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Anti-listerial synergism of leaf essential oil of *Metasequoia glyptostroboides* with nisin in whole, low and skim milks

Vivek K. Bajpai[1], Jung In Yoon[2], Monika Bhardwaj[2], Sun Chul Kang[2*]

[1]*Department of Applied Microbiology and Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Republic of Korea*
[2]*Department of Biotechnology, Daegu University, Gyeongsan, Gyeongbuk 712-714, Republic of Korea*

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ABSTRACT

Objective: To examine the individual and synergistic anti-listerial effect of nisin and leaf essential oil of *Metasequoia glyptostroboides* (*M. glyptostroboides*) against one of the leading foodborne pathogens *Listeria monocytogenes* (*L. monocytogenes*) ATCC 19116 in milk samples. **Methods:** The whole (8%), low (1%) and skim (no fat content) milk samples were inoculated with *L. monocytogenes* ATCC 19116 along with leaf essential oil of *M. glyptostroboides* or nisin alone as well in combinations. **Results:** In this study, the leaf essential oil at the concentrations of 2% and 5% revealed strong anti-listerial effect against *L. monocytogenes* ATCC 19116 in all categories of milk samples. Nisin at the concentrations of 250 and 500 IU/mL displayed a strong inhibitory effect against ATCC 19116 as compared to the control group. Additionally, synergistic combinations of leaf essential oil (1%) and nisin (62.5, 125, 250 and 500 IU/mL) also had a remarkable anti-listerial synergism in all the tested milk samples including whole, low and skim milk after 14 days. **Conclusions:** As a major finding, the leaf essential oil of *M. glyptostroboides* might be a useful candidate for using in food industry to control the growth of foodborne pathogenic bacteria as confirmed by its potent anti-listerial synergistic effect with nisin against *L. monocytogenes* ATCC 19116 in different milk samples.

1. Introduction

Since decades, pasteurization of milk samples has been found to play a significant role on the inactivation of a major foodborne pathogens including *Listeria monocytogenes* (*L. monocytogene*), however, unsatisfactory concerns on the efficacy of pasteurization to inactivate this organism have also been reported[1,2]. *L. monocytogenes* can survive at low pasteurization treatment and can grow under aerobic and microaerophilic conditions[3]. It has also been found to be more thermotolerant than other non-spore-forming bacteria[3]. Although an optimal temperature of 35 to 37 ℃ is normally required for the growth of *L. monocytogenes*, it can also grow under refrigerated conditions, over a wide range of pH (4-9), and up to a 10% salt solution[4,5]. Nowadays, food industry is very much concerned about the occurrence of this foodborne pathogenic bacterium in dairy products such as cheese, milk and yogurt. In addition, other sources for the proliferation of this harmful pathogen may also include a variety of food products and processed meat samples[5]. Hence, it has become obvious to inhibit the proliferation of such foodborne pathogenic bacteria that cause decay and transmutation in stored and processed foods. Although numbers of different chemicals and synthetic compounds have been used at commercial level in order to control the growth of *L. monocytogenes* by food processors and in food system, they might be detrimental to human health, raising a great food safety concern to consumers. Hence, potent and effective antibacterial compounds are being explored in order to provide higher levels of food safety standards as natural antibacterial agents of plant origin to combat against major foodborne pathogens[6].

In order to sustain the food quality, plant-based

*Corresponding author: Prof. Sun Chul Kang, Department of Biotechnology, Daegu University, Gyeongsan, Gyeongbuk 712-714, Republic of Korea.
Tel.: +82 53 850 6553
Fax: +82 53 850 6559
E-mail: sckang@daegu.ac.kr

compounds such as essential oils have been used individually or synergistically with other chemicals or natural antibacterial agents using a variety of treatments[7-9]. Previously synergistic effects of essential oils and nisin on the inactivation of foodborne pathogens including Bacillus cereus and *L. monocytogenes* have been reported[10]. Also it has been found that the addition of lysozyme as a third preservative factor can enhance the synergistic effect between essential oil component and nisin when tested against foodborne pathogens in combination[11]. Accordingly, a similar synergistic effect on other appropriate preservatives in combination with essential oils and nisin can be expected. Among the variety of bacteriocins, nisin is known to be a potent antimicrobial agent against a broad spectrum of pathogenic bacteria associated with food contamination. However, low efficacy against some of the selected bacterial pathogens and its being developing resistant in few cases of sensitive gram positive bacteria, its application as a food preservative has been limited[12]. Mode of action of different bacteriocins (nisin) along with other natural food preservatives on the reduction of resistance to bacteriocins in target strains and/or to extend its inhibitory effect on Gram-negative bacteria has been reported previously[13]. Also the efficacy of nisin against Gram-negative bacteria can be increased by using it in combinations with other natural antimicrobial agents[14].

Metasequoia glyptostroboides (*M. glyptostroboides*) is a deciduous conifer belonging to Cupressaceae family which is propagated and distributed in various regions of Eastern Asia, North America and Europe. A plethora of biological activities of *M. glyptostroboides* derived secondary metabolites including essential oils and terpenoid compounds have been reported previously[15-19]. Also the cone essential of *M. glyptostroboides* has been found to exert inhibitory effect against foodborne pathongen in milk samples[20]. However, there is no report available in the literature on the synergistic effect of the leaf essential oil of *M. glyptostroboides* and nisin against *L. monocytogenes* in milk samples. Therefore, this study was conducted to determine the anti-listerial synergistic effect of nisin and leaf essential oil of *M. glyptostroboides* against *L. monocytogenes* ATCC 19116 in different categories of whole, low and skim milk samples.

2. Materials and methods

2.1. Plant materials

The leaves of *M. glyptostroboides* were collected from the Pusan area of South Korea, in September 2009. The leaves were initially identified by the morphological features and in-house data base by Prof. Man Kyu Huh. A voucher specimen number has been deposited in the Herbarium of the College of Engineering, Department of Biotechnology, Daegu University, Republic of Korea.

2.2. Reagents and chemicals

Nisin was purchased from Sigma-Aldrich Chemical Co. (N5764; Sigma, St. Louis, Missouri, USA) that contains 2.5% nisin with minimum potency of 106 U/g. Nisin (100 mg) was dissolved in 100 mL 0.02 N HCl to give the concentration of 103 U/mL. Then the solution was sterilized by autoclaving at 121 ℃ for 15 min, and kept in a refrigerator at 4 ℃ until used. All other chemicals and reagents were of the highest commercial grade.

2.3. Isolation of leaf essential oil

The air-dried powdered leaves (250 g) of *M. glyptostroboides* were subjected to hydrodistillation for 3 h using a Clevenger type apparatus. The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4 ℃ until further analysis[16].

2.4. Preparation of milk samples

Pasteurized and homogenized grade A milk samples consisting of whole (8% fat), low (1% fat) and skim milk (no fat) were purchased from a local market of Daegu city, Republic of Korea.

2.5. Preparation of culture of L. monocytogenes

A foodborne pathogenic strain *L. monocytogenes* ATCC 19116 used in this study was collected from the Korean Agricultural Culture Collection, Suwon, Republic of Korea. The strain was maintained on BHI agar (brain heart infusion, Difco) at 4 ℃ and was grown in BHI broth at 37 ℃ for 24 h.

2.6. Anti-listerial effect of leaf essential oil

Different concentrations of leaf essential oil (1%, 2% and 5%) were added to whole, low and skim milks and inoculated with initial population of approximately 1.8×10^6 CFU/mL of *L. monocytogenes* ATCC 19116 strain, respectively. Controls were inoculated with *L. monocytogenes* ATCC 19116 strain without leaf essential oil, and stored at 0, 2, 4, 6, 8, 10, 12 and 14 days at 4 ℃. The colonies were counted in nutrient agar (NA), Difco at 37 ℃ after 24 h of incubation.

2.7. Anti-listerial effect of nisin

Different concentrations of nisin (62.5, 125, 250 and 500 IU/mL) were added to whole, low and skim milk samples and were inoculated with an initial population of approximately 1.8×10^6 CFU/ml of *L. monocytogenes* ATCC 19116 strain, respectively, in order to determine the anti-listerial inhibitory effect of nisin. Control samples were inoculated with *L. monocytogenes* ATCC 19116 strain without nisin, and stored at 0, 2, 4, 6, 8, 10, 12 and 14 days at 4 oC. The colonies were counted in NA at 37 oC after 24 h of incubation.

2.8. Anti-listerial synergistic effect of leaf essential oil and nisin in milk samples

To determine the synergistic effect of leaf essential oil and nisin, two different concentrations of essential oil (1% and 2%) in combination with 62.5, 125, 250 and 500 IU/mL of nisin were added to whole, low and skim milks, and inoculated with initial population of approximately 1.8×10^6 CFU/mL of *L. monocytogenes* ATCC 19116 strain, respectively. Control samples were inoculated with *L. monocytogenes* ATCC 19116 strain and without essential oil and nisin, and stored at 0, 2, 4, 6, 8, 10, 12 and 14 days at 4 ℃. The colonies were counted in NA (nutrient agar, Difco) at 37 ℃ after 24 h of incubation.

2.9. Statistical analysis

Each experiment was run in triplicate, and mean values were calculated. The statistical analysis was carried out by employing one way ANOVA ($P<0.05$). A statistical package (SPSS version 11.0) was used for the data analysis.

3. Results

3.1. Anti-listerial effect of leaf essential oil

In this assay, it was found that the leaf essential oil showed potent anti-listerial effect at the tested concentrations (1%, 2% and 5%). However the higher concentration of the leaf essential oil had highest anti-listerial effect against *L. monocytogenes* ATCC 19116 in all kinds of milk samples tested as compared to the control (Figure 1). The initial population of *L. monocytogenes* ATCC 19116 in whole, low and skim milk samples was 6.23 log CFU/mL. The leaf essential oil showed strong anti-listerial effect against the growth of *L. monocytogenes* ATCC 19116 in low and skim milk samples as compared to control groups, whereas, moderate anti-listerial activity was observed in whole milk (Figure 1). The controls were counted at 8.15, 7.99 and 7.88 log CFU/mL in whole, low and skim milks, respectively, after 14 days. In all treated group, the initial cell number was rapidly decreased after 2 days at the concentration of 5% essential oil. In case of low and whole milk samples, 1% essential oil showed the cell count differences of 0.99 and 1. 51 log CFU/ mL, respectively, after 14 days as compared to control groups. For whole milk, 2% essential oil showed the cell count differences of 2.25 CFU/mL, after 14 days as compared to control groups. However, 5% essential oil completely inhibited the growth of *L. monocytogenes* ATCC 19116, with no cell count formation, at 14 day observation in all kinds of milk samples including whole, low and skim milks (Figure 1).

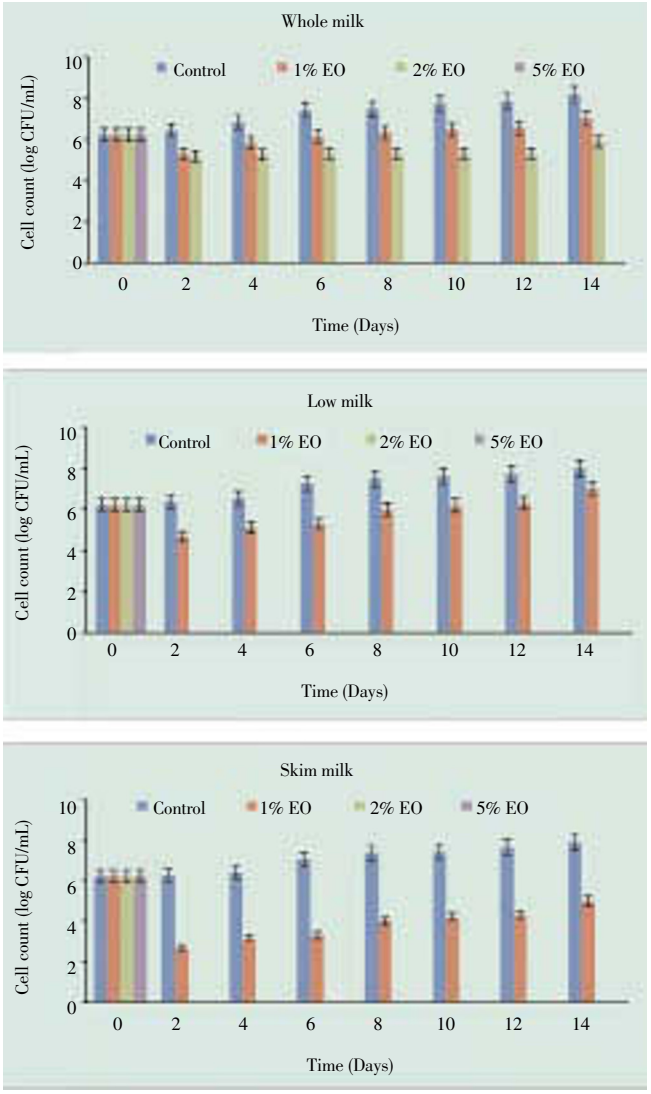

Figure 1. Anti-listerial effect of leaf essential oil of *M. glyptostroboides* against *L. monocytogenes* ATCC 19116 in whole, low and skim milk samples for 14 days.
CT: Control; EO: Essential oil.

3.2. Anti-listerial effect of nisin

As shown in Figure 2, on the anti-listerial activity of nisin, it was found that nisin at the used concentrations (62.5, 125, 250 and 500 IU/mL) showed remarkable anti-listerial effect against the growth of *L. monocytogenes* ATCC 19116 in low fat, whole and skim milk samples as compared to control groups[20]. In all treated groups, the initial cell number was rapidly decreased after 2 days at 500 IU/mL nisin concentration and no cell count numbers were observed until 14 days in case of low fat and skim milk samples. However, complete growth inhibition on colony forming units against *L. monocytogenes* ATCC 1916 was observed for 10 day at 500 IU/mL nisin concentration. The differences on cell count numbers in whole, low and skim milk samples at the concentration of 250 IU/mL nisin were observed to be 0.84, 2.20 and 3.32 CFU/mL until 14 day as compared to controls. It was found that the decrease in cell count numbers of *L. monocytogenes* was dependent on fat contents present in whole, low and skim milk samples.

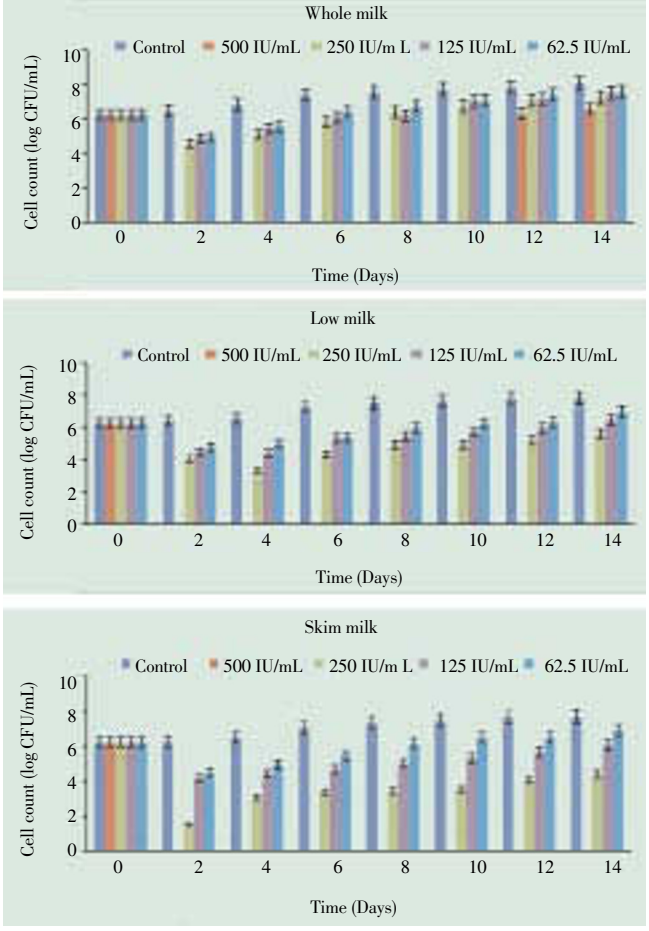

Figure 2. Anti-listerial effect of nisin against *L. monocytogenes* ATCC 19116 in whole, low and skim milk samples for 14 days.

3.3. Synergistic effect of leaf essential oil and nisin in milk samples

In this assay, it was found that *M. glyptostroboides* leaf essential oil (1% and 2%) in combination with different concentrations of nisin (62.5, 125, 250 and 500 IU/mL) had a potential synergistic effect on the anti-listerial activity in whole, low and skim milk samples for 14 days. A strong anti-listerial effect was confirmed when milk samples were treated with 1% essential oil in combination with various concentrations of nisin. In tests with whole milk, the combined groups had a stronger inhibitory ability against *L. monocytogenes* ATCC 19116 as compared to the control (Figure 3). Both 1% and 2% essential oil and 62.5 IU/mL nisin as controls were found to display cell count differences in anti-listerial effect as compared to the negative control. The combined action of the leaf essential oil with various concentrations of nisin (62.5, 125, 250 and 500 IU/mL) essentially decreased the cell count numbers in whole milk. Although individual concentration of leaf essential oil (1% and 2%) had considerable amount of anti-listerial effect against *L. monocytogenes* ATCC 19116 in whole milk sample as compared to control, 1% leaf essential oil in combination with 500 IU/mL nisin completely inhibited the growth of *L. monocytogenes* ATCC 19116 in whole milk sample and no cell count numbers were observed.

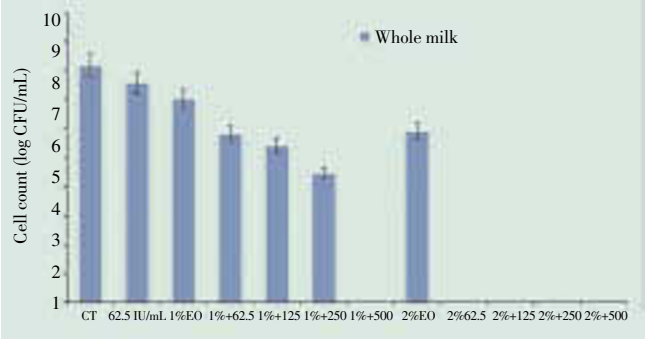

Figure 3. Anti-listerial synergistic effect of various combinations of *M. glyptostroboides* leaf essential oil and nisin on *L. monocytogenes* ATCC 19116 in whole milk at14 days.
CT: Control; EO: Essential oil.

As shown in Figure 4, addition of 1% oil alone to the low milk reduced cell count numbers about 0.99 log CFU/mL, as compared to the control. The addition of nisin 62.5 IU/mL alone had partial anti-listerial effect as compared to 1% essential oil alone. The combined groups of *M. glyptostroboides* leaf essential oil and nisin had a potent anti-listerial effect in low fat milk. In particular, 1% leaf

essential oil in combination with 500 IU/mL nisin completely inhibited the growth of *L. monocytogenes* ATCC 19116 at 14 day, and no cell count formation was observed. Interestingly 2% leaf essential oil alone and in combination with all the tested concentrations of nisin completely eradicated the cell count numbers of *L. monocytogenes* ATCC 19116 for 14 days in low milk sample.

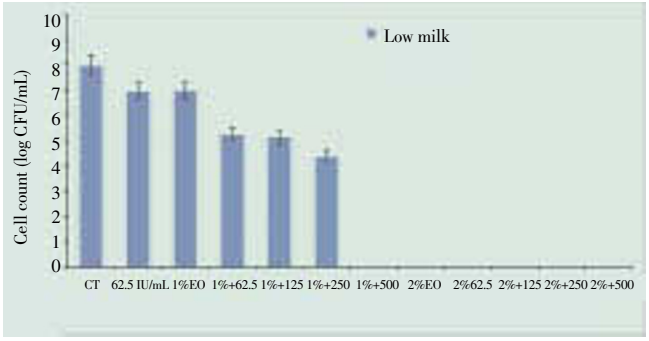

Figure 4. Anti-listerial synergistic effect of the various combinations of *M. glyptostroboides* leaf essential oil and nisin on *L. monocytogenes* ATCC 19116 in low milk at14 days.
CT: Control; EO: Essential oil.

The results of the combined or individual effect of essential oil or in combination with nisin have been shown in Figure 5. The combined group of leaf essential oil (1%) and nisin (62.5, 125 and 250 IU/mL) produced a great reduction in the growth of *L. monocytogenes* ATCC 19116 in skim milk. However, the leaf essential oil (1%) in combination with 500 IU/mL completely inhibited the growth of *L. monocytogenes* ATCC 19116 in skim milk at 14 day. Moreover, 2% leaf essential oil alone and/or in combination with all the tested concentrations of nisin completely inhibited the cell count numbers of *L. monocytogenes* ATCC 19116 for 14 days in skim milk sample.

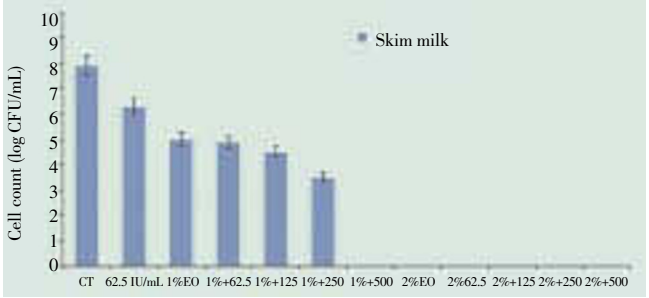

Figure 5. Anti-listerial synergistic effect of the various combinations of *M. glyptostroboides* leaf essetntial oil and nisin on *L. monocytogenes* ATCC 19116 in skim milk at14 days.
CT: Control; EO: Essential oil.

4. Discussion

Food contamination or diseases caused by a foodborne pathogen *L. monocytogenes* has upsurge the demand on using effective antimicrobials to control the propagation of this bacterium in food commodities. Also there is great food safety concern among the consumers on food or food products that contain chemical preservatives, and are less processed, resulting in severe cases of food contaminations. Hence, more preference has been given by the consumers on using foods or food products secured by natural antimicrobials which are less toxic to human health[21]. This study showed that *M. glyptostroboides* leaf essential oil with various combinations of nisin had a great influence on anti-listerial effect in whole, low and skim milk samples. The leaf essential oil of *M. glyptostroboides* revealed remarkable anti-listerial effect at the used concentrations at day 2 in all kinds of tested milk samples including whole, low and skim milks as compared to control group. In particular, 5% essential oil displayed strong anti-listerial activity with no dependency of fat content present in all categories of milk samples. Also 2% leaf essential oil showed potent inhibitory effect against *L. monocytogenes* ATCC 19116 in case of low and skim milk samples.

On the other hand, addition of nisin in whole, low and skim milk samples displayed significant amount of anti-listerial effect at the tested concentrations 62.5, 125, 250 and 500 IU/mL. Nisin showed strong anti-listerial effect in low fat and skim milk samples at higher concentration. However, nisin at low concentrations displayed a weaker anti-listerial effect against *L. monocytogenes* ATCC 19116 in whole milk as compared to the control. These results are in strong agreement with previous findings other researchers as they also confirmed individual inhibitory effect of nisin to inhibit the growth of other foodborne pathogens in variety of milk samples. These variations may be correlated to the different ratio of fat contents in varied milk samples[22]. Previously it was reported that the inhibitory effect of nisin against *L. monocytogenes* in fluid milk sample was directly dependent upon the fat contents[23]. Similarly *L. monocytogenes* was found to be the most sensitive bacterial pathogen to the nisin in skim milk, which showed a rapid decline in the cell numbers up to <10 CFU/mL after 12 days[24]. However, nisin showed an initial decline in cell number of *L. monocytogenes* in whole milk at the concentration of 2%.

The synergistic effect of nisin at the used concentrations (62.5, 125, 250 and 500 IU/mL) was found to be increased by

a combined action of 1% leaf essential oil in whole milk. However, 2% leaf essential oil alone could not reveal the significant anti-listerial effect against *L. monocytogenes* ATCC 19116 in whole milk. The combined effect of 1% leaf essential oil with 250 IU/mL nisin revealed significant anti-listerial efficacy. However, complete growth inhibition of *L. monocytogenes* ATCC 19116 in whole milk was observed at 1% leaf essential oil in combination with 500 IU/mL nisin after 14 days. Other combined groups also exhibited a strong anti-listerial effect as compared to control group.

In addition, the leaf essential oil (1%) with low fat milk at 250 IU/mL nisin severely decreased the cell count numbers, resulting in no cell growth formation of *L. monocytogenes* ATCC 19116 at 1% leaf essential oil in combination with 500 IU/mL of nisin. Also 62.5 and 125 IU/mL treatments of nisin with 1% leaf essential oil exhibited strong anti-listerial effect in low fat milk as compared to control group. No growth of *L. monocytogenes* ATCC 19116 was observed at further combined concentrations of oil and nisin in low milk sample.

The synergistic effect of leaf essential oil in combination with nisin against *L. monocytogenes* ATCC 19116 in skim milk has been shown in Figure 5. The essential oil (1%) in combination with nisin (62.5, 125 and 250 IU/mL) synergistically decreased the cell count numbers of *L. monocytogenes* ATCC 19116 ranging from 3.01 to 4.38 CFU/mL in skim milk, whereas, 1% essential oil along with nisin (500 IU/mL) completely inhibited the growth of *L. monocytogenes* ATCC 19116 in skim milk and no cell counts were observed.

These findings demonstrated that 1% essential leaf oil in combination with 500 IU/mL nisin had a very strong synergistic effect against *L. monocytogenes* ATCC 19116 in whole, low fat and skim milk samples as compared to the control groups. Further combined concentrations of leaf essential oil and nisin did not allow the growth of *L. monocytogenes* ATCC 19116 in all kinds of milk samples tested. Based on the above results, it was confirmed in this study that *M. glyptostroboides* leaf essential oil and nisin possessed strong synergistic effect as an antlisterial potential against *L. monocytogenes* in all categories of milk samples.

In fact, food preservation using small quantity of different preservatives in combination might exert better results than to a larger amount of a single preservative which may secure both microbial stability and safety, thereby maintaining the sensory, nutritive and economic properties of the foods[25,26]. Hence, use of essential oil and nisin in combinations could become an alternative approach to synthetic bactericides for using in food industry. Previously synergistic effects of the nisin in combinations with various essential oils have been confirmed and it has been found that the activity of the essential oils or their constituents can be enhanced by the use of bacteriocin while using in combination[9-11].

As demonstrated previously, the reaction between nisin and the cell membrane of *L. monocytogenes* was caused by hydrophobic interaction between the amino acid residues of nisin and the fatty acids of the membrane phospholipids[27]. Later on it was confirmed that the charged membrane phospholipids are involved in the anti-listerial effect[28]. Moreover, it was reported that the phospholipids present in 2% fat market milk that was pasteurized and homogenized appeared to bind a large portion of the added nisin, resulting in a reduced nisin available to react with the cell membrane of *L. monocytogenes*, thereby reducing the anti-listerial activity[24]. This was not the case in skim milk, where similar nisin concentrations were sufficient to cause disruption of the listerial cell membrane. In this study, it was found that *M. glyptostroboides* leaf essential oil and nisin synergistically and significantly inhibited the growth of foodborne pathogen *L. monoctogenes* ATCC 19116 strain in all categories of milk samples.

In conclusion, the results of this study showed that synergistic combinations of *M. glyptostroboides* leaf essential oil along with nisin have significant anti-listerial efficacy in the tested milk samples. Hence, it is concluded that the leaf essential oil of *M. glyptostroboides* in combination with nisin can be used as an alternative approach of natural food preservative in food industry to inhibit the growth of food spoilage and/or foodborne pathogens which severely cause food deterioration and affect food qualities.

Conflict of interest statement

We declare that we have no conflict of interest.

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