Antibacterial Mode of Action of the Essential Oil Obtained from *Chamaecyparis obtusa* Sawdust on the Membrane Integrity of Selected Foodborne Pathogens

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Summary

The present study examines the possible antibacterial mechanism of action of the essential oil obtained from *Chamaecyparis obtusa* (COEO) sawdust against foodborne pathogenic bacteria. The COEO was obtained by microwave-assisted hydrodistillation of *C. obtusa* sawdust. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of COEO against the tested foodborne pathogens including *Bacillus cereus* ATCC 13061, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 12600, *Salmonella* Typhimurium ATCC 43174 and *Escherichia coli* ATCC 43889 were found in the range from 62.5 to 500 µg/mL and from 125 to 1000 µg/mL, respectively. At the MIC concentrations, the COEO had potential inhibitory effect on the cell viability of the tested bacteria. In addition, the scanning electron microscopic analysis confirmed the inhibitory effect of COEO by revealing significant morphological alterations or rupture of the cell membranes of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889. Moreover, the mode of action of COEO on the cell membrane of both Gram-positive *B. cereus* ATCC 13061 and Gram-negative *E. coli* ATCC 43889 bacteria was confirmed by marked release of extracellular adenosine 5′-triphosphate (ATP) and cellular material that absorbs at 260 nm, and by efflux of potassium ions. These findings suggest that COEO holds a broad-spectrum antibacterial efficacy, confirming its influence on the membrane integrity and morphological characteristics of tested foodborne pathogens.

Key words: Chamaecyparis obtusa, sawdust, essential oil, antibacterial activity, foodborne pathogens

Introduction

Food safety is a known problem worldwide, affecting millions of people who suffer from the consequences of chemical and microbial spoilage of food. World Health Organization (WHO) characterizes this issue as one of the most prevalent health problems and a major cause of the reduction in economic output (*1*). Furthermore, Centers for Disease Control and Prevention (CDC) have reported that acute foodborne diseases cost a developed country about $52 billion per year in healthcare, work-place and other economic losses (*2*). Undoubtedly, changes in the standard of living and the consequent changes in dietary habits have increased the demand for ready--to-eat products. Moreover, cold distribution of unpreserved food can help, but it cannot guarantee the overall safety and quality of the product. In addition, expansion of the industry and trade of fruit and vegetables has been followed by increasing reports of foodborne pathogens because of their presence in raw materials (*3*).

Frequent outbursts of foodborne diseases have raised the demand for preservation systems that limit the pro-

liferation of foodborne pathogens in processed foods. Currently, many food additives of chemical origin, such as benzoic and sorbic acids, are used in the food industry. Although these synthetic preservatives are effective, they can be detrimental to human health, and consequently increasing number of consumers prefer to choose food products which are preservative-free or contain only trace amounts of preservatives (*4*). Historically, the plant-based essential oils have been used in food preservation, cosmetics, and in pharmaceuticals, as alternative medicines and natural therapies (*5*). Hence, it is worthwhile to scientifically explore these traditional medicinal plants to improve the quality of healthcare systems. Also, increasing consumers' demand for safe and effective natural products means that more research on plant-based essential oils is needed.

Many food products are perishable and require protection against spoilage during preparation, storage and distribution to achieve the desired shelf-life (*6*). Consumption of foods contaminated with foodborne pathogenic microorganisms is a threat to human health; the percentage of people that falls ill from foodborne diseases each year has been reported to reach rates of up to 30 % (*1*). The development of the food preservation process started from the need to extend the shelf-life whilst ensuring its safety and quality. Food preservation is a constant struggle against microorganisms that spoil foods and make their consumption unsafe. Conventional preservation techniques like control of pH, drying, freezing, heating, and adding antimicrobial compounds can be used to reduce the risk of food contamination, although these conservation techniques also cause changes of sensory and nutritional characteristics of food (*6*). Although there is a variety of conventional techniques available for conservation, food industry is investigating other techniques to replace them due to the high demand by consumers for nutritious, tasty and natural food. Greater consumer awareness and concern regarding synthetic preservatives, which have been used in foods for decades, and their negative health consequences, have led researchers and food process industry to look for natural, efficient and non-toxic food additives with a broad spectrum of antimicrobial activity, including the extracts and essential oils of plant origin (*6*).

Essential oils are mixtures of natural volatile organic compounds derived from the plant secondary metabolites, mainly terpenes and their oxygenated derivatives, as well as phenol-derived aromatic compounds and aliphatic hydrocarbons. Essential oils possess several biological activities including antibacterial, antiviral, antifungal, insecticidal, repellant, anti-inflammatory, spasmolytic and antioxidant properties (*6,7*). In addition, essential oils and their components are gaining increasing importance because of their Generally Recognized as Safe (GRAS) status, wide acceptance by consumers, safety for the environment and less chance for pathogens to develop resistance to chemical components, due to diverse modes of mechanisms of action.

Chamaecyparis obtusa, commonly known as hinoki, is a conifer in the cypress family, Cupressaceae, native to Northeast Asia. It is known to be rich in a variety of active pharmaceutical ingredients such as flavonoids and other essential components (*8,9*). The oils extracted from the leaves and twigs of *C. obtusa* have been commercially used in soap, toothpaste and cosmetics as functional additives due to their potent fragrance (*8*). The essential oil from *C. obtusa* leaves possesses a wide spectrum antimicrobial activity against various fungal pathogens and pathogenic bacteria, as well as allelopathic and insecticidal potentials (*10–12*).

Previously, we reported the chemical composition analysis, antioxidant and free radical scavenging efficacy of *Chamaecyparis obtusa* sawdust essential oil (COEO) (*13*). Although antimicrobial efficacy of various essential oils has been reviewed previously, to the best of our knowledge, no systematic research on the antibacterial mechanism of COEO against a wide range of foodborne microorganisms has been conducted so far. Therefore, this study was undertaken in order to investigate the effectiveness of COEO in the control of selected foodborne pathogens using *in vitro* models. Furthermore, antibacterial mechanism of action was investigated by determining the release of extracellular ATP, potassium ions and cellular materials as well as morphological alterations using scanning electron microscopic analysis.

Materials and Methods

Chemicals and instruments

The standard tetracycline and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (Rahway, NJ, USA). Spectrophotometric measurements were done by using a 96-well microplate reader (Infinite M200, Tecan, Mannedorf, Switzerland) and a luminometer (Synergy HT Multi-Mode Microplate Reader, BioTek, Gen5™, Winooski, VT, USA).

Extraction of essential oil from the sawdust of C. obtusa

The sawdust of *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. was purchased from a local timber company specialized in processing timber materials. The sawdust material was dried in shade at room temperature. The dried sawdust sample (200 g) was immersed in water (2 L) which was subjected to hydrodistillation using a microwave-assisted extraction apparatus for a period of 2 h. The microwave-assisted extraction apparatus was especially manufactured by the KMD Engineering (Yangju-si, Korea). The commercial microwave apparatus (*13*) was fitted with an automated thermo controller system with oven power capacity of 40 W, operating at the frequency of 15 THz. The distillate was collected and mixed with dichloromethane, shaken and kept in a separating funnel. The lower layer of dichloromethane containing the essential oil was collected and evaporated using rotary evaporator at room temperature to give essential oil. Eventually, the oil was dried using anhydrous sodium sulphate (Na_2SO_4) and preserved in a sealed vial at 4 °C for a week until tested and analyzed (*13*).

Gas chromatography-mass spectrometry analysis

A detailed chemical composition analysis of sawdust essential oil of *C. obtusa* was performed using a gas chro-

matography-mass spectrometry (GC-MS) system (Jeol JMS 700 mass spectrometer, Jeol Korea Ltd., Seoul, Korea) as described previously (*13*).

Microbial strains

The following foodborne pathogenic bacteria were used in this study: *Bacillus cereus* ATCC 13061, *Escherichia coli* ATCC 43889, *Listeria monocytogenes* ATCC 7644, *Salmonella* Typhimurium ATCC 43174 and *Staphylococcus aureus* ATCC 12600. The bacterial pathogens were obtained from the Korea Food and Drug Administration (KFDA) and maintained on nutrient agar medium at 4 °C.

Determination of minimum inhibitory and minimum bactericidal concentrations

The minimum inhibitory concentration (MIC) of the COEO was tested by a twofold broth macrodilution as per method reported previously (*14*). The COEO was first dissolved in dimethyl sulphoxide (DMSO) and incorporated into nutrient broth (NB) to obtain a bacterial pathogen concentration of 2000 µg/mL, and serially diluted to attain 1000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/mL, respectively. A standardized suspension of 10 µL of each tested bacteria (approx. 10^7 CFU/mL) was transferred to each tube. The control tubes contained only bacterial suspensions. All tubes were incubated at 37 °C for 24 h. The lowest concentration of COEO that did not show any visible growth of test organisms after macroscopic evaluation was determined as MIC, which was expressed in µg/mL. Further, the concentrations showing complete inhibition of visual growth of bacterial pathogens were identified, and 50 µL of each culture broth were transferred onto the agar plates and incubated for specified time at a determined temperature as mentioned above. The lowest concentration of the sample at which complete absence of the growth of bacterial colonies was observed was defined as the minimum bactericidal concentration (MBC). All experiments were conducted in triplicate.

Effect of COEO on cell viability of bacterial pathogens

Active cultures for viable count assay were prepared in NB and grown at 37 °C for 24 h. Simultaneously, stock bacterial suspensions to determine the number of viable counts were prepared by microbial spread plate count method so that the final suspension contained approx. 10^7 CFU/mL. For each strain, 1 mL of active stock solution (approx. 10^7 CFU/mL) was transferred to a 2-mL Eppendorf tube. The cultures were then centrifuged at 10 000×*g* for 10 min. The pellets were collected and resuspended in 1 mL of phosphate-buffered saline (PBS). For viable counts, each of the tubes containing resuspended bacterial suspension (approx. 10^7 CFU/mL) of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 was inoculated with 100 µL of COEO at MIC concentration in 900 µL of NB, and incubated at 37 °C. Samples for viable cell counts were taken out at 0, 40, 80, 120, 160 and 200 min. The viable cell counts were monitored as follows: after incubation, 100 µL of the resuspended

culture were diluted into 900 µL of PBS, thereby diluting it tenfold. A 100-µL bacterial suspension of each treatment was diluted and spread on the surface of nutrient agar. The plates were incubated at 37 °C for 24 h, and then the colonies were counted (*14*). The controls were inoculated without COEO for each bacterial strain under the same experimental conditions as mentioned above. Each assay in this experiment was performed in triplicate.

Scanning electron microscopic analysis

Scanning electron microscopic (SEM) observations were performed to determine the effect of COEO at MIC concentration on the morphology of the tested bacteria, *B. cereus* ATCC 13061 and *E. coli* ATCC 43889. Control samples were prepared without the COEO. To observe the morphological changes, the SEM protocol was modified from the method reported previously (*14,15*). The bacterial samples were washed gently with 50 mM phosphate buffer solution (pH=7.2), fixed with 100 mL of glutaraldehyde (2.5 %) and 100 mL of osmic acid solution (1 %). The specimen was dehydrated using sequential exposure to ethanol fractions ranging from 50 to 100 %, and then the ethanol was replaced by *t*-butanol. After dehydration, the specimen was dried with CO_2. Finally, the specimen was sputter-coated with gold in an ion coater for 2 min, followed by the examinations with a SEM analyzer (S-4300; Hitachi, Hitachi City, Japan).

Measurement of extracellular ATP concentration

The extracellular adenosine 5′-triphosphate (ATP) concentrations were measured in order to determine the effectiveness of COEO on membrane integrity (*16*). Stock bacterial suspensions for determining the number of viable counts were prepared by microbial spread plate count method so that the final suspension contained approx. 10^7 CFU/mL. Cells of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 were centrifuged for 10 min at 1000×*g* and the supernatants were removed. The cell pellets were spread and washed three times with 0.1 M of sodium phosphate buffer (pH=7.0), and then the cells were collected by centrifugation under the same conditions. A cell suspension (10^7 CFU/mL) was prepared with sodium phosphate buffer (0.1 M, pH=7.0) and 0.5 mL of cell solution was taken into an Eppendorf tube for the treatment with COEO at the MIC concentrations. Controls were tested without the essential oil. Samples were maintained at room temperature for 30 min, centrifuged for 5 min at 2000×*g*, and incubated on ice immediately to prevent ATP loss until measurement. The extracellular (upper layer) ATP concentrations were measured using an ATP bioluminescent assay kit (Sigma-Aldrich) which comprised ATP assay mix containing luciferase, luciferin, $MgSO_4$, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA) and tricine buffer salts. The ATP concentration in the supernatants, which represented the extracellular concentration, was determined using the luminometer after the addition of 100 µL of ATP assay mix to 100 µL of supernatant. The excitation and emission wavelengths were 520 and 420 nm, respectively, while excitation band pass and emis-

sion band pass were 1 and 2 nm, respectively (Synery HT luminometer, BioTek, Gen5™).

Measurement of the release of the cellular material that absorbs at 260 nm

The measurement of the release of the cellular material that absorbs at 260 nm from *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells was carried out in aliquots of 2 mL of the bacterial inocula in sterile peptone water (0.1 g per 100 mL) to which COEO was added at the MIC concentrations. The mixture was incubated at 37 °C. After 0, 30 and 60 min, the cells were centrifuged at 3500×*g*, and the absorbance of the obtained supernatant was measured at the microplate reader (*17*). Control flasks containing bacterial supernatant without COEO treatments were tested similarly (*17*). Results were expressed in terms of absorbance at 260 nm in each interval with respect to the ultimate time.

Assay of potassium ion efflux

Potassium ion efflux was determined according to a previously described method (*16*). The concentration of free K$^+$ ions in suspensions of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 was measured after the exposure of bacterial cells to the MIC concentrations of COEO in sterile peptone water (0.1 g per 100 mL) for 0, 30, 60 and 120 min. The mixture was incubated at 37 °C. At each pre-established interval, the extracellular potassium concentrations were measured by a photometric procedure using the Kalium/Potassium kit (Quantofix, GmbH, Wiesbaden, Germany). Control flasks without COEO were tested similarly. Results were expressed as the amount of extracellular free K$^+$ ions in the growth medium (mmol/L) in each interval of incubation.

Statistical analysis

Results were expressed as the mean values±standard deviations (S.D.) by measuring three independent replicates. Analysis of variance using one-way ANOVA followed by Duncan's test was performed to test the significance of differences between means obtained among the treatments at the 5 % level of significance using a SAS software (v. 9.2, SAS Institute Inc., Cary, NC, USA). Differences were considered significant at p<0.05.

Results

MIC and MBC concentrations

In this assay, the COEO showed potent inhibitory effect expressed in terms of MIC and MBC values against all the tested foodborne pathogenic bacteria. As presented in Table 1, the MIC and MBC values of COEO against the tested Gram-positive bacteria, including *B. cereus* ATCC 13061, *L. monocytogens* ATCC 7644 and *S. aureus* ATCC 12600 were found in the range from 62.5 to 500 μg/mL, while for Gram-negative bacteria such as *S.* Typhimurium ATCC 43174 and *E. coli* ATCC 43889, they ranged from 250 to 1000 μg/mL. In this assay, *B. cereus* ATCC 13061, *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 12600 were found extremely susceptible to the

Table 1. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Chamaecyparis obtusa* sawdust essential oil (COEO) against foodborne pathogens

Bacterial pathogen	COEO	
	MIC	MBC
	μg/mL	μg/mL
Bacillus cereus ATCC 13061	62.5	125
Listeria monocytogenes ATCC 7644	250	500
Staphylococcus aureus ATCC 12600	125	250
Salmonella Typhimurium ATCC 43174	250	500
Escherichia coli ATCC 43889	500	1000

COEO with MIC and MBC values of 62.5, 250 and 125 μg/mL and 125, 500 and 250 μg/mL, respectively. Although both Gram-positive and Gram-negative bacteria were found susceptible to COEO in this study, *S.* Typhimurium ATCC 43174 and *E. coli* ATCC 43889 showed relatively weaker susceptibility to COEO.

Cell viability

Based on the sensitivity of the tested foodborne pathogens, one Gram-positive (*B. cereus* ATCC 1306) and one Gram-negative (*E. coli* ATCC 43889) bacteria were selected as the model organisms for further studies to confirm the mechanism of antibacterial action of COEO at MIC concentration. In this regard, additional study was carried out to evaluate the effect of COEO on the viable counts of the selected bacteria. The effect of COEO on the growth of the tested bacterial pathogens demonstrated reduced viability at the used concentration (Fig. 1). In the case of Gram-positive *B. cereus* ATCC 13061 bacterium, exposure to COEO for 0 to 40 min did not cause severe decline in the inhibition of cell viability of the tested pathogens. Afterwards, a sharp decline in the viable count numbers was observed. On the other hand, 0 to 80-minute exposure to COEO had no significant effect on the inhibition of viable count numbers of Gram-negative bacterium *E. coli* ATCC 43889; however, a considerable inhibitory effect was observed in the inhibition of the cell viability of the test pathogen upon extended exposure to COEO. Eventually, the exposure to the COEO for 120 and 200 min caused complete inhibition of *B. cereus* ATCC 13061 (Fig. 1a) and *E. coli* ATCC 43889 (Fig. 1b), expressed in colony forming unit (CFU) per mL, respectively.

Morphological analysis by SEM

Physical and morphological alterations may happen on the cell surface of bacterial pathogens when treated with a suitable antimicrobial agent. Hence, a SEM analysis was carried out to further visualize the effect of COEO on the morphological and surface characteristics of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells as compared to the control group (Fig. 2). Control cells (not exposed to COEO) of the tested food spoilage and foodborne pathogens showed a regular and smooth surface, indicating the absence of morphological alterations (Figs. 2a and d). On the contrary, *B. cereus* ATCC 13061 and *E. coli*
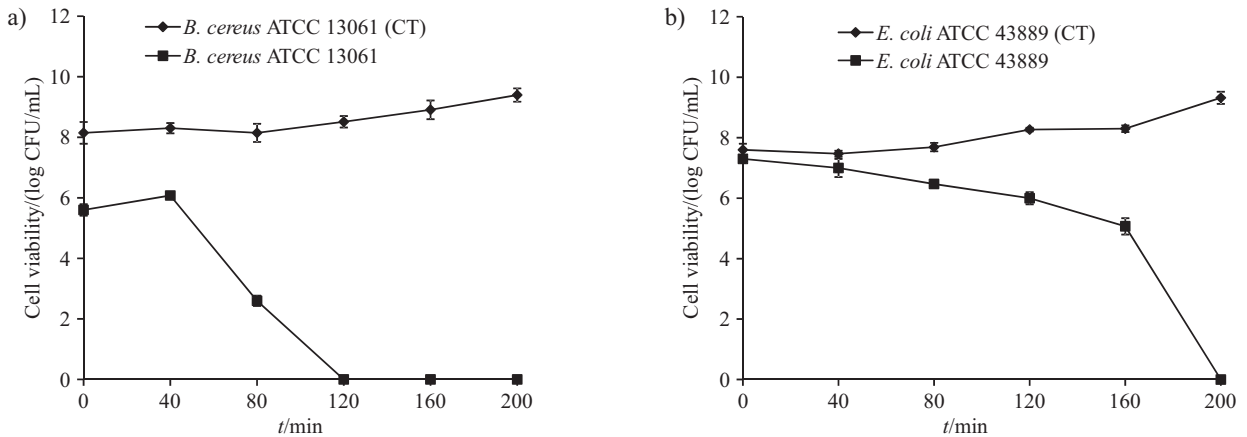
Fig. 1. Effect of *Chamaecyparis obtusa* sawdust essential oil at MIC concentration on the cell viability of the tested foodborne pathogenic bacteria: a) *B. cereus* ATCC 13061, and b) *E. coli* ATCC 43889; CT=control without treatment

ATCC 43889 cells treated with COEO at MIC concentrations (62.5 and 500 μg/mL, respectively) revealed severe detrimental effect of the COEO on the morphology of the tested pathogens, showing disruption of cell membrane and swelling of the cells (Figs. 2b and e). Moreover, initial exposure of the tested foodborne pathogenic bacteria to COEO revealed large surface collapse and abnormal cell breaking, as well as complete lysis or formation of dead cells (Figs. 2c and f).

Extracellular ATP concentration

Deterioration of the cytoplasmic membrane and/or leakage of ions are expected to have a significant impact on the membrane-associated energy-transduction system. Hence, the effect of COEO on the extracellular ATP concentrations in *B. cereus* ATCC 13061 and *E. coli* ATCC

43889 cells was studied. The extracellular ATP concentration in the untreated *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells (controls) was 0.81 and 0.29 pg/mL, respectively (Fig. 3). *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells treated with COEO at MIC concentration (62.5 and 500 μg/mL, respectively) showed significant ($p<0.05$) increase in the extracellular ATP concentration. At the MIC concentration of COEO, extracellular ATP concentrations in *B. cereus* ATCC 13061 (Fig. 3a) and *E. coli* ATCC 43889 (Fig. 3b) cells were 1.68 and 1.42 pg/mL, respectively (Fig. 3). These results showed that COEO had a potential inhibitory effect on *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells, which was proven by the increased release in the extracellular ATP concentration. It was confirmed that COEO had detrimental effect on the cell membrane integrity, resulting in the increased extracellular ATP concentration of the treated cells.
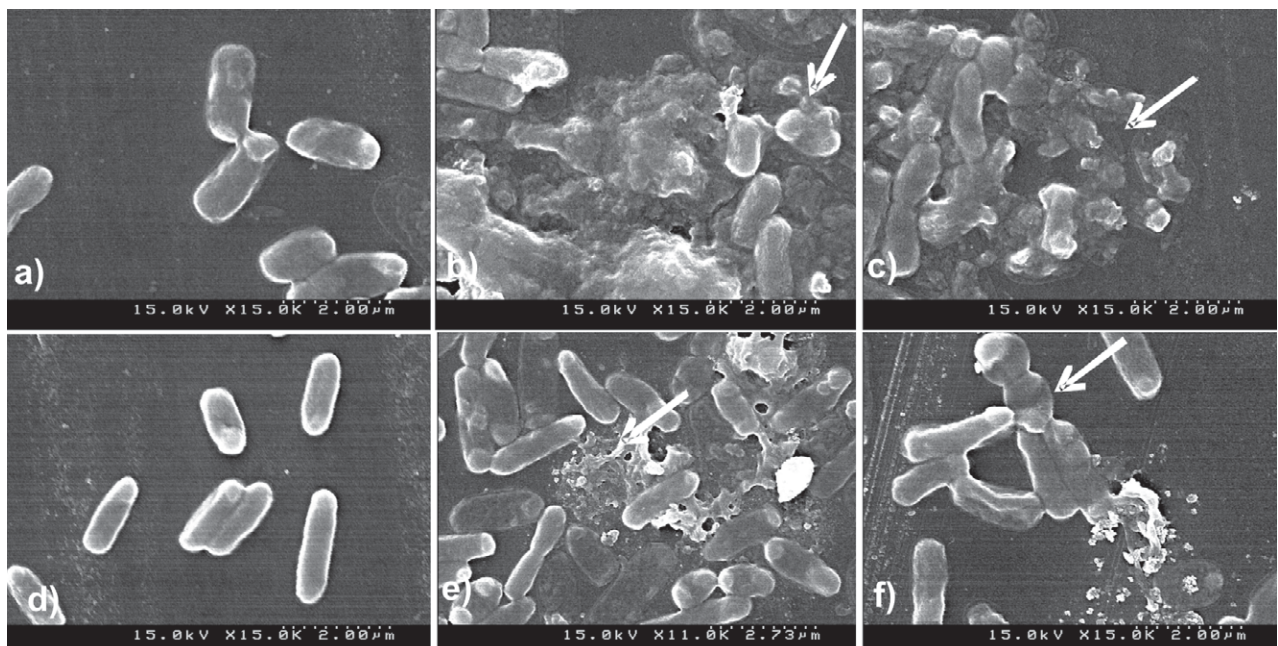

Fig. 2. Results of the scanning electron microscopy of *B. cereus* ATCC 13061 (a, b, c), and *E. coli* ATCC 43889 (d, e, f) treated with *Chamaecyparis obtusa* sawdust essential oil; a and d): controls, showing a regular and smooth surface; b and e): disruption and swelling of the cells; c and f): surface collapse or formation of lysed cells
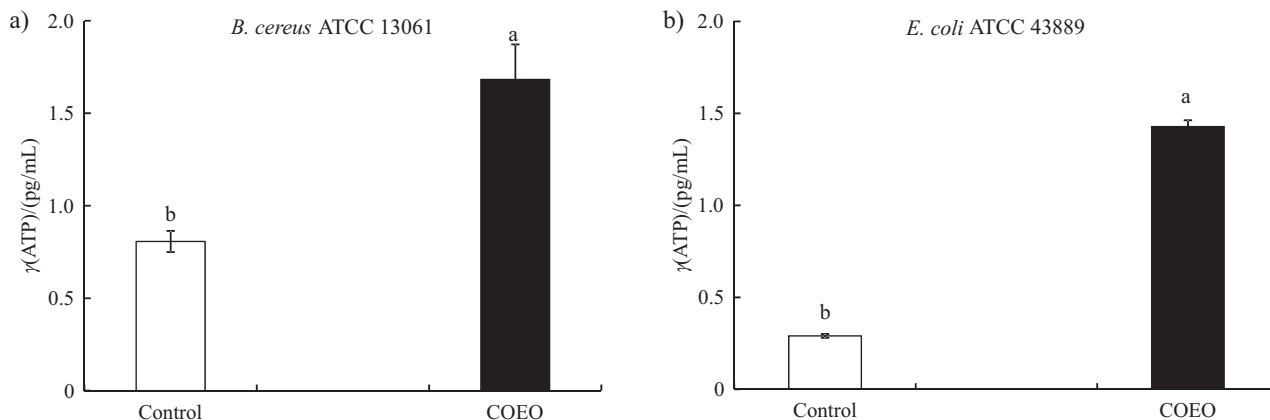
Fig. 3. Effect of *Chamaecyparis obtusa* sawdust essential oil at MIC concentration (62.5 and 500 µg/mL, respectively) on extracellular ATP concentration in: a) *B. cereus* ATCC 13061, and b) *E. coli* ATCC 43889. Data are expressed as mean values±standard deviations (S.D.), N=3. Values with different letters are significantly different (p<0.05)

Potassium ion efflux

The mechanism of antibacterial action of COEO against the tested foodborne pathogens was further confirmed using the assay for the release of K^+ ions from the treated cells of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 (Fig. 4). Since COEO is capable of membrane potential ($\Delta\Psi$) dissipation, it must conduct ion or proton movements across the membrane. Therefore, we investigated the ability of COEO to elicit transmembrane ion movement. In this study, treatment of bacterial cells with COEO at the MIC concentration induced a major efflux of intracellular K^+ ions, following a sturdy loss along the specified intervals (Figs. 4a and b). However, no K^+ ion efflux was observed in control cells of the tested bacterial pathogens of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 during the study.

Release of cellular material that absorbs at 260 nm

The leakage of cytoplasmic membrane was analyzed by determining the release of cellular materials including nucleic acids, metabolites and ions, which were absorbed at 260 nm into the bacterial suspensions. This strategy for determining the mechanism of antibacterial action of COEO was applied against foodborne and food spoilage bacterial pathogens *B. cereus* ATCC 13061 and *E. coli* ATCC 43889. The results of the release of cellular material at the absorbance value of 260 nm from *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells exposed to COEO at the MIC concentrations indicated that the higher exposure time led to higher cell leakage of nucleic acids, critical molecules and ions (Fig. 5). However, no changes in the absorbance of untreated cells (control) of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 were observed during the study. At 30 and 60 min of treatment, significant increase in the absorbance of the bacterial cell culture filtrates treated with COEO was observed (Figs. 5a and b). This directly confirms the leakage of the material absorbing at 260 nm from the bacterial cells treated with COEO.

Discussion

Some essential oils exert greater inhibitory effect against a broad range of pathogenic microorganisms. In this study, the Gram-positive bacteria were found to be
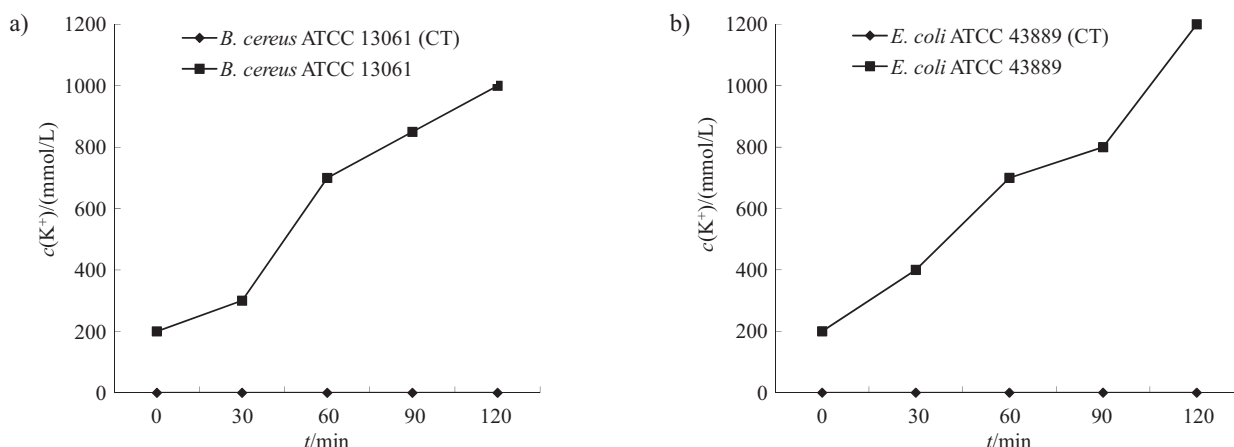

Fig. 4. Effect of *Chamaecyparis obtusa* sawdust essential oil at MIC concentration (62.5 and 500 µg/mL, respectively) on the leakage of potassium ions from the tested foodborne pathogenic bacteria: a) *B. cereus* ATCC 13061, and b) *E. coli* ATCC 43889. Data are expressed as mean values±standard deviations (S.D.), N=3; CT=control without treatment
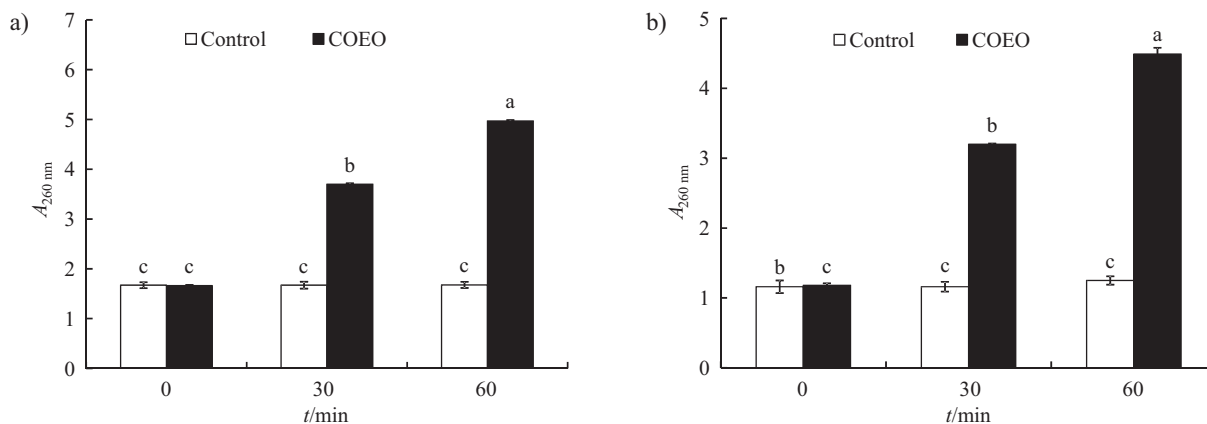
Fig. 5. Effect of *Chamaecyparis obtusa* sawdust essential oil at MIC concentration (62.5 and 500 µg/mL, respectively) on the release rate of material that absorbs at 260 nm from: a) *B. cereus* ATCC 13061, and b) *E. coli* ATCC 43889. Data are expressed as mean values±standard deviations (S.D.), N=3. Values with different letters are significantly different (p<0.05)

more susceptible to the COEO than the Gram-negative bacteria. The hydrophilic cell wall structure of Gram-negative bacteria is constituted essentially of lipopolysaccharides that block the penetration of hydrophobic components and prevent the accumulation of essential oils in target cell membrane (18). This membrane acts as an endotoxin, and protects the bacteria from several antibiotics, which would normally damage the inner membrane or cell wall (peptidoglycan). The outer membrane provides these bacteria with resistance to lysozyme and antibiotics. The single membrane of the Gram-positive bacteria is considerably more accessible to permeation by hydrophobic components of essential oil in the target sites. This might be the reason why Gram-positive bacteria were found to be more sensitive to the COEO than Gram-negative bacteria. Similar findings on the susceptibility of foodborne pathogenic bacteria, including Gram-positive and Gram-negative bacteria, to various essential oils have been reported (14,19,20).

In this study, the results of MIC and MBC assays showed that COEO had a strong and consistent inhibitory effect. However, COEO showed higher antibacterial effect against Gram-positive bacteria than against Gram-negative bacteria. Essential oils are volatile and odorous produts of plant secondary metabolism which have wide applications in food flavouring and preservation industries (19). 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 (7,20,21). These findings strongly support the outcomes of this study as the COEO was also found to contain oxygenated monoterpenes, sesquiterpenes and their respective hydrocarbons, confirming its efficacy as natural antimicrobial agent. Furthermore, the results of viable count assay revealed that exposure to COEO had a detrimental effect on the cell viability of the tested bacterial pathogens. The COEO 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* ATCC 43889 cell viable counts at 120 and 200 min of exposure, respectively. Previously we had confirmed the inhibitory

effects of various plant-based essential oils on the cell viability of foodborne pathogenic bacteria (14,19,20,22). Similar to our findings, other essential oils also exerted inhibitory effects against various foodborne pathogens (21).

The SEM analysis investigated physical and morphological alterations in the cell wall of the tested bacterial pathogens, *B. cereus* ATCC 13061 and *E. coli* ATCC 43889. The inhibitory effect of COEO was confirmed by the severe morphological alterations on the cell wall of the tested foodborne pathogens, leading to disruption and cell lysis. Such morphological alterations have been observed in various types of test organisms when treated with different essential oils (14,23). Also, the morphological changes observed in this study were different from those reported previously, where the treatment of *B. cereus* with resveratrol led to the change in the form of cells, from the typical long rod shape to short rods (24). This could be explained on the basis of different mechanisms of action of different antimicrobial compounds: resveratrol tends to stop the cell division and affect the bacterial cell cycle, while COEO may induce bactericidal effect through membrane damage, thereby resulting in the lysis of bacterial cell wall followed by the loss of intracellular dense material on the surface of the treated cells, as evident by the previous findings (25). Changes in membrane fluidity usually occur due to alterations in membrane lipid composition (26) and are thought to be a compensatory mechanism to counter the lipid disordering effects of the treatment agent. The literature suggests that the active components of the essential oil might bind to the cell surface and then penetrate to the target sites, possibly the cytoplasmic membrane and membrane-bound enzymes, resulting in the disruption of cell wall structure (14,23). 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 (27). Leakage of intracellular material is a general phenomenon induced by many antimicrobial substances, which results in cell death (27).

ATP is used for many cell functions including transport of substances across cell membranes, which might

be a potential parameter for understanding the mode of action of antimicrobial agents. In prokaryotes, the cell membrane takes care not only of the cell energy conversion needs, but also of nutrient processing, synthesizing structural macromolecules and secretion of the many enzymes needed for life. The results of our study of the extracellular ATP concentration showed an increasing rate of extracellular ATP concentrations after *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells were exposed to COEO at the MIC concentration. This may have occurred because of significant destruction in membrane permeability of the tested bacteria by the COEO, which caused the intracellular ATP leakage through defective cell membrane. Furthermore, reduction in intracellular ATP may also be due to decreased rate of ATP synthesis and increased rate of ATP hydrolysis inside the cells. Similar findings on this phenomenon have also been reported for various antibacterial agents (*28*). In addition, Burt (*6*) reported that exposure of *B. cereus* cells to some monoterpene alcohols resulted in decreased level of intracellular ATP and disproportionately increased level of extracellular ATP. Similarly, Helander *et al.* (*29*) found that the treatment of *B. subtilis* ATCC 6633 cells with essential oil components resulted in decreased level of intracellular ATP pool and increased level of extracellular ATP pool. In addition, the significant reduction in intracellular ATP can be due to the loss of inorganic phosphate through the highly compromised permeable cell membrane (*30,31*). Subsequently, the antimicrobial effect might be established by the inhibition of proton motive force, inhibition of mitochondrial respiratory and electron transfer chain, or the disruption of synthesis of DNA, RNA, proteins, lipids and polysaccharides. Leakage of intracellular material is a general phenomenon induced by many antimicrobial substances, resulting in cell death (*27,32*).

Moreover, the mechanism of antibacterial action of COEO was also confirmed on the basis of leakage of the K^+ ions from *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells when exposed to COEO at the MIC concentration. The internal ionic environment of bacterial cells is generally potassium rich, so leakage of this ion has been used to monitor membranolytic events (*33*). The bacterial cell membrane provides a permeability barrier to the passage of small ions such as potassium ions, which are the most prevalent cations in the cytoplasm, facilitating cell membrane functions, charge balancing and protein synthesis, and maintaining proper enzyme activity that allows the cell to adapt to elevated osmolarity (*33*). This impermeability to small ions is maintained and even regulated by the structural and chemical composition of the membrane itself. Increases in the leakage of K^+ ions indicate a disruption of this permeability barrier. Therefore, even relatively slight changes in the structural integrity of cell membranes can detrimentally affect cell metabolism and lead to cell death (*33*).

The bacterial membrane serves as a structural component which may become compromised during a biocidal challenge such as exposure to anionic, cationic or neutral biocides. Therefore, release of intracellular components is a good indicator of membrane integrity. Small ions such as potassium and phosphate tend to leach out first, followed by large molecules such as DNA, RNA, and other materials, when treated with a suitable antimicrobial. Since these nucleotides have strong UV absorption at 260 nm, they are described as '260-nm absorbing materials' and this method is widely used in determining membrane integrity parameters (*27,34*). In this study, exposure of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells to COEO caused rapid loss of materials that absorb at 260 nm (nucleic acids, ions and some metabolites) and release of free K^+ ions. These findings indicate that the accumulation of the essential oil components in the cytoplasm membrane caused instant loss of their integrity, which became increasingly 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 COEO may decrease or deplete the proton motive force of sensitive cells which depends on the membrane potential ($\Delta\Psi$) and the pH gradient (ΔpH) that are used to generate ATP *via* the membrane-bound ATPase (*35*). These gradients serve as the driving force for many vital energy-dependent cellular processes. Our data suggest that the COEO induces cell death by making sensitive cell membrane permeable, allowing for the efflux of materials that absorb at 260 nm such as K^+ ions, inorganic phosphate and ATP, resulting in a collapse of $\Delta\Psi$ and ΔpH (*36*). Moreover, the loss of materials that absorb at 260 nm was as extensive as the leakage of K^+ ions, which might indicate that the membrane structural damage sustained by *B. cereus* ATCC 13061 and *E. coli* ATCC 43889 cells resulted in the release of biomolecules (*33*). The effect of various essential oil components on the proton motive force of bacteria has been strongly correlated with leakage of various substances, such as ions, ATP, nucleic and amino acids (*29*). Maintaining homeostasis is vital to maintain the energy status of the cells as well as membrane-coupled and energy-dependent cellular processes such as solute transport, regulation of metabolism, maintenance of turgor pressure and motility (*33*). Therefore, even minor changes in the structural integrity of the cell membrane can adversely affect the synthesis of macromolecules. This suggests that, in the case of *B. cereus* ATCC 13061 and *E. coli* ATCC 43889, monitoring K^+ efflux and release of materials that absorb at 260 nm may be more sensitive indicators of disruption of membrane integrity (*33*).

In view of our data, it is proposed that the antibacterial action of COEO takes place *via* a two-step process. The first step involves the entry of the oil components into the cell membrane in order to initiate membrane disruption, while the second step is the accumulation of oil in the cell membrane, resulting in the inhibition of cell growth. This can be attributed to permeabilization and depolarization of the cytoplasmic membrane (*37*). Permeabilization of the membrane enhances the leakage of protons from cells, disrupts membrane electric potential, and reduces proton motive force and finally ATP synthesis. In addition, reduced membrane potential enhances the leakage of ions, ATP, amino acids and proteins from cells. These changes disturb the osmotic balance of the cell, making membrane-associated proteins inefficient, eventually leading to the inhibition of cell growth or cell death.

Conclusion

Briefly, this study showed that *Chamaecyparis obtusa* essential oil (COEO) possesses antibacterial efficacy against both Gram-positive and Gram-negative bacteria of food origin. The oil contains terpenes and phenolic (thymol) compounds as the most important phytoconstituents, which are well known for their antimicrobial potential. We conclude that COEO exerts its antibacterial effect through permeabilization of the cell membrane associated with generalized membrane-disrupting effects, and this corresponds to a simultaneous reduction in cell viability, loss of the materials that absorb at 260 nm, and potassium ion efflux with decreased pool of extracellular ATP being indicative of the loss of cell membrane integrity. Moreover, the SEM observation also supported the above hypothesis, and strongly indicated the membrane disrupting activity of COEO. This should lead to effective application of the COEO as a natural antimicrobial agent to control foodborne and food spoilage pathogens in food industry. The heterogeneous composition of COEO and the antimicrobial activities of many of its components indicate that many of them exert antibacterial action *via* several mechanisms. Further research regarding safety and toxicology is required to fully understand the mechanisms involved in order to justify the real applications of COEO in food industry as a natural antibacterial agent.

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