direct or indirect exposure to animals [2,3]. *Brucella* spp. are Gram-negative facultative intracellular bacteria and the major site of residence of the *Brucella* in both the natural hosts and humans is the macrophage. After entry into the host, they are taken up by macrophages and display a remarkable resistance to be killed by these phagocytes [3,4]. Indeed the capacity of these bacteria to maintain long-term residence within macrophages serves as the basis for their ability to establish and maintain chronic infection.

In numerous experiments, cell culture of macrophages of a variety of hosts including cattle, humans and mice has been use to show a correlation between the ability of *Brucella* to resist the activity of phagocytes by virulence [4-6]. In human, brucellosis is a chronic, debilitating febrile illness that can last for months and is characteristically difficult to treat with antimicrobial agents. Typically prolonged treatment (four weeks or more) with two antibiotics is employed to treat human brucellosis. Even with the prolonged combined antibiotic therapy, relapses are not uncommon (5-10%) as well as no safe and effective vaccine exists for human brucellosis. To treat animal brucellosis which is similar to human brucellosis, multiple antibiotic regimens are used but generally these are not effective [1,7-11].

Hop (*Humulus* spp.), is a small genus of flowering plants native to temperate regions of the Northern Hemisphere. The hop is part of the family *Cannabaceae*, which also includes the genera *Cannabis* (hemp), and *Celtis* (hackberries). The leaves are opposite, with a 7 to 12cm leafstalk and a heart-shaped, the edges are coarsely toothed. Male and female flowers of the hop plant develop on separate plants. The female flowers (often called "cones") of *H. lupulus* are known as hops, and are used as a culinary flavoring and stabilizer, especially in the brewing of beer, and grown in the absence of male plants. There are three species and *H. lupulus* var. *lupulus* isolate from Europe and western Asia [12].

The female inflorescences (hop cones or hops) rich in polyphenolic compounds and acylphloroglucides are widely used to preserve beer and to give it a characteristic taste and flavour [12,13]. Also hop cones have been used in medicine including for the treatment of sleeping disorders. The antimicrobial properties of hop are mainly due to the hops acids. They are shown antibacterial, antimycobacterial, antifungal, antiviral and antiparasitic activities [12,13]. Thus, the objective of the present study was to determine the antimicrobial activity of hops extracts against *B. abortus* 544 and *B. melitensis* 16M in cell culture of mice macrophages.

Materials and methods

Bacterial strains

In this study we used of *B. abortus* 544, *B. melitensis* 16M strains obtained from the Department of Bacterial Vaccine and Antigen Production of institute Pasteur of Iran. Both strains are virulent for human and animals [14].

Preparation of hops extracts

The process of making hops extract normally involves drying and powdering the hops flowers or cones, then adding the powder (50g) to solvent (500ml) (in this study water, ethanol and acetone) then combination is sealed and stored in a cool dark place. After 4h, the extracts were centrifuged and filtered, then extracts were

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collected and concentrated up to 10ml in vacuum pump at 40°C then concentrated extracts sterilized by 0.22µm filters and stored at -20°C until further use [15-17].

Antibacterial activity of hops extracts
On Mueller-Hinton (Merck, Germany) that supplemented with 1% of sheep Hb, 5mm diameter wells were prepared and then from 1.5×10^8cfu/ml of *Brucella* suspension cultivated on plates with sterile swab, then wells loading with hops extracts dilutions (70µl of 1:10 to 1:2560 dilutions). Plates incubated at the same conditions as mentioned above for three days and the zone of growth inhibition was measured. Finally residual activity of extracts was measured as a broth macrodilution method [18-22].

Determination of minimum inhibitory concentration and minimum bactericidal concentration
Serial dilutions (1:10 to 1:2560) of hops extracts were prepared in Mueller-Hinton broth (Merck, Germany) supplemented with 1% of sheep blood. Then 5×10^5cfu/ml of *Brucella* suspension was added to each tube and incubated at 37°C under 7-10% CO_2 for five days, then the tubes were examined for turbidity, indicating growth of the microorganisms. The lowest concentration of the extracts that inhibits growth of the *Brucella*, as detected by lack of visual turbidity, is designated as the minimum inhibitory concentration (MIC). For calculation of minimum bactericidal concentration (MBC) of extracts, after the MIC has been determined, 0.1ml of inoculums from each of the tubes of broth was sub-cultured on Mueller-Hinton agar (Merck, Germany) plates supplemented with 1% of sheep blood [14,18-21].

The number of colonies on agar after five days of incubation at the same conditions as mentioned is then counted and compared to the number of cfu/ml in the original inoculums. The lowest concentration of extracts that allowed less than 0.1% of the original inoculums to survive were determined as a MBC. Also the extracts were heated at 80°C for 20, 40 and 60mins and residual activity was measured by the following method [14,18-21].

Isolation of peritoneal macrophages
Thioglycollate-elicited peritoneal exudates cells were obtained from 6-8 weeks old female BALB/c mice following intraperitoneal injection of 1ml thioglycollate broth (4.05g/100 ml) (Difco, USA) and lavage of peritoneal cavity with 5ml of medium (RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS) four days later. The cells were washed twice and resuspended in RPMI 1640 with 10% FCS and placed in 96-well polystyrene micro titer plates at 0.27×10^4 macrophage per well. The plates were incubated for 2h at 37°C with 5% CO_2, allowed adhering. Then the supernatant was aspirated and the adherent monolayer was washed three times with medium [2,4,23,24].

Treatment of peritoneal macrophages with hops extracts
Peritoneal exudate cells were counted for in vitro viability by dye exclusion test with trypan blue, before and 24h after incubation of macrophages with various dilutions of extracts (1:20 to 1:160) and normal saline was used as negative control [2,4,23,24].

Effect of hops extracts on intramacrophages Brucella spp.
Brucella abortus 544 and *B. melitensis* 16M, were grown at 37°C in *Brucella* agar (Merck, Germany) under 7-10% CO_2 for 48h, resuspended in phosphate-buffured saline (PBS), washed and resuspended in the same buffer. Bacterial numbers were

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determined by comparing the optical density at 600nm with a standard curve. Then bacterial suspension (5×10^5cfu/ml) was added to peritoneal macrophages and incubated for 2h at the same conditions as mentioned above, allowing the macrophages to ingestion the *Brucella* spp. [2,4,23,24]. Then, gentamicin at a concentration of 50µg/ml was added and micro plates incubated for 1h (at the same conditions) to kill extracellular bacteria. Then the monolayer was washed three times with media and various dilutions of hops extracts (1:40 to 1:320) were added. After 24h incubation Triton X-100 was used to lysis the macrophages. The number of CFU in laysates was determined by serial dilutions and plating on *Brucella* agar and normal saline was used as negative control [2,4,23,24].

Results

Antibacterial activity of hops extracts

The agar-well diffusion method showed that *B. abortus* 544 was more sensitive than *B. melitensis* 16M to all of hops prepared extracts and all of extracts were shown inhibition zone further than 3mm between 1:10 to 1:320 of dilutions for both *Brucella* strains. Among prepared hops extracts, ethanolic extract was shown the most effective on inhibition of both *Brucella* strains and acetonic and aquatic extracts effectiveness were in subsequent ranks (Table 1). Similar to residual activity assay in broth macrodilution method, the same inhibition zone with unheated extracts was shown in agar-well diffusion method (see MIC and MBC section of results).

Table 1: Means of zone of growth inhibition (mm) of hops extracts on *B. abortus* 544 and *B. melitensis* 16M

Extract	Dilution of extract									
	Control (DW)	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Aqueous on *B. abortus*	-	18	15	12	7	5	4	2	-	-
Aqueous on *B. melitensis*	-	17	15	10	7	4	4	1	-	-
Acetonic on *B. abortus*	-	23	19	15	11	6	6	3	1	-
Acetonic on *B. melitensis*	-	20	17	12	9	5	4	2	1	-
Ethanolic on *B. abortus*	-	24	21	16	12	8	7	4	1	-
Ethanolic on *B. melitensis*	-	22	20	13	11	6	5	2	1	-

Determination of MIC and MBC

Results showed that MIC of aqueous hops extract for *B. abortus* 544 and *B. melitensis* 16M was equal to 1:80 (0.625mg/ml) extract dilution and MBC was equal to 1:40 (1.2mg/ml) extract dilution. Whereas MIC and MBC results of acetonic and ethanolic extracts for *B. abortus* and *B. melitensis* were the same and MIC was equal to 1:160 (0.05mg/ml) and MBC was equal to 1:80 (0.1mg/ml) extracts dilutions. Also results of residual activity at 80°C for 20, 40 and 60mins of each extracts showed similar results to the above mentioned broth macrodilution results.

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Effect of hops extracts on macrophages

To determine the effect of hops extracts on viability of macrophages, murine peritoneal macrophages were treated *in vitro* with various dilutions of hops extracts for 24h. Viability of macrophages before and after incubation with dilutions of extracts were determined and expressed (Table 2). Results indicated that 1:10 and 1:20 dilutions of all extracts (4.8 and 2.4mg/ml for aqueous and 0.8 and 0.4mg/ml for ethanolic and acetonic extracts, respectively) had inhibitory effect on peritoneal macrophages while this effect was not seen by other dilutions of extracts. Therefore we can use 1:40 to 1:160 dilutions of hops extracts for studying their effects on intramacrophages *Brucella* strains.

Table 2: Means of viable macrophages at 0 and 24h after treatment with hops extracts

Dilution of extract	Viable of macrophages (average of percentages ± SD)		
	Aqueous extract	Acetonic extract	Ethanolic extract
Lack of extract at 0 time	86±3	85±1	83±4
Control (normal saline) after 24h	79±6	81±1	77±2
1:20 of dilution after 24h	26±4 *	20±5*	21±1*
1:40 of dilution after 24h	70±6	71±5	74±2
1:80 of dilution after 24h	78±1	80±3	82±4
1:160 of dilution after 24h	85±1	80±1	80±3

*indicates a significant differences comparing with the control group with one way ANOVA analysis ($p<0.01$). SD: Standard Deviation.

Effect of hops extracts on intramacrophages Brucella strains

In order to assess the number of CFU of *B. abortus* 544 and *B. melitensis* 16M in macrophage lysates for both treated and untreated macrophages, both lysate were cultured on *Brucella* agar. The results showed (Table 3) a significant decrease in the number of colony forming on the test plates comparing with control group. We also noticed that at the 1:40 and 1:80 (or 1.2 and 0.625mg/ml) for aqueous extract and 1:40, 1:80 and 1:160 (or 0.2, 0.1 and 0.05 mg/ml, respectively) for acetonic and ethanolic extracts hops extracts dilutions cause complete elimination of intracellular *Brucella* spp. after 24h.

Discussion

The inhibitory effect of hops aqueous, acetonic and ethanolic extracts shown on *B. abortus* 544 and *B. melitensis* 16M were suitable in both broth macrodilution and agar-well diffusion methods, especially for alcoholic extract that showed the most inhibitory effect. Moreover, this anti-*Brucella* activity of extracts was heat resistant and heating at 80°C up to 60min did not eliminate or reduce their antibacterial activity.

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Table 3: Means numbers of CFU of intramacrophages *Brucella* per ml lysates by plating (primary inoculation was 5×10^5 bacteria)

Extract	Dilution of extract					
	Control (DW)	1:10	1:20	1:40	1:80	1:160
Aqueous on *B. abortus*	$(116\pm2)\times10^3$	No growth*	No growth*	83±1 *	*(173±10)×10	*$(63\pm3)\times10^3$
Aqueous on *B. melitensis*	$(220\pm3)\times10^3$	No growth*	No growth*	113±7*	*(250±4)×10	$(95\pm2)\times10^3$*
Acetonic on *B. abortus*	$(120\pm1)\times10^3$	No growth*	No growth*	No growth*	*152±7	$(24\pm7)\times10^3$*
Acetonic on *B. melitensis*	$(219\pm4)\times10^3$	No growth*	No growth*	No growth*	*242±9	$(33\pm1)\times10^3$*
Ethanolic on *B. abortus*	$(101\pm6)\times10^3$	No growth*	No growth*	No growth*	*111±3	$(21\pm5)\times10^3$*
Ethanolic on *B. melitensis*	$(231\pm1)\times10^3$	No growth*	No growth*	No growth*	*198±2	$(28\pm6)\times10^3$*

*indicates a significant differences comparing with the control group with one way ANOVA analysis ($p<0.01$). SD: Standard Deviation.

Other investigators showed antimicrobial effect of hops extracts on some bacteria such as *Listeria monocytogenes*, *Bacillus* spp., *Helicobacter pylori*, toxigenic Clostridia, *Lactobacillus* spp., *Streptococcus mutans*, *Staphylococcus* spp., fungi, viruses and some protozoa [25-30]. It was also demonstrated that hops extracts exhibited antimicrobial activity when stored at 4, 10 and 25°C and this activity was enhanced with increasing concentration and storage at the lower temperature of 4°C. Therefore, hops extracts can be used in food systems as a preservative. However, further studies are needed to show application in the food industry [26]. In addition the most effective method of their application in food products, such as dipping, spraying or emulsification, needs to be investigated.

Macrophages are particularly important for the survival and spreading of *Brucella* spp. during brucellosis [4-6]. In this study, the effect of extracts dilutions on viability of macrophages was determined (Table 2) and these results indicated that MIC and MBC of hops extracts for *Brucella* spp. were not toxic for murine macrophages. Therefore, we used these dilutions for the treatment of intramacrophages *Brucella* spp. But, 1:10 and 1:20 dilutions of hops extracts were also toxic for macrophages and should not be used in macrophage culture.

Study of antimicrobial effect of hops extracts on intramacrophages bacteria and other microbes have not been carried out, as yet and this is for the first time we have shown that hops extracts have antimicrobial activity on intramacrophages *Brucella* spp., and MBC, MIC and 1:160 dilutions of all prepared extracts have significant effect on elimination of intramacrophage *Brucella* for a period of 24h (Table 3). Therefore, prepared hops extracts are able to penetrate into eukaryotic and immune system cells to kill the microbes in addition to their broth dilution and ager-well diffusion antimicrobial activity. Also they may be useful for the treatment of diseases caused by intracellular agents such as *Brucella*, that some useful antibiotic at laboratory was not effective *in vivo*.

Since there are several essential nutrients in hops extract, further experiments and complementary studies are needed. Significant amounts of the B vitamins, especially niacin, as well as potassium are present in the product. In

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addition, there are moderate amounts of calcium, magnesium, and vitamin C, as well as trace amounts of zinc and vitamin A [15-17] that showed that hops extracts had several useful effects.

Conclusion

In conclusion, the present study clearly shows that the aqueous, acetonic and ethanolic extracts of hops were effective on *Brucella* at laboratory and intramacrophage and therefore, they can be used to treat brucellosis. They can also be useful for food preservation.

Acknowledgement

This study was financially supported by Islamic Azad University, Zanjan-branch (Grant no: 88.31054).

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