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Antibacterial mode of action of seed essential oil of *Eleutherococcus senticosus* against foodborne pathogens

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Summary This study investigated the antibacterial mechanism of action of the seed essential oil of *Eleutherococcus senticosus* (ESEO) against foodborne pathogenic bacteria. Preliminarily, the ESEO (1000 μg $disc^{-1}$) showed potential antibacterial effect as diameter of inhibition zones (12.0 ± 0.2–37.0 ± 2.0 mm) against the tested foodborne pathogens. The MIC and MBC values of ESEO against the tested bacteria were found in the range of 125–500 and 500–1000 μg mL^{-1}, respectively. At MIC concentration, the ESEO had potential inhibitory effect on the cell viability of the tested pathogens. In addition, SEM analysis showed the inhibitory effect of ESEO as confirmed by considerable morphological alterations on the cell wall of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889. Moreover, the ESEO revealed its mode of action against foodborne pathogens on membrane integrity as confirmed by release of extracellular ATP, 260-nm absorbing materials and leakage of potassium ions. These findings confirm that the ESEO can be used as a potential antibacterial agent in food industry to inhibit the growth of various foodborne pathogens.

Keywords Action mechanism, *Eleutherococcus senticosus*, essential oil, food safety, membrane integrity, surface characteristic.

Introduction

Reported outbreaks of foodborne illnesses involving fruits and vegetables have increased dramatically during the last decade. A broad spectrum of microbial pathogens can contaminate human foods and water supplies and cause illness after the organisms and their toxins are consumed (Tauxe, 2002). The most common bacteria causing foodborne illness are *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Listeria monocytogenes*, *Bacillus cereus* and others (Busani *et al*., 2006). The epidemiology of foodborne diseases is changing, and reports from different parts of the world indicate that the resistant strains of foodborne pathogens have emerged as public health problem (Slutsker *et al*., 1998). Foodborne diseases create a significant hazard to human health and the economy of individuals. Food safety is an essential concern of both consumers and the food industry, especially as the number of reported cases of foodborne infections continues to increase (Alzoreky & Nakahara, 2003).

In addition, increasing rate of resistance development for commonly used antibiotics have led to search for newer, more effective, affordable and easily available medicine. Also, greater consumer awareness and concern regarding synthetic chemical additives, which can be detrimental to human health, has also led researchers and food processors to look for natural food additives with a broad spectrum of antimicrobial activity (Marino *et al*., 2001; Burt, 2004). In this context, plant essential oils are gaining more interest for their potential as preservative ingredients or decontaminating treatments, as they have found to be with 'generally recognised as safe (GRAS)' status by the Food and Drug Administration (FDA) and are considered as safer food additives by the European Union (Marino *et al*., 2001).

Essential oils are fairly complex mixtures, which are well known to possess varying degree of antimicrobial effects (Bajpai *et al*., 2013). They generally show selective toxicity towards various pathogens and are relatively safe both to animals and humans. In complex mixtures, synergism of individual components is also expected so that micro-organisms hardly can develop resistance towards essential oils. Moreover, plant-based essential oils have been long used as flavouring agents or preservatives in foods, beverages and confectionary products (Marino *et al*., 2001). Recently, some researchers have reported the efficacy of plant essential oils as antimicrobial agents against foodborne pathogens and

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spoilage microflora in food system (Carraminana *et al*., 2008). Thus, the application of plant-based essential oil for the control of foodborne pathogens and food spoilage bacteria requires the evaluation of efficacy within food products or in model systems that closely simulate food composition.

In general, studies on the mechanism of action of essential oils have used a common methodology that attempts to illustrate deleterious effects on cellular membranes (Denyer *et al*., 1991; Cox *et al*., 1998; Helander *et al*., 1998). However, different antibiotics or antimicrobials have different modes of action, owing to the nature of their structure and degree of affinity to certain target sites within bacterial cells. Cell wall structure is critical for the life and survival of bacterial species. A drug that targets cell walls can therefore selectively kill or inhibit bacterial organisms. Cell membranes are important barriers that segregate and regulate the intra- and extracellular flow of substances. A disruption or damage to this structure could result in leakage of important solutes essential for the cell's survival (Denyer *et al*., 1991; Cox *et al*., 1998; Helander *et al*., 1998). On the other hand, enzymes and cellular structures are primarily made of proteins. Protein synthesis is an essential process necessary for the multiplication and survival of all bacterial cells. Several types of antibacterial agents target bacterial protein synthesis by binding the subunits of the intracellular ribosomes. This activity then results in the disruption of the normal cellular metabolism of the bacteria and consequently leads to the death of the organism or the inhibition of its growth and multiplication (Denyer *et al*., 1991; Cox *et al*., 1998; Helander *et al*., 1998). Besides, DNA and RNA are keys to the replication of all living forms, including bacteria. Some antimicrobials work by binding to components involved in the process of DNA or RNA synthesis, which causes interference of the normal cellular processes that will ultimately compromise bacterial multiplication and survival (Denyer *et al*., 1991; Cox *et al*., 1998; Helander *et al*., 1998).

Eleutherococcus senticosus Rupr. (Araliaceae) is a woody medicinal plant, geographically distributed in North-East Asia (Umeyama *et al*., 1992). The cortical root and stem tissues of *E. senticosus* are used as tonic and sedative as well as to treat rheumatism and diabetes (Umeyama *et al*., 1992; Davydov & Krikorian, 2000). Noroleanane-type and oleanane-type saponins isolated from *E. senticosus* have been denoted as ciwujianosides, which possess important pharmacological activities, including hypoglycaemia and reducing acute myocardial infarct size (Sui *et al*., 1994). Saponins derived from *E. senticosus* have been found to exhibit a wide range of structural diversity and biological activity and have been shown to serve potential role in chemical defences against pathogens and herbivores (Davydov & Krikorian, 2000).

Previously, we reported the chemical composition analysis and antioxidant efficacy of *E. senticosus* seed essential oil (Bajpai *et al*., 2013). However, the antimicrobial activity of *E. senticosus* seed essential oil (ESEO) has not been explored so far. Therefore, this study was undertaken to investigate the effectiveness of ESEO on survival and growth of selected foodborne pathogens using *in vitro* models. Furthermore, antibacterial mode of action was investigated by determining the release of extracellular ATP, potassium ions and cellular materials as well as changes in membrane characteristics were confirmed using scanning electron microscopy.

Results

Chemical Composition of ESEO

Previously, we reported the GC-MS chemical composition analysis of the ESEO which led to the identification of 21 different components (Bajpai *et al*., 2013). The major components detected in ESEO were found to be ethylpthalate (31.73%), D-mannitol (20.06%), germane (9.37%), sulfamide (7.38%), boric acid (7.20%), didesethylflurazepam dehydration product (3.97%), 2-cyclohexen-1-one (3.77%), levomenol (1.75%), torreyol (1.33%), 2-(3-Indolyl)-5-aminopyrido [2, 3-d] pyrimidine (1.11%) and T-cadinol (0.67%) (Bajpai *et al*., 2013).

Antibacterial activity

The *in vitro* antibacterial activity of ESEO against the tested foodborne pathogenic bacteria was qualitatively and quantitatively assessed by the presence or absence of diameters of inhibition zones. Data obtained from the disc diffusion method indicate that the ESEO (1000 μg $disc^{-1}$) displayed a variable degree of antimicrobial activity against the tested foodborne pathogenic bacteria (Table S1). In this assay, *B. cereus* ATCC 13061, *S. aureus* ATCC 12600 and *L. monocytogenes* ATCC 7644 were found to be the most inhibited bacterial pathogens by the ESEO with their respective diameters of inhibition zones of 37.0 ± 2.0, 37.0 ± 0.4, 36.0 ± 0.5 mm, respectively, whereas *S.* Typhimurium ATCC 43174 and *E. coli* O157:H7 ATCC 43889 were inhibited moderately with diameters of inhibition zones of 12.0 ± 0.4 and 12.0 ± 0.2 mm, respectively (Table S1). In this assay, ESEO exhibited significantly higher antibacterial effect against Gram-positive bacteria, whereas almost similar antibacterial effect of ESEO was observed against Gram-bacteria bacteria as did by the standard compound tetracycline at the used concentration. The diameters of inhibition zones of ESEO against Gram-positive bacteria were found to be higher than Gram-negative bacteria (Table S1). The DMSO,

as a negative control, had no inhibitory effect at the used concentration.

In recent years, several researchers have reported that monoterpene or sesquiterpene hydrocarbons and their oxygenated derivatives, which are the major components of essential oils, exhibit potential antimicrobial activity (Cakir *et al*., 2004). These findings strongly support the outcomes of this study as the ESEO was also found to contain oxygenated sesquiterpenes and their respective hydrocarbons, representing 4.3% of total essential oil, confirming its efficacy as natural antimicrobial agent. Previously, we reported a detailed chemical composition analysis of ESEO, and it was found that ESEO contained phenolic compounds (phenol), aromatic acid (boric acid), oxygenated sesquiterpenes (torreyol, T-cadinol, levomenol, guaiazolene) and polyols (D-mannitol), which exert potent antimicrobial and antioxidant activities (Randrianarivelo *et al*., 2009; Bajpai *et al*., 2013).

Determination of MIC and MBC

The results of the antibacterial screening including the results from MIC and MBC values illustrated that ESEO had strong and consistent inhibitory effect against the tested foodborne pathogens. In this assay, the ESEO showed potent inhibitory effect as MIC and MBC values against all the tested foodborne pathogenic bacteria. As shown in Table S1, the MIC and MBC values of ESEO against the tested Gram-positive bacteria, *B. cereus* ATCC 13061, *L. monocytogens* ATCC 7644 and *S. aureus* ATCC 12600, were found in the range of 125–500 μg mL^{-1}, while for Gram-negative bacteria such as *S.* Typhimurium ATCC 43174 and *E. coli* O157:H7 ATCC 43889, the MIC and MBC values were ranged from 500 to 1000 μg mL^{-1}. Among the bacteria, *B. cereus* was found to be the most sensitive bacterial pathogen to the ESEO with MIC and MBC values of 125 and 500 μg mL^{-1}, respectively. Although both Gram-positive and Gram-negative bacteria were found susceptible to ESEO in this study, *S.* Typhimurium ATCC 43174 and *E. coli* O157:H7 ATCC 43889 showed less susceptibility to ESEO. Previously, several studies have confirmed the antibacterial efficacy of various essential oil as MIC and MBC values against different foodborne pathogens (Bajpai *et al*., 2009, 2012).

Effect of ESEO on viable counts

Based on the sensitivity of the test foodborne pathogens, one Gram-positive (*B. cereus* ATCC 1306) and a Gram-negative (*E. coli* O157:H7 ATCC 43889) bacteria were selected as the model organisms for further studies to confirm the antibacterial mode of action of ESEO. In this regard, further study was carried out to evaluate the effect of ESEO on the viable counts of the selected bacteria such as *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889. At MIC concentration, the effect of ESEO on the growth of tested bacterial pathogens demonstrated reduced viability (Fig. S1). The exposure of 0–120 min of ESEO did not cause severe decline on the inhibition of cell viability of the tested pathogens; however, considerable amount of inhibitory effect was observed on the inhibition of the cell viability of the tested bacteria of *E. coli* O157:H7 ATCC 43889 and *B. cereus* ATCC 13061 at the exposure time of 160 min. Interestingly, the exposure of the ESEO for 200 min revealed complete inhibition of CFU numbers against *B. cereus* ATCC 13061 (Fig. S1a) and of *E. coli* O157:H7 ATCC 43889 (Fig. S1b), and no CFU formation was observed.

The results from viable count assay revealed that exposure of ESEO had a rigorous effect on the cell viability of the tested foodborne bacterial pathogens. The ESEO exerted its maximum bactericidal activity as evident by the significant reduction in microbial counts and complete inhibition of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cell viable counts at 200-min exposure. Previously, the inhibitory effects of various plant-based essential oils on the cell viability of various foodborne pathogenic bacteria were reported by several co-workers (Bajpai *et al*., 2008, 2009, 2012).

SEM analysis

Physical and morphological alterations may encounter the cell wall surface deterioration of bacterial pathogens when treated with a suitable antimicrobial agent (Kockro et al., 2000). Hence, an SEM analysis was carried out to further visualise the effect of ESEO on the morphology of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells as compared with control group (Fig. S2). Control cells of the test foodborne pathogens in the absence of ESEO showed a regular and smooth surface (Fig. S2a,d). In contrast, *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells treated with ESEO at MIC concentrations (125 and 500 μg mL^{-1}, respectively) revealed severe damaging effect on the cell morphology of the tested pathogens, showing disruption of cell membrane and swelling of the cells (Fig. S2b,e). Moreover, initial exposure of ESEO to the tested foodborne pathogenic bacteria revealed large surface collapse and abnormal cell breaking, as well as complete lysis or dead cell formation (Fig. S2c,f).

The ESEO revealed its inhibitory effect as confirmed by the severe morphological alterations on the cell wall of the tested foodborne pathogens leading to disruption and lysed cell formation. Such types of physical and morphological alterations to the pathogens treated

with various essential oil were reported previously (Zhu *et al*., 2005). Also, the morphological observations made here are quite different from those reported previously where the treatment of *B. cereus* with resveratrol (200 mg mL^{-1}) leads to the change in the cells, from the typical long rod shape to short rod shape (Paulo *et al*., 2010). This could be explained on the basis of different mechanism of actions of the two antimicrobial compounds as resveratrol tends to stop the cell division and affects the bacterial cell cycle while ESEO may induce bactericidal effect through membrane damage. It is also suggested that the active components of the essential oil might bind to the cell surface caused deformity like holes to the cell surface and then penetrate to the target sites possibly the cytoplasmic membrane and membrane-bound enzymes (Zhu *et al*., 2005).

Measurement of extracellular ATP concentration

Destabilisation of the cytoplasmic membrane and/or leakage of ions are expected to have an impact on the membrane-associated energy-transducing system (Lee et al., 2002). Hence, the effect of ESEO on the extracellular ATP concentrations in *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells was studied. The extracellular ATP concentration in the untreated *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells (controls) was noted to be 0.94 and 0.20 picogram mL^{-1}, respectively (Fig. S3). *E. coli* O157:H7 ATCC 43889 and *B. cereus* ATCC 13061 cells treated with ESEO at MIC concentration (125 and 500 μg mL^{-1}, respectively) showed significant ($P < 0.05$) increase in extracellular ATP concentration. At MIC concentration of ESEO, extracellular ATP concentrations for *B. cereus* ATCC 13061 (Fig. S3a) and *E. coli* O157:H7 ATCC 43889 (Fig. S3b) cells were measured to be as 6.90 and 10.16 picogram mL^{-1}, respectively (Fig. S3). These results showed that ESEO had potential inhibitory effect on *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells, which have been proved by the increased release in extracellular ATP concentration. This might be occurred due to the consequences of cell wall damage, induced by ESEO.

Membrane perturbation, decrease in respiration and loss of K^+ and H^+ ion gradients suggest that energy generation within the cells would be detrimentally affected. ATP can be used to determine the amount of viable microbial cells present and quantified using a bioluminescence assay comprised of the enzyme luciferase from *Photinus pyralis*, which has got a high sensitivity to ATP and D-luciferin, the enzyme's substrate. Luciferin is converted into oxyluciferin in an ATP, Mg^{2+} and oxygen-dependent reaction, which generates yellow-green light. Hence, the amount of emitted light is directly proportional to the ATP content (Han *et al*., 2011), a linear function of the number of living cells in the suspension. Burt (2004) has reported that the level of ATP within the cell decreased, while there was no proportional increase outside the cell after *B. cereus* cell was exposed to monoterpene alcohols. Therefore, it is likely that the rate of ATP synthesis decreased or the rate of hydrolysis increased. Conversely, Helander *et al*. (1998) reported that essential oil components resulted in a decrease in the intracellular ATP pool of *B. subtilis* ATCC 6633, while inducing an increase of extracellular ATP pool on the cytoplasm membrane. Similarly, our results showed an increase in extracellular ATP concentrations when *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells were treated with ESEO. Interactions between both lipids and membrane-embedded proteins with the essential oil components result in the destabilising of the membrane and loss of integrity. Because we observed that the membrane structure of the bacteria was significantly impaired by ESEO, which caused the intracellular ATP leakage through imperfect membrane as an explanation of lethality. Moreover, the fact that internal ATP may be strongly reduced in the presence of weak ATP efflux suggests that it is hydrolysed inside sensitive cells. This effect has been also reported for several antibacterial agents (Herranz *et al*., 2001).

Leakage of potassium ions

Further antibacterial mode of action of ESEO against the tested foodborne pathogens was confirmed using the assay for leakage of potassium ions from the treated cells of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 at MIC concentration (125 and 500 μg mL^{-1}, respectively) (Fig. S4). In this assay, the leakage of potassium ions from the bacterial cells occurred immediately after the addition of ESEO following a sturdy loss along the specified intervals (Fig. S4a,b). However, no leakage of potassium ion was observed in control cells of the tested bacterial pathogens of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 during the study. It was found that potassium ion efflux of Gram-positive bacterium was higher than Gram-negative bacterium.

Potassium is the major intracellular cation in bacteria, which acts as cytoplasmic signalling molecule, activating and/or inducing enzymes and transport systems that allow the cell to adapt to elevated osmolarity. The effect of monoterpene alcohol on proton motive force of bacteria has been strongly correlated with leakage of various substances, such as ions, ATP, nucleic acids and amino acids (Helander *et al*., 1998). Maintaining homoeostasis is vital to maintain the energy status of the cells as well as membrane-coupled and energy-dependent processes such as solute

transport, regulation of metabolism, control of turgor pressure and motility (Cox *et al*., 2001). Therefore, even minor changes to the structural integrity of cell membrane can adversely affect the synthesis of macromolecules. These results are supported by the observation that terpenes alter cell permeability causing changes in membrane properties and functions by increasing membrane fluidity and altering membrane permeability (Denyer, 1990). Leakage of intracellular material is a general phenomenon induced by many antimicrobial substances results in cell death (Denyer, 1990).

Release of 260-nm absorbing materials

Another strategy for determining the mode of action of ESEO against Gram-positive and Gram-negative bacteria of foodborne origin was performed on the basis of release of 260-nm absorbing materials from the treated cells of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 (Carson et al., 2002). The optical density (OD_{260}) of the culture filtrates of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells exposed to ESEO at MIC concentration (125 and 500 μg mL^{-1}, respectively) revealed an increasing release of 260-nm absorbing materials with respect to exposure time (Fig. S5). However, no changes in the optical density of untreated (control) cells of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 were observed during the study. After 60 min of treatment, approximately more than twofold increase in the optical density of the bacterial cell culture filtrates of *B. cereus* ATCC 13061 (Fig. S5a) and *E. coli* O157:H7 ATCC 43889 (Fig. S5b) treated with ESEO was observed (Fig. S5). This directly indicates the confirmation of leakage of 260-nm absorbing materials from the bacterial cells treated with ESEO.

Measurement of specific cell leakage markers such as 260-nm absorbing materials is an indicative of membrane sensitivity to specific antimicrobial agent in relationship to unexposed cells. Exposure of *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells to ESEO caused rapid loss of 260-nm absorbing materials (nucleic acids, ions and some metabolites) and release of free potassium ions. These findings assume that the accumulation of the essential oil components in the cytoplasm membrane causing instant loss of their integrity and become increasingly more permeable to protons and ions that might be responsible for the establishment of the antibacterial activity. In addition, the changes in membrane permeability induced by ESEO may deplete the proton motive force (PMF) of sensitive cells, which allows the efflux of 260-nm absorbing materials, resulting in a collapse of membrane potential and gradient pH (Cox *et al*., 1998). Moreover, the observation that the amount of loss of 260-nm absorbing materials was as extensive as the leakage of potassium ions might indicate that the membrane structural damage sustained by *B. cereus* ATCC 13061 and *E. coli* O157:H7 ATCC 43889 cells resulted in release of macromolecular cytosolic constituents (Cox *et al*., 2001).

Conclusions

The results of this study indicate that the *E. senticosus* seed essential oil (ESEO) is able to disrupt membrane functions of both Gram-positive and Gram-negative bacteria, exerting its inhibitory effect through permeabilisation of the cell membrane associated with generalised membrane-disrupting effects. This corresponds to a simultaneous reduction in the number of viable bacteria, loss of 260-nm absorbing materials and leakage of potassium ions with decreased pool of extracellular ATP being indicative of loss of membrane integrity. Moreover, the SEM observation also supports the above hypothesis and strongly indicates the membrane activity of ESEO. The results obtained in this study confirm the possible use of biologically active ESEO to control foodborne pathogens. Thus, recent findings strengthen the suggestions that ESEO and/or *E. senticosus*-derived antibacterials might act against broad spectrum of bacteria for their practical applications in the food industry. However, further studies regarding safety, toxicology, combined use with traditional medicines and legislation are warranted.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effect of the seed essential oil of *Eleutherococcus senticosus* on the viability of the tested food-borne pathogenic bacteria of *B. cereus* ATCC 13061 (a) and *E. coli* O157:H7 ATCC 43889 (b).

Figure S2. Scanning electron microscopy of *B. cereus* ATCC 13061 (a, b, c) and *E. coli* O157:H7 ATCC 43889 (d, e, f) treated with seed essential oil of *Eleutherococcus senticosus*.

Figure S3. Effect of the seed essential oil of *Eleutherococcus senticosus* on extracellular ATP concentration of *B. cereus* ATCC 13061 (a) and *E. coli* O157:H7 ATCC 43889 (b).

Figure S4. Effect of the seed essential oil of *Eleutherococcus senticosus* on the leakage of potassium ions from the tested food-borne pathogenic bacteria of *B. cereus* ATCC 13061 (a) and *E. coli* O157:H7 ATCC 43889 (b). CT: control without treatment.

Figure S5. Effect of the seed essential oil of *Eleutherococcus senticosus* on the release rate of 260-nm absorbing material from *B. cereus* ATCC 13061 (a) and *E. coli* O157:H7 ATCC 43889 (b). Data are expressed as mean ± SD (n = 3).

Table S1. Antibacterial activity of the seed essential oil of *Eleutherococcus senticosus* against food-borne pathogens.