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Antibacterial and Cytotoxic Effects of *Urtica dioica* Hydro-alcoholic Extract on Causative Bacteria of Mastitis and Epithelial Cells of Bovine Mammary Gland in vitro

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Abstract

Background & Objectives: Nowadays, owing to positive features of herbal plants, they are widely being applied to treat different diseases. The current study was designed to assess antibacterial activities and cytotoxic effects of hydro-alcoholic extract of *Urtica dioica* (*U. dioica*) on causative bacteria of mastitis and epithelial cells of bovine mammary gland *in vitro*.
Materials & Methods: Hydro-alcoholic extract of *U. dioica* was provided, then minimum inhibitory concentration (MIC) 50 and MIC90 of the extract (µg/mL) was determined against both *Escherichia coli* and *Staphylococcus aureus*. Finally, after isolation and culture of epithelial cells of bovine mammary gland, cytotoxic effects of MIC50 and MIC90 concentrations of the hydro-alcoholic extract were evaluated using MTT assay during 24 and 48 h.
Results: The hydro-alcoholic extract could inhibit growth of both *E. coli* and *S. aureus*, with higher inhibitory effect on *S. aureus* than that of *E. coli*. For both species, MIC50 concentration was 312.5µg/mL concentration of hydro-alcoholic extract and MIC90 was 39.06 µg/mL. The results of MTT assay confirmed that viability of the epithelial cells for MIC50 concentration during 24 and 48 h was 68% and 28%, respectively. Additionally, cell viability for MIC90 concentration in 24 and 48 h was 50% and 6 %, respectively.
Conclusion: According to our results, it can be concluded that despite bactericidal activity of *U. dioica* extract, cytotoxic effects was observed at antibacterial levels. Hence, further formulations are required to treat mastitis using this herbal ointment.

Keywords: Mammary gland, Antibacterial traits, Epithelial cells, Mastitis, Herbal extract, Cytotoxicity

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Introduction

In most countries mastitis or inflammation of mammary gland is known as a prevalent disease which can infect cattle during lactation and dry period. Mastitis not only can change nature of mammary gland tissue but also is able to alter physical and chemical features of milk (1). It has been reported that mastitis can extremely decrease quality and quantity of milk. Moreover, the disease causes huge losses to the dairy industry by increasing the cost of treatment and even causing the death of animals (2). In general, disease pathogens and environmental factors play a crucial role in the prevalence of mastitis, so these parameters must be considered to combat this

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disease (3). A wide variety of bacteria species are known as a causative agent of mastitis in dairy cattle, based on origin, these bacteria are categorized in two important groups including contagious and environmental agents (4). The members of contagious group such as *staphylococcus aureus*, *Streptococcus agalactiae*, *mycoplasma Bovis* and *Corynebacterium* mainly grow on mammary gland skin and they are able to make colonies in ducts of mammary gland (4). In addition to herd of cattle, contagious agents can infect human societies through pollutant milk. In contrary to contagious agent, environmental agents such as *Escherichia coli* found in barn and bed can lead to mastitis during milking (5). It is important to note that *staphylococcus aureus* is gram-positive bacteria related to different types of mastitis (e.g. acute and chronic) (6). Use of beta-lactam antibiotics has been recommended to treat mastitis attributed to *S. aureus*. New studies have reported that this bacterium using beta-lactamase enzyme can decompose beta-lactam antibiotics and consequently resist against them (7, 8). Besides, *S. aureus* is able to resist against environmental pressure using production of an appropriate biofilm (9-12). *E coli* is a gram-negative bacterium which can usually cause weak mastitis with moderate symptoms, but sometime this bacterium can lead to acute mastitis and in some cases, it can even kill the infected animals (13). In general, endotoxin, production of capsule in serum, elimination of iron, and ability of biofilm production are the most important virulence factors of *E coli* which trigger inflammatory responses (13, 14). Today, due to advent of resistant strains and presence of antibiotics in milk, consumption of antibiotics for treatment of mastitis has been limited (14, 15). Consequently, a new strategy must be considered as an alternative for antibiotics. Derivatives of herbal plants can be introduced as a new and promising method instead of antibiotic treatment, because many researches have reported that these agents have antimicrobial features, hence, they can control a wide variety of microbial infection without advent of bacteria resistance (16-18). The Nettle is a plant with scientific name of *U. dioica* extensively grown in shadowy and moist regions of Asia and Europe. *U. dioica* is a rich source of minerals (e.g., iron, manganese, potassium, calcium, copper and nickel), acetylcholine, histamine, flavonoids, pantothenic acid, carotenoids, proteins, essential oils, phenolic acids, alkaloids, chlorophyll, tannins, sterols, polysaccharides and isoelectins (19-22). It must be considered that anti-bacterial and anti-inflammatory activities of *U. dioica* are related to its phenolic, flavonoids and derivatives of caffeic acid (19-22). As a result, this plant can be applied for microbial infection such as mastitis. In view of the importance of mastitis and therapeutic features of *U. dioica*, the current study was designed to investigate bactericidal activities and cytotoxic effects of hydro-alcoholic extract of *U. dioica* on causative bacteria of mastitis and epithelial cells of bovine mammary gland.

Material and Methods

Preparation of hydro-alcoholic extract To make this extract, first, *U. dioica* leaves powder were prepared using air drying with sun exposure at 25°C for 65 hours (23). Then, 100g of leaves powder were added to 200mL distilled water and then the volume of solution was upped to 1000mL using methanol (this solvent owing to its high polarity leads to achievement of more extract yield) (24). The solution was poured in a dark jar and shaken for 72h at room temperature. The solution was filtered using a Whatman paper and solvent of the solution was removed by heating at 37°C in 72h. Finally, 5 grams of the dry extract were solved in 10mL distilled water (final concentration was 500mg/mL) (25).

Determination of Minimum inhibitory concentrations (MICs)

Initially, standard strains of *S. aureus* (ATCC code: 25923) and *E coli* (ATCC code: 25922)

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were cultured in nutrient agar medium for 16h at 35°C along with shaking. Then, both bacteria were rejuvenated using Mueller–Hinton agar medium. To determine MIC50 and MIC90 concentrations (MIC90 and MIC50 values are defined as the minimum concentration of the extract which can inhibit growth of 90 and 50% of the bacteria, respectively), broth microdilution was applied. In this case, 200µL of prepared hydro-alcoholic extract (500mg/mL) was added in first well of the 96 wells plate and 100µL of broth Mueller–Hinton medium was added to other wells. To make a serial dilution, 100µL of the extract poured in first well, was added to second well and 100µL of the second well was added to third well and this process was repeated until the last well. Eventually, 100µL of bacterial suspension (15× 10^5 cfu/mL) were added to each well including medium and hydro-alcoholic extract. The plates were located in an incubator in 37°C for 24h (26).

Primary culture of mammary gland epithelial cells

The primary cell culture was conducted based on a study by Forouharmehr et al (27). In this case, a 1 cm^2 sample was sterilely cut from healthy mammary gland immediately after slaughtering, the isolated sample was transferred to laboratory for subsequent processes. In laboratory, to provide cell suspension, the isolated sample was scratched in medium using sterile blade for 15 min, then the cell suspension was filtered using a 70 µm cell strainer. the filtered suspension was centrifuged at 5000 rpm for 5min, the supernatant was discarded and the pellet of cells was resuspended using fresh medium including 88% DMEM, 10% FBS, 1% amphotericin B and % 1 Penicillin-Streptomycin. The cells were seeded in 25cm flasks and incubated in 37 °C with 5%CO_2 and 99% humidity.

Isolation of epithelia cells

Isolation of epithelial cells from fibroblast cells was performed based on protocol of *Wellnitz* et al 2004 (28). According to this protocol, 30 min after primary cell culture, the medium of flasks which contained epithelial cell were collected (the empty flask which contained adhered fibroblasts were discarded). the collected mediums were centrifuged at 5000 rpm for 5 min, the supernatants were discarded and the epithelial cells were resuspended in fresh medium containing 88% DMEM, 10% FBS, 1% amphotericin B and % 1 Penicillin-Streptomycin. The epithelial cells were seeded in 25 cm flasks and incubated in 37°C with 5% CO_2 and 99% humidity.

Cytotoxicity assay

To investigate the effect of *U. dioica* hydro-alcoholic extract on viability of mammary gland epithelial cells, 36×10^3 epithelial cells were seeded in a 96-well plate and incubated in 37 °C with 5% CO_2 and 99% humidity for 24h. Then, to investigate cell viability after 24 and 48h exposure with extract, two concentrations of extract including 312.5µg/mL, 39.06 5µg/mL determined based on MIC test were (each concentration had 5 replications for 24h incubation and 48h incubation, also control group had 5 replications) added to wells. After exposure, the old medium was discarded and 50 µL(5mg/mL) MTT reagent was added to each well, then incubated in 37°C for 4h. The wells were evacuated and 50 µL DMSO was added to each well and incubated in 37°C for 30 min. Finally, the plate was read at wavelength of 570nm (29).

Results

Determination of MIC50 and MIC90 concentrations

As shown in the Table1, the results of MIC revealed that, the MIC50 concentrations for both bacteria (*S. aureus and E coli)* were approximately 312.5µg/mL. Moreover, these results demonstrated that MIC90 concentration for both *S. aureus* and *E coli* was 39.06 µg/mL (Table1).

Table1. Minimum inhibitory concentrations (MIC) of *Urtica dioica* hydro-alcoholic extract for inhibition 50 and 90 % growth of *S. aureus* and *E coli.*

Bacteria	MC50 (µg/mL)	MIC90 (µg/mL)
S. aureus	312.5µg/mL	39.06 µg/mL
E coli	312.5µg/mL	39.06 µg/mL

Isolation of mammary gland epithelial cells

To investigate results of cell isolation, three days after isolation process, the cultured flasks were monitored using an invert microscope with 100X magnification. The results showed that epithelia cells adhered to culture flask with 80 % confluency without fibroblast contamination (Figure 1). It is important to note that neither fungal nor bacterial contamination were observed in the cultured flasks.

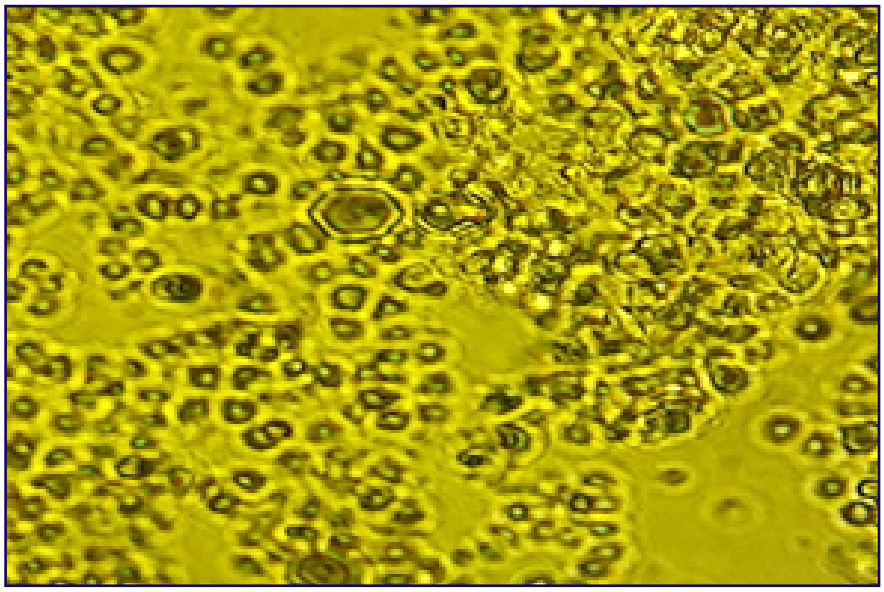

Figure 1. The isolated bovine mammary gland epithelial cells, the cells were illustrated with 100X magnification

Cytotoxicity assay

As mentioned earlier, to investigate the viability of epithelial cells in exposure with *U. dioica* hydro-alcoholic extract, MTT assay was employed. The results of MTT assay revealed that, compared to control group (without treatment), the concentration of 312.5µg/mL of extract was able to induce death to 32% and 74% of cell after 24 and 48h after treatment, respectively (Figure 2). Also, the results of MTT assay showed that concentration of 39.06 µg/mL (compared to control group) could induce death to 50% and 94% of the cell 24h and 48h after exposure (Figure 3).

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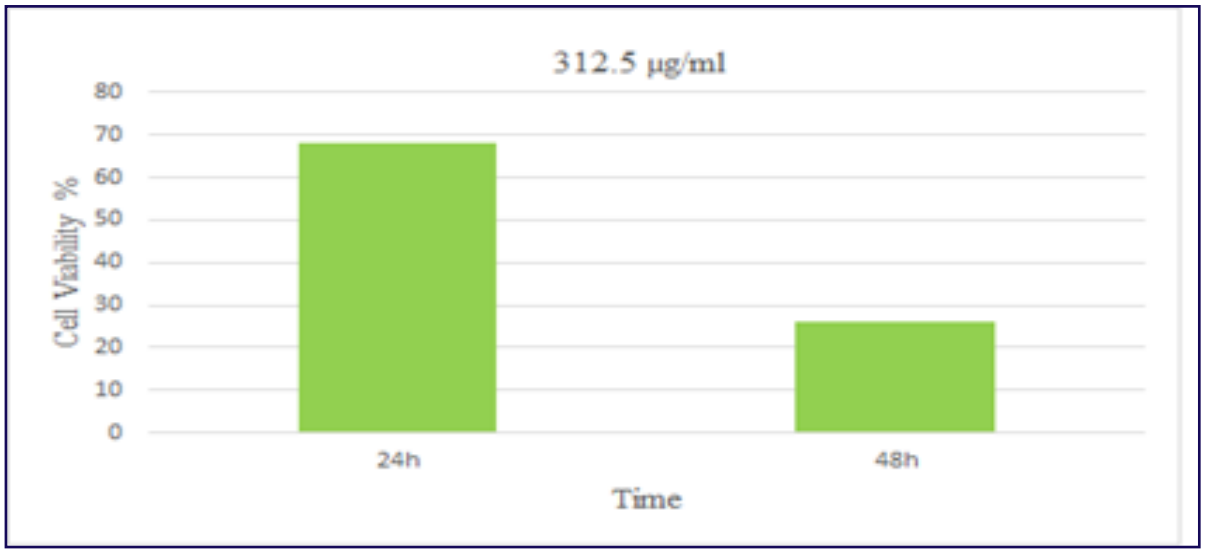

Figure 2. Chart of MTT assay 24h and 48h after exposure with 312.5µg/mL of *Urtica dioica* hydro-alcoholic extract

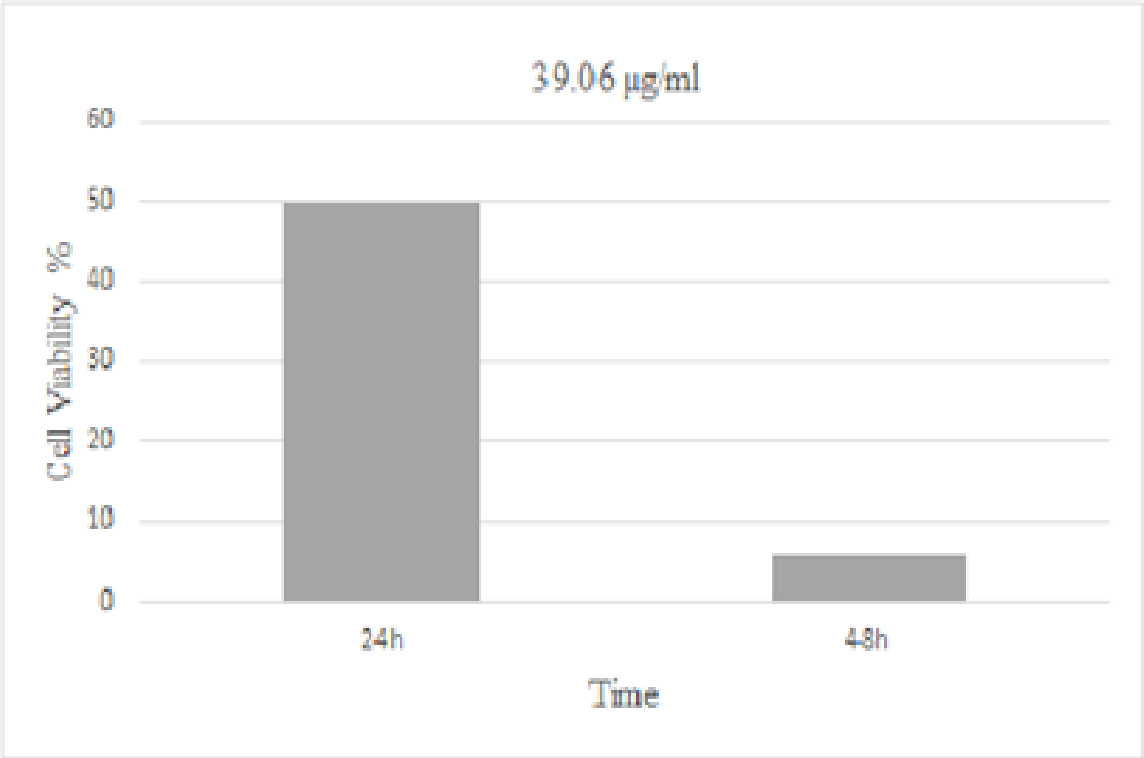

Figure 3. Graph of MTT assay 24h and 48h after exposure with 39.06 µg/mL of *Urtica dioica* hydro-alcoholic extract

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Discussion

Mastitis is defined as an inflammatory response which is triggered because of mammary gland infection (30). This disease is observed in different diary domestic animals such as dairy cattle. Mastitis is classified as an expensive and prevalent disease that not only can lead to increase in mortality rate but also can negatively impress quality and quantity of milk (30, 31). Therefore, owing to importance of mastitis, many studies have focused to treat this disease (14). So far, different strategies such as antibiotics therapy, animal breeding and antimicrobial peptides have been employed to control mastitis, but it has been reported that these strategies cannot effectively control mastitis (32). Hence, many attempts are being done to find new strategies as a promising alternative for conventional methods. In view of the importance of herbal medicines in traditional medicine, these plants were quickly taken into account to treat mastitis (2). Generally, plant extracts show antimicrobial activity and are able to neutralize inflammation of a pathogen using inactivation of NF-kB pathways. Despite, Positive features of herbal medicines, due to existence of chemical components such as alkaloids, terpenes and phenol, excessive consumption of this plants can lead to damage of different part of a cell and consequently induce apoptosis (33). Therefore, during the selection of a plant extract to treat mastitis, this point must be considered that the selected plant has no cytotoxicity for mammary gland cells. As a result, the current study was designed to investigate antibacterial and cytotoxic effects of *U. dioica* hydro-alcoholic extract as a potential herbal medicine to treat mastitis. In this case, antibacterial effect of *U. dioica* hydro-alcoholic extract was assessed using MIC test on *S. aureus* and *E coli* as the most important pathogens which lead to mastitis. Also, for investigation of cytotoxic effect of this herbal medicine on mammary gland epithelial cells, MTT assay was conducted. As mentioned, the results of the current study demonstrated that *U. dioica* hydro-alcoholic extract was able to inhibit the growth of *S. aureus* and *E coli* with MIC50 and MIC90 of 312.5µg/mL. and 39.06 µg/mL, respectively. Majd et al (2001) reported that seed extract of *U. dioica* showed antibacterial activity against gram-positive bacteria, whereas leaves extract of *U. dioica* was able to inhibit growth of gram-negative bacteria which these results confirmed our results (33). Moreover, research results of Ilhami Gülçin et al (2004) revealed that *U. dioica* extract could inhibit growth of both gram-negative and gram-positive bacteria, hence the results of this study completely confirmed the results of the current study (34). Recently, the data released by Mirtagi et al (2016) confirmed that leaves extract of *U. dioica* had antibacterial activity against *S. aureus*, *S. epidermidis*, *S. saprophyticus,* consequently the results of this research completely confirmed our results (22). In a project conducted by Motamedi et al (2014), antibacterial effects of *U. dioica* extract were investigated on both gram-negative and gram-positive bacteria species. They reported that this extract displayed significant antibacterial effects against those pathogenic bacteria. Although, the mentioned projects like our project confirmed antibacterial effects of *U. dioica* extract, in contrast to our study, they did not asses cytotoxic effects of U. dioica hydro-alcoholic extract (35). The MTT assay results showed that 68% and 26% of epithelial cell could evade from death while exposed 24h and 48h (respectively) with 312.5µg/mL (MIC50) of *U. dioica* hydro-alcoholic extract. Also, our results confirmed that only 50% and 6% of the epithelial cells were able to evade from cytotoxic effect of *U. dioica* hydro-alcoholic extract with concentration of 39.06 µg/mL during 24hand 48h exposure, respectively. Taqyzadeh-Kashani et al (2014) reported that *U. dioica* extract showed cytotoxic effect on T47D cell line that these results completely confirmed our results (36). In general, the results of the current project revealed that

although *U. dioica* hydro-alcoholic extract could show antibacterial activity and inhibit growth of *S. aureus* and *E coli*, due to induction of death to mammary gland epithelial cells cannot be applied to treat mastitis as a promising drug (e.g., topical ointment).

Conclusion

The current study was designed to assess antibacterial and cytotoxic effects of *U. dioica* hydro-alcoholic extract on causative bacteria of mastitis (*S. aureus* and *E coli* bacteria) and mammary gland epithelial cells, respectively. The results of the current project demonstrated that *U. dioica* hydro-alcoholic extract played a role in the antibacterial effects for treatment of mastitis. However consumption of this plant extract could be restricted owing to its cytotoxic effects on mammary gland epithelial cells.

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Conflict of interest

The authors declare that there is no conflict of interest.

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