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Research Article

Effects of *Ziziphora clinopodioides* Essential Oil and Nisin on the Microbiological Properties of Milk

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ABSTRACT

Background: Recently, because of increasing concern regarding food safety containing chemical additives, remarkable attempts have been made and progress to develop natural antibacterial compounds such as essential oils.
Methods: This study was conducted to determine antibacterial activities of *Ziziphora clinopodioides* essential oil (ZEO) alone and in combination with different concentrations of nisin to increase shelf life and control *Salmonella typhimurium* and *Staphylococcus aureus* in milk stored at refrigerated temperature.
Results: The dominant constituents of ZEO in our study were carvacrol (65.22%) and thymol (19.51%). The microbial population was decreased significantly with addition of ZEO and nisin, increased their concentrations and longer storage time. It was found that milk samples containing ZEO at 0.2% and nisin at 500 IU mL^{-1} had significantly lower microbial population (p<0.05).
Conclusions: Based on our findings, ZEO and nisin extended the shelf life of milk and may be used as natural antibacterial compounds in food products such as milk and dairy products.

Introduction

It is generally agreed upon that pasteurization of milk play an important role on the inactivation of major food-borne pathogenic bacteria. However, concerns of dairy industries increased about the efficacy of pasteurization to inactivate bacterial pathogens.[1] In order to control and eliminate food-borne pathogens, various chemicals and synthetic compounds such as antibiotics as preservative has been used.[2] In recent years, because of increasing concern regarding food safety containing chemical additives, remarkable attempts have been made and progress to develop natural antibacterial compounds such as essential oils (EOs).[3]

Several bacteriocins have been evaluated for their activities against wide variety of bacterial pathogens especially *Yersinia enterocolitica*, *Listeria monocytogenes*, *Bacillus cereus*, *B. subtilis*, *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Staphylococcus aureus*.[4-6] Among them, nisin has been considered as the most widely effective antimicrobial agents. Nisin is a well-known broad spectrum bacteriocin, and it is only the approved bacteriocin by the US Food and Drug Administration (FDA) and World Health Organisation (WHO) for using in food and food products especially milk and milk-based products.[7] The principle mechanism of nisin is through binding electrostatically to the cytoplasmic membrane and formation of numerous pores in the bacterial cell wall.[8] Despite the inhibitory effect of nisin on Gram-positive bacteria, it has been reported that this bacteriocin is not able to inhibit some Gram-positive bacteria such as *L. monocytogenes*, Gram-negative bacteria and also spore forming bacteria.[9] To enhance the inhibitory effect of nisin against bacterial food-borne pathogens, combination of this antimicrobial agent with other antimicrobials such as EOs should be evaluated.[10]

Ziziphora clinopodioides is an edible medicinal plant belonging to the family of *Laminaceae* which is widely distributed in various regions of Asia and Europe especially Iran and Turkey.[11,12] In Iran, the most important traditional use of *Z. clinopodioides* plant is to cure stomach tonic and expectorant, carminative, wound healing material, sedative and antiseptic.[13] *Z. clinopodioides* essential oil (ZEO) is used for diarrhea, intestinal gas, nausea and vomiting. Moreover, it is commonly and widely applied as a flavoring agent in foods such as dairy and meat products.[14]

Attempts have been done to growth inhibition and/or elimination of *S. typhimurium* and *S. aureus*

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in raw and processed foods by various antimicrobial agents.[2,6] However, in the scientific literature, there was no data available about the antibacterial effects of the ZEO with nisin against *S. typhimurium* and *S. aureus* in milk. Hence, this study was conducted to determine antibacterial activities of the ZEO alone and in combination with different concentrations of nisin to increase shelf life (mesophilic, *Enterobacteriaceae* and *Psychrotrophic* microorganisms) and control *S. typhimurium* and *S. aureus* in milk stored at refrigerated temperature.

Materials and Methods

Plant material

The leaves samples gathered from wild population of *Z. clinopodioides* plant growing in the Gilane Gharb city, Kermanshah province, west of Iran were used in the current study. A total of three replicate samples were obtained during the full flowering stage of growth (March-July 2014). The plant was identified and authenticated by a taxonomist and a representative voucher specimen (No. 6816) was deposited in the botany herbarium of the Research Center of Natural Resources of Tehran, Iran.

Isolation and Gas chromatography–mass spectrometry (GC–MS) analysis of essential oil

The isolation method and GC-MS analysis of the ZEO were described in our previous study.[10]

Preparation of nisin

Preparation of nisin was conducted according to the previously method reported by Shahbazi et. al., 2015.[15]

Test microorganisms

The microorganisms used in the current study included *S. typhimurium* (ATCC 14028) and *S. aureus* (ATCC 6538). Lyophilized cultures of the microorganisms were obtained from the culture collection of the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The strains were maintained in Brain Heart Infusion broth (BHI; Merck, Darmstadt, Germany) medium containing 20% v/v glycerol at -80 °C. For activating, two consecutive subcultures were incubated in BHI broth overnight at 37 °C. The density of bacterial cultures needed for the inoculation of milk samples examined by using a spectrophotometer at 600nm. The determination of inoculum dose (1×10^5 CFU mL^{-1}) of milk also was assessed using plate count on Brain Heart Infusion agar (BHI; Merck, Darmstadt, Germany) medium in triplicate.

Experimental design

Raw milk samples were obtained from a local market of Kermanshah city, west of Iran. The milk samples were subdivided three groups: the first group was without inoculated pathogenic bacteria; the second and third groups were inoculated with *S. aureus* and *S. typhimurium*, respectively. In the case of groups two and three, before inoculating selected pathogens, the milk samples were boiled in a water bath. Then, for each group, nine batches were designed as follows: Control: no ZEO or nisin added, 1: 0.1% ZEO, 2: 0.2% ZEO, 3: 250 IU mL^{-1} nisin, 4: 500 IU mL^{-1} nisin, 5: 0.1% ZEO+250 IU mL^{-1} nisin, 6: 0.1% ZEO+500 IU mL^{-1} nisin, 7: 0.2% ZEO+250 IU mL^{-1} nisin and 8: 0.2% ZEO+500 IU mL^{-1} nisin. In the case of inoculated samples with pathogenic bacteria, firstly the milk samples were boiled and then inoculated with *S. typhimurium* and *S. aureus* cultures at the level of 10^5 CFU mL^{-1}. Control milk without pathogenic bacteria and ZEO or nisin was also considered. After homogenization for 30 s, all samples were stored at refrigerated temperature (4±1 °C) before microbial analysis. The bacterial examination of milk samples were conducted at 0, 1, 3, 5, 7 and 9 days. All experimental data were conducted at three replicates.

Microbiological analysis

10 mL of each milk samples was sampled with 90 mL of 0.1% peptone water in a stomacher bag. The sample was homogenized in a stomacher and diluted it ten-fold serial dilution for enumeration. In the current study, several culture media including Plate count agar (total mesophilic and *Psychrotrophic* counts), Violet Red Bile agar (*Enterobacteriaceae* family), Baird parker agar with egg yolk tellurite (*S. aureus*) and *Salmonella Shigella* agar (*S. typhimurium)* were used. The experiments were repeated in triplicates. Results were expressed as log CFU mL^{-1}.

Statistical analysis

All experiments were conducted in triplicate. Data was transformed into logarithms of the number of CFU mL^{-1}, mean and standard deviations were calculated and then subjected to the analysis. Tukey's test at 95% confidence interval of the SPSS package (SPSS 16, Chicago, IL, USA) was used to determine mean differences among the treatments.

Results and Discussion

Chemical composition of Z. clinopodioides essential oil

The major chemical constituents identified in the ZEO by GC and GC-MS were published in our previous study.[10] Based on the findings, a total of 24 constituents were identified which represent 99.65% of the ZEO extract. The dominant compounds of ZEO in our study were carvacrol

(64.22%), thymol (19.22%), γ-terpinene (4.63%) and *p*-cymene (4.86%).

Change in microbial content of milk during storage at 4 °C

Figure 1, 2 and 3 show the microbial changes (mesophilic, *Enterobacteriaceae* and *Psychrotrophic* microorganisms) of milk during 9 days. In detail, the microbial population (mesophilic, *Enterobacteriaceae* and *Psychrotrophic* microorganisms) was reduced significantly by adding ZEO and nisin, increased their concentrations and longer storage time ($p<0.05$).

Total Viable Count (TVC), the most commonly quality and acceptability index, of the milk samples treated with ZEO alone and in combination with different concentrations of nisin during refrigerated storage are exhibited in Figure 1. Although, slightly lower TVC was determined in the samples containing the ZEO in combination with nisin during storage, significant difference was observed between the untreated and treated groups ($p<0.05$). In control sample, the initial population of mesophilic population was 5.01 log CFU mL^{-1} and increased to 7.11 log CFU mL^{-1} on day 9. In the group treated with ZEO at 0.2% and nisin at 500 IU mL^{-1}, TVC was decreased 2 log CFU mL^{-1} at the end of storage. This result is good in accordance with previous researches that showed a reduction in TVC with the addition of some EOs and nisin, alone and in combination, in food model systems during storage at refrigerated condition.[2,6,16,17]

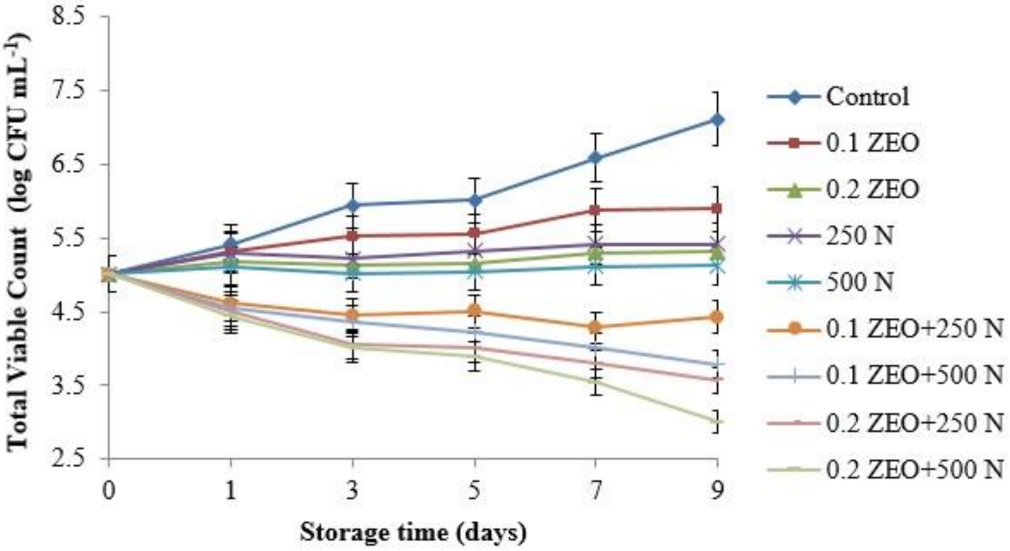

Figure 1. Effect of *Z. clinopodioides* essential oil (ZEO), nisin (N) and their combination on Total Viable Count in raw milk stored at 4 °C.

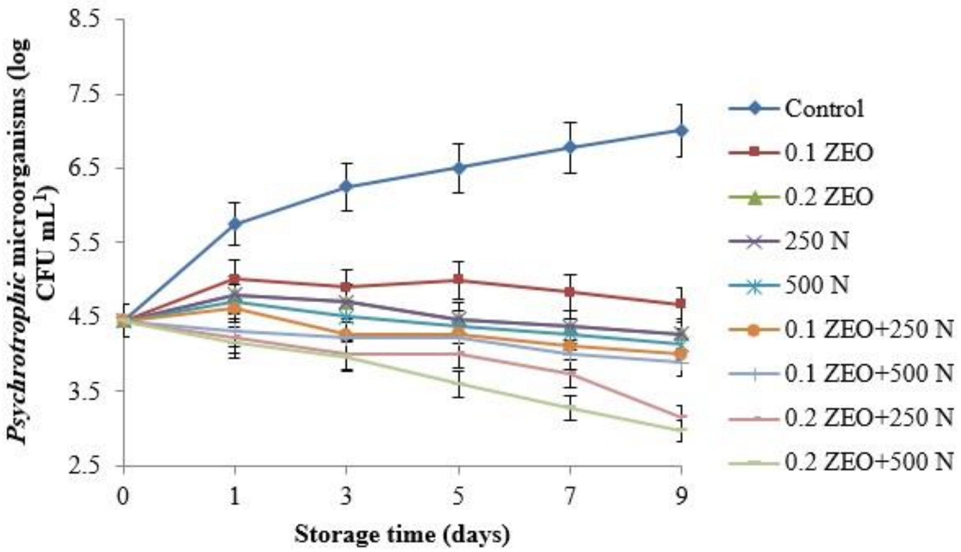

Figure 2. Effect of *Z. clinopodioides* essential oil (ZEO), nisin (N) and their combination on *Psychrotrophic* microorganisms in raw milk stored at 4 °C.

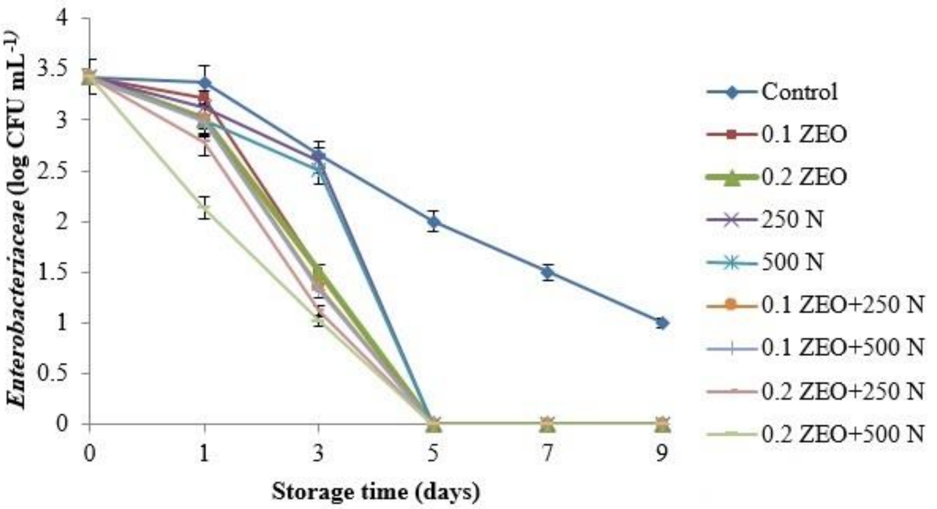

Figure 3. Effect of *Z. clinopodioides* essential oil (ZEO), nisin (N) and their combination on *Enterobacteriaceae* in raw milk stored at 4 °C.

The ZEO and nisin, alone and in combination, showed good inhibitory effects against *Psychrotrophic* microorganisms, the most important group of microorganisms responsible for spoilage of milk at refrigerated condition, in treated milk samples (Figure 2). The initial count of *Psychrotrophic* microorganisms was 4.45 log CFU mL^{-1} and reached as 7.01, 4.67, 4.27, 4.27, 4.13, 4, 3.89, 3.15 and 2.97 log CFU mL^{-1} for control, 0.1%, 0.2%, 250 IU mL^{-1}, 500 IU mL^{-1}, 0.1%+250 IU mL^{-1}, 0.1%+500 IU mL^{-1}, 0.2%+250 IU mL^{-1} and 0.2%+500 IU mL^{-1} samples, respectively after 9 days. The most reduced count of *Psychrotrophic* microorganisms was found for milk samples containing 0.1%+500 IU mL^{-1}, 0.2%+500 IU mL^{-1} and 0.2%+250 IU mL^{-1} until the end of refrigerated storage.

Finally, regarding *Enterobacteriaceae* (Figure 3) the initially recorded population of 3.42 log CFU mL^{-1} was reduced most rapidly during the first day of storage and it was reached to below 1.0 log CFU mL^{-1} until the end of refrigerated storage for untreated samples. This results agreed with previous published studies.[18-21]

Control of S. typhimurium and S. aureus in milk during storage at 4 °C

As it can be seen in Figure 4 and 5, it was observed that the ZEO and nisin had high inhibitory activities against *S. aureus* and *S. typhimurium* in milk compared to the control. The initial inoculum population of *S. aureus* and *S. typhimurium* was 5 log CFU mL^{-1} that was counted at 3.12 and 3.25 log CFU mL^{-1} after 9 days in control samples, respectively.

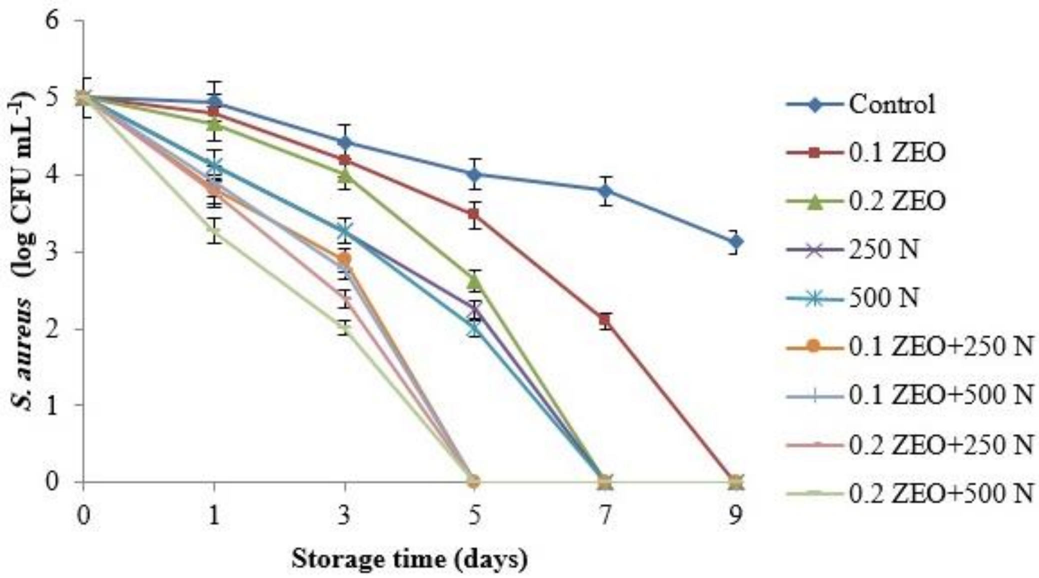

Figure 4. Effect of *Z. clinopodioides* essential oil (ZEO), nisin (N) and their combination on *S. aureus* in pasteurized milk stored at 4 °C.

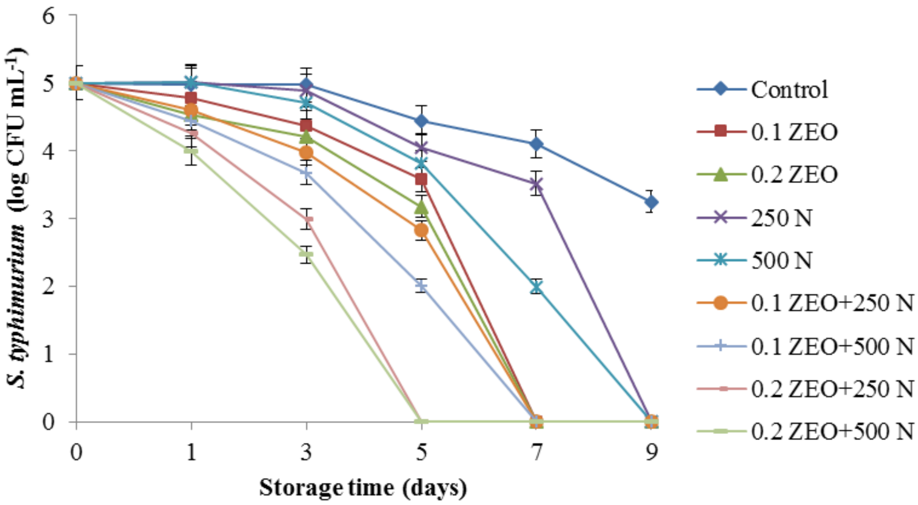

Figure 5. Effect of *Z. clinopodioides* essential oil (ZEO), nisin (N) and their combination on *S. typhimurium* in pasteurized milk stored at 4 °C.

With respect to *S. aureus*, it was found that nisin and the ZEO, alone and in combination, had inhibitory effects against *S. aureus* in milk. A good antibacterial activity was found in the milk sample containing 0.1 and 0.2% ZEO in combination with 250 and 500 IU mL^{-1} nisin. Sample containing ZEO at 0.2% and nisin at 500 IU mL^{-1} had significantly lower population of *S. aureus* ($p<0.05$). Moreover, both 1% and 2% ZEO and 250 and 500 IU mL^{-1} nisin, alone and in combination, completely inhibited the growth of *S. aureus* at day 9. Regarding *S. typhimurium*, containing the ZEO at 0.2% and nisin at 250 and 500 IU mL^{-1}, had significantly lower bacterial population ($p<0.05$). Based on our knowledge, the combination effect of ZEO and nisin against *S. typhimurium* and *S. aureus* in milk had not been examined until now. Previous published researches indicated that the combination of EOs and nisin had a higher effect than the ZEO or nisin alone against food-related bacteria such as *L. monocytogenes S. typhimurium*, *S. aureus*, *S. enteritidis* and *E. coli* O157:H7.[5,6,8,16] Numerous *in vitro* studies reported that ZEO had strong antibacterial effect against some Gram-positive and Gram-negative bacteria such as *S. aureus*, *Bacillus subtilis*, *B. cereus*, *L. monocytogenes*, *S. typhimurium* and *E. coli* O157 : H7.[12,13,22] Our previously studies showed that ZEO alone and in combination with nisin had good antibacterial activities against *S. aureus*, *B. subtilis*, *B. cereus*, *L. monocytogenes* and *E. coli* O157:H7 and also could extend shelf life of food model systems including raw beef patty, commercial barley soup and doogh (Iranian yogurt based drink).[15,23,24] The antibacterial effect of the ZEO could be attributed to its major chemical compounds (carvacrol and thymol). It has been reported that carvacrol is one of the main antimicrobial agents of medicinal plants.[11] The mechanism of carvacrol action may be due to the destabilization of the phospholipid bilayer structure and interaction with membrane enzymes. However, previous published studies indicated that other antibacterial compounds of the EOs such as *α*-pinene, myrcene and *α*-terpineol possess antibacterial effects against numerous pathogenic bacteria and enhance the effects of carvacrol and thymol.[2] The mechanism of combination effects of nisin and various EOs is not fully understood. It seems that EO enhances the effect of nisin through increasing the number and size of pores in the cytoplasmic membrane structure.[3,4,25] Based on our results, the microbial population was decreased significantly with longer storage time ($p<0.05$). Several factors such as acidic property (pH) and the presence of lactic acid bacteria may be contributed to the reduction of the microorganisms during longer storage time. Some authors found that reduction of pH due to production of acid by lactic acid bacteria present in dairy products such as milk and cheese could inhibit the growth of food borne pathogens.[26-28]

Conclusion

Based on our results, the ZEO alone and in combination with nisin showed good antibacterial activity against two common food-borne pathogens, *S. typhimurium* and *S. aureus* in milk. Therefore, ZEO and nisin extended the shelf life of milk and may be used as natural antibacterial compounds in food products such as milk and dairy products.

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Conflict of interests

The author claims that there is no conflict of interest.

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