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Inactivation of foodborne pathogens using Sargent juniper-inspired sesquiterpenoid as synergistic phytochemicals

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파이토케미컬로서의 눈향나무 정유 기반
세스퀴터페노이드의 활용

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By Da-Song Lee

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지도교수 최 인 규

이 논문을 농학석사 학위논문으로 제출함
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위 원 장 이 학 래 (인)
부 위 원 장 최 인 규 (인)
위 원 곽 효 원 (인)

Abstract

Inactivation of foodborne pathogens using Sargent juniper-inspired sesquiterpenoid as synergistic phytochemicals

Da-Song Lee
Program in Environmental Materials Science
Department of Agriculture, Forestry, and Bioresources
The Graduate School
Seoul National University

With the emergence of antibiotic-resistant microbes (ARM), developing new antibiotics that exhibit a prompt response to varying foodborne pathogenic bacteria has become crucial. In recent years, phytochemicals derived from plant essential oils (EOs) have drawn attention as natural antimicrobials. Crude EOs have been suggested as excellent antimicrobial agents, although data on each phytochemical present in the EOs and their interactions are insufficient. Of the plant-derived active phytochemicals such as terpenoid and phenolics, some sesquiterpenoid are difficult to isolate, hindering the analysis of their antimicrobial activity and interaction with other phytochemicals.

This study aimed to identify the active sesquiterpenoid from new plant resources by using thin layer chromatography-direct bioassay (TLC-DB) and a multi-criteria analysis approach of preparative. The EOs were extracted from the leaves of two Sargent juniper varieties harvested at different altitudes in the Republic of Korea. The antibacterial activities of Sargent juniper EOs against

gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and gram-negative bacteria (*Salmonella typhimurium* and *Escherichia coli* O157:H7) were first screened using the disk diffusion method. The active Sargent juniper EOs were quantitatively analyzed by measuring the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The active phytochemicals in the EOs were screened by TLC assay, and the active fractions were collected using preparative TLC (prep. TLC). Scanning electron microscopy (SEM) revealed structural deformation of the bacterial cell wall after treatment with the active fractions. The antibacterial activity of each active compound and the combinational interactions between the active compounds were measured using the checkerboard and growth curve assay.

In this study, Sargent juniper EO containing sabinene, elemol, terpinen-4-ol, α-copaen-11-ol, and γ-terpinene exhibited higher antibacterial activities against the tested foodborne pathogens. In particular, antibacterial screening assay indicated that the active EO (NFSV EO) exhibited prominent growth inhibitory activity against the tested gram-positive bacteria, but not as much against the tested gram-negative bacteria.

The two dimensional (2D) TLC-DB-guided screening showed growth inhibition of gram-positive bacteria on the fractionated TLC plate, but not of gram-negative bacteria. SEM images showed the destruction of the bacterial cell wall of gram-positive bacteria in the inhibition zone by the active compounds. The results of 2D TLC-DB revealed that the three most abundant compounds—elemol, γ-eudesmol, and terpinen-4-ol—were fractionated and identified using GC-MS. The combinational effects of the three major compounds were investigated against the gram-positive bacteria. Elemol and γ-eudesmol exerted remarkable antibacterial activities against *L. monocytogenes* and *S. aureus*. These results indicated that elemol and γ-eudesmol might

directly cause bacterial growth inhibition, whereas terpinen-4-ol exhibited relatively low activity and indirectly affected bacterial growth.

According to the isobologram obtained using the checkboard assay, terpinen-4-ol applied with elemol and γ-eudesmol exhibited synergism, as the fractional inhibitory concentration index (FICI) of each combination was 0.375 and 0.25 against *L. monocytogenes*, respectively. In particular, the combination of elemol and terpinen-4-ol synergistically reduced the MIC against both *L. monocytogenes* and *S. aureus*.

The bacterial growth inhibition activity was boosted by adding a trace amount of elemol to EOs containing terpinen-4-ol, the monoterpenoid commonly available in nature. These combinations might be utilized as potential natural antibacterial agents in the food and beverage industry.

Keywords: Sargent juniper, sesquiterpenoid, foodborne pathogens, antibacterial activity, synergistic effect, elemol

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List of Abbreviations

ARMs = Antibiotic-resistant microbes

CAMHB = Cationic-adjusted Mueller Hinton broth

CF = Seoul National University Chilbosan Forest

DMSO = Dimethylsulfoxide

ELE = Elemol

γ-EUD = *γ*-Eudesmol

EO = Essential oil

FICI = Fractional inhibitory concentration index

GC-MS = Gas chromatography–Mass spectrometry

MAPs = Medicinal and aromatic plants

MBC = Minimal bactericidal concentration

MIC = Minimum inhibitory concentration

MHA = Mueller Hinton agar

NFSV = National Forest Seed Variety Center in Gangneung branch

Prep. TLC = Preparative thin layer chromatography

Rf = Retention factor

SEM = Scanning electron microscopy

TER = Terpinen-4-ol

TLC = Thin layer chromatography

TLC-DB = Thin layer chromatography–direct bioassay

1. Introduction

1.1. Microbial foodborne illness: Phenomena and responses

The top 10 health threats announced by World Health Organization (WHO) in 2019 include air pollution, non-infectious diseases, influenza epidemic, antimicrobial resistance, Ebola virus infection, and infections caused by high-risk pathogens (WHO, 2019). Besides, concerns about viral or microbial infections have become serious because of the recent viral pandemic. Millions of people die annually due to viral and bacterial infections, which accounts for 5% of the total deaths (Jeong *et al*., 2016). In particular, an estimated 1 in 10 people in the world—almost 600 million—is affected by the consumption of food contaminated with harmful bacteria, viruses, parasites, or chemicals (WHO, 2020). According to the Center for Disease Control (CDC), 31 known foodborne pathogens cause an estimated 48 million illnesses (CDC, 2018). Most (58%) foodborne illnesses are caused by norovirus, followed by *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* (Sadekuzzaman *et al*., 2018; Scallan *et al*., 2011). Therefore, the Hazard Analysis and Critical Control Point (HACCP), which is a system that regulates the production of microbiologically safe foods, has implemented sanitation standards and pathogen testing programs for several pathogenic bacteria.

With the emergence of these global regulations, the demand for the development of antibiotics against foodborne pathogens has increased. Foodborne illnesses due to bacterial infections can be treated with antibiotics that kill or interfere with the metabolism of the pathogens (Hashempour-Baltork *et al*., 2019). Various synthetic antibiotics, ampicillin, tetracycline,

chloramphenicol, streptomycin, and sulphonamides, have been used to inhibit the growth of pathogens (Tshipamba *et al.*, 2018). However, several studies have revealed the adverse effects of these synthetic antibiotics on animals and humans, such as bovine pneumonia, shipping fever, diarrhea, anaphylaxis, cardiotoxicity, and hepatotoxicity (Granowitz & Brown, 2008; McEwen & Fedorka-Cray, 2002). In addition to the adverse effects of synthetic antibiotics, another critical problem is that pathogens adapted to these synthetic antibiotics evolve into antibiotic-resistant microbes (ARMs).

Accordingly, anxiety over the negative perception of patients regarding synthetic antibiotics has motivated the pharmaceutical industry to develop new alternatives to existing antibiotics. The alternatives to synthetic antibiotics, i.e., natural antimicrobial substances, include animal-derived (lysozyme, lactoperoxidase, and serum), microorganisms-derived (nisin and pediocin), and plant-derived antimicrobials (essential oils, volatile compounds, phenolics, and phytoalexins; Merritt, 1998). In particular, antimicrobial phytochemicals derived from plant essential oils (EOs) have attracted attention in recent years as natural antimicrobials (Seow *et al*., 2014). Their increasing application in the food industry for fragrances or preservatives is also likely to promote their market growth (G.V.R., 2017). In some countries such as the Republic of Korea and Singapore, 86% and 76% of the population rely on natural active substances (primarily plant-based) owing to the adverse effects of synthetic antibiotics (Qi, 2013). More research on new EO-derived phytochemicals is required to understand the exact antimicrobial mechanism of EOs and determine their effective levels to counteract the constant emergence of ARMs.

1.2. Inactivation of foodborne pathogens by using active phytochemicals

Conventional synthetic antibiotics, including penicillins, streptovaricins, tetracyclines, polymyxins, and sulfonamides, etc., inhibit the synthesis of cell wall, nucleic acids, and proteins; change the cell membrane permeation; and interfere with folic acid metabolism (Fig. 1; Merritt, 1998). This disrupts the growth of the microorganisms or kills them. Natural antimicrobials, especially plant-based antimicrobial compounds, also have similar mechanisms. Several phytochemicals derived from plants EOs exhibit potent antimicrobial activity (Fig. 2).

Extractives from herbaceous plants rich in phenolics and terpenoid show selective antimicrobial activities against foodborne pathogens: thyme oil exerts inhibitory activity on *Clostridium perfringens* (Svoboda *et al*., 2005). In particular, EOs—volatile extractives among secondary metabolites from plants—have been reported to exhibit excellent activities against specific microorganisms. Thus far, EOs have been extracted from more than 3,000 plant species, and research on their physiological activity is being actively conducted (Kim *et al*., 2011). Because of their bactericidal, fungicidal, and medicinal properties, these EOs are used as food preservatives, pesticides, disinfectants, pharmaceutical products, and cosmetic additives (Bakkali *et al*., 2008). Oregano (*Origanum vulgare*), cinnamon (*Cinnamomum zeylanicum*), and tea tree EOs or extracts are generally recognized as antibacterial substances (Burt, 2004; Lin *et al*., 2005; Sadekuzzaman *et al.*, 2018; Seaberg *et al.*, 2003).

The main components of EOs are terpenes and terpenoid, which are synthesized in the cytosol and plastids, based on C5 isoprene units (Croteau *et al*., 2000). In this study, the volatile mixture of terpenes and terpenoid from plant resources is collectively referred to as terpenoid which are the main components of EOs. Terpenoid compounds are innate phytochemicals used as

antimicrobial chemicals in plant defense mechanisms against external microorganisms (Zwenger & Basu, 2008). The distinct functional groups of these terpenoid, such as aldehyde or ketone group, are closely related to their antimicrobial activity. A compound having an aldehyde or oxygenated terpene structure, as well as a mixture of such compounds, exhibits fairly high antimicrobial activity (de Barros *et al*., 2009; Dorman & Deans, 2000; Miladi *et al.,* 2017). Some compounds containing a ketone group or alkyl with the nonphenolic ring were reported to increase the antimicrobial properties of terpenoid (Naigre *et al*., 1996). For instance, menthone, which has a ketone group, exhibited modest activity against foodborne pathogens. Limonene, as well as *p*-cymene, was revealed to be an antimicrobial active monoterpenoid having an alkyl substituent (Fig. 2; Dorman & Deans, 2000). In addition, the antimicrobial activity of several sesquiterpenoid has also been investigated. Fractions including elemol and *β*-eudesmol, which are known as important sesquiterpene alcohols, showed moderate to high antimicrobial activities (Ayaz *et al*., 2017). Moreover, nerolidol, farnesol, bisabolol, and apritone were found to increase permeability and susceptibility to the bacterial cell wall and membrane (Brehm-Stecher & Johnson, 2003). As mentioned above, EOs with excellent physiological and antimicrobial activities have numerous industrial applications. However, the components of EOs extracted from the same species vary remarkably depending on their external environmental conditions. Because of this characteristic, several efforts and refining processes are required to retain the quality of EOs in the industry. Fundamentally, physiological data and optimum combinations of each chemical constituent of EOs are demanded extending their industrial applications.

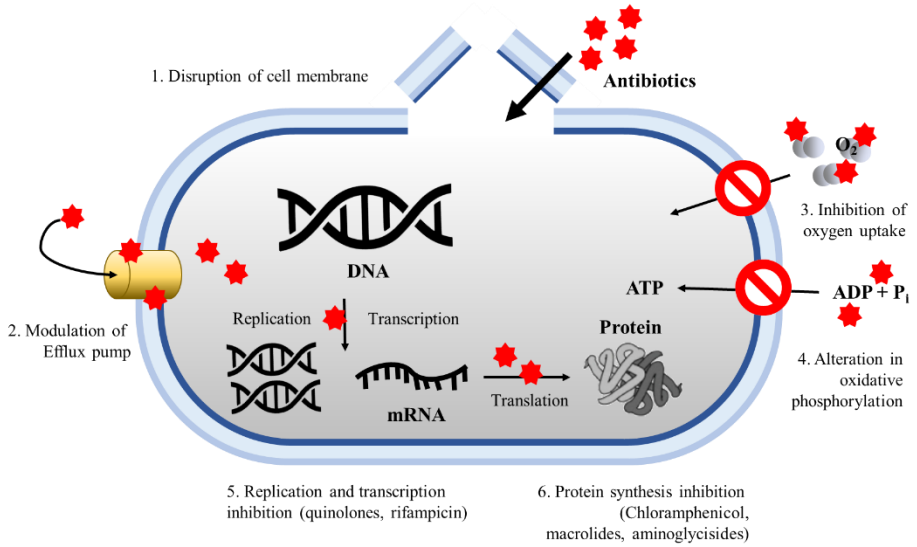
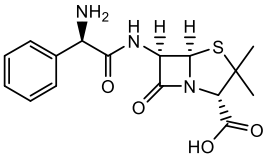
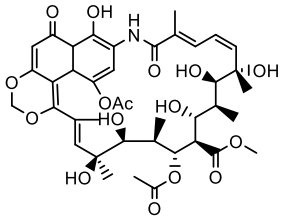


Figure 1. The main mechanisms of action and cellular targets of antibiotics within a bacterial cell (Chifiriuc *et al.,* 2016).

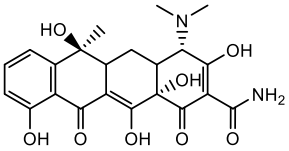
General Antibiotics



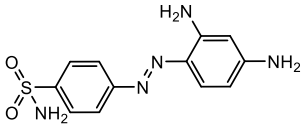
aminobenzylpenicillin



streptovaricin

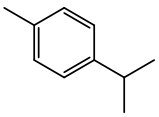


tetracycline

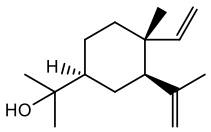


sulfonamide

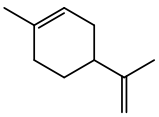
Natural antimicrobial chemicals



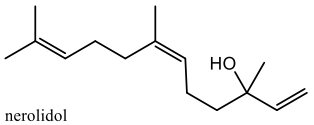
p-cymene



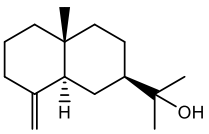
elemol



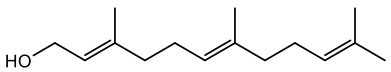
limonene



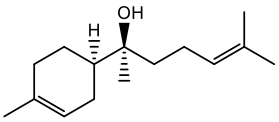
nerolidol



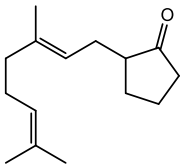
β-eudesmol



farnesol



bisabolol



apritone

Figure 2. Chemical structures of synthetic antibiotics and potential natural antimicrobial compounds.

1.3. Synergistic effects of phytochemicals from EO

Plants with antimicrobial active substances are called medicinal and aromatic plants (MAPs). Activities of EOs from MAPs against pathogens are not superior to those of synthetic antibiotics; nonetheless, EOs have fewer adverse effects and high safety (Pandey *et al.*, 2000). As natural antimicrobial agents have attracted considerable attention in recent years, strategies involving the use of the combination of plant-derived terpenoid have been applied in various fields to suppress foodborne pathogens and other pathogenic microorganisms (Bassolé & Juliani, 2012). This antimicrobial activity is adjusted by the distinct interactions between each component (Bassolé & Juliani, 2012). Several studies have shown that crude EOs consisting of inherent component ratio generally show higher antibacterial activity than the simple sum of the activity of individual substances. Some compounds are assumed to have an impact on the synergistic activity along with antagonistic and additive effects (Mourey & Canillac, 2002). The interaction between EO compounds against target microorganisms can be divided into four types of effects: antagonistic, additive, synergistic, and indifferent activity (Nychas, 1995; Toroglu, 2007). Antagonism is when the activity of compounds is lower when they are applied together than when they are applied individually (Bassolé & Juliani, 2012). The concept of additive and synergistic effects is only slightly different: the former is observed when the effect of a mixture is equal to the sum of the individual effects, whereas the latter is observed when the effect of the mixture is greater than the sum of the individual effects (Edris, 2007). Indifferent effects refer to the absence of interactions between the substances in EOs.

In plant-based EOs, various terpenoid and phenolics interact by either reducing or increasing the antimicrobial efficacy of each other (Delaquis *et al.,*

2002). Interestingly, the mixture of monoterpenoid and phenolics containing other minor compounds increased the antimicrobial activity; this kind of interaction between compounds is called synergistic effect (Bassolé & Juliani, 2012). For instance, the combination of carvacrol (phenolic monoterpenoid) with linalool (terpenoid) showed synergistic effects on antimicrobial activities against *L. monocytogenes, Enterobacter aerogenes, E. coli, P. aeruginosa, S. aureus, Bacillus. cereus* (Bassolé *et al.*, 2010). Most studies have focused on the interaction of phenolic monoterpenes and phenylpropanoids, specifically with other phenolic compounds, and monoterpene alcohols. In contrast, studies on monoterpene hydrocarbon and sesquiterpenoid are sparse (Bassolé & Juliani, 2012). Moreover, one of the limitations is with regard to the identification of the exact substances in EOs that have a critical impact on the physiological activity owing to the complexity of EO components (Filipowicz *et al*., 2003). In this regard, the physiological activity and the synergistic effect of sesquiterpenoid, which are known to have remarkable antimicrobial activity, need to be investigated.

1.4. EOs from Sargent juniper

The cytotoxicity of plant-derived EOs against microorganisms has been extensively studied since the 1980s (Andrews *et al*., 1980). Recently, EOs from *Chamaecyparis obtuse* and *Phebalium squamulosum* (Rutaceae) subspecies have shown high antimicrobial activities. The presence of eudesmol and, particularly elemol, played a crucial role as active components exhibiting these antimicrobial activities (Burt, 2004; Yang *et al.,* 2015). Sesquiterpenoid such as elemol and eudesmol isomers were reported to exhibit high antimicrobial activities (Ayaz *et al.,* 2017). These chemicals can be easily found in juniper species. Monoterpenes (α-pinene, sabinene, and β-myrcene) and sesquiterpenes (β-caryophyllene, germacrene D, and α-humulene) contained in juniper species produce antibacterial activities against *C. perfringens* (foodborne pathogen), *Candida glabrata* (opportunistic pathogen), and *S. aureus* (respiratory or foodborne pathogen; Zheljazkov *et al*., 2018).

Sargent juniper, member of the genus *Juniperus* L., which has over 70 species distributed worldwide and spread across Northeast Asia—Japan, Manchuria, and Siberia—(Adams & Demeke, 1993; Choe, 2011). In Korea, it grows above the timberline of 700 m above sea level, such as Halla Mountain and Seorak Mountain (Song *et al*., 2010). At present, most research on Sargent juniper has been focusing on planting methodologies for the propagation of trees or the analysis of genetic characteristics (Adams & Demeke, 1993; Himejima *et al*., 1992). Studies have compared the components of the extracts and EOs of Sargent juniper with those of other juniper species; sabinene (28.35%) and bornyl acetate (15.26%) were found to be the common components (Kim *et al*., 2015). However, as the composition of EOs considerably varies depending on plant species, geographical conditions, and climate, which affect the organoleptic properties and physiological activities of

EOs, further research is needed to determine the composition for the same species at various sites (Nerio *et al*., 2010).

Studies on the bioactivity of EOs from Sargent juniper are insufficient compared to those for other juniper species. A database on its properties is required to increase the value of this domestic species. Elemol and eudesmol, which are estimated to be active phytochemicals in juniper species, are difficult to isolate, and hence, research on their physiological activity is scarce. Characteristics of each active chemical and its interaction with other compounds in Sargent juniper EOs might form the basic data for industrial use. Furthermore, determining the efficacy of highly effective mixtures based on the distinctive component ratio of Sargent juniper EOs can allow their use as new antimicrobial substances. In this point of view, this study aimed to comprehensively build a foundation for a database about EO properties for the utilization of native species.

1.5. Objectives

At present, the food and beverage industry needs to focus on health problems caused by foodborne pathogens, especially ARMs. Plant-derived EOs, a complex mixture of terpenoid and phenolic compounds with synergistic antimicrobial activities, could be the solution to these problems. Because of the difficulty in isolation and availability of various combinations of EOs in new plants, the interactions between active compounds are required to be determined by comparing the activity of a single substance with that of combined substances with a unique composition of phytochemicals from EOs.

Thus, this study focused on a rare species in Korea, *Juniperus chinensis* var. *sargentii* (Sargent juniper). Compared to the potency of phytochemicals from Sargent juniper on the authority of the chemical composition, research on the characteristics of their physiological activity and applicability in industries is lacking. This study aims to improve the efficiency of the identification of active fractions by applying rapid and intuitive thin layer chromatography–direct bioassay (TLC-DB).

The specific aims of this study are as follows:

1. To evaluate the antimicrobial activities of phytochemicals from Sargent juniper EOs against foodborne pathogens by applying TLC-DB.

2. To explore the interactions among sesquiterpenoid from Sargent juniper EOs by applying checkerboard assay and bacterial growth curve analysis.

3. To broaden the range of application of Sargent juniper-inspired terpenoid mixture as a potential antimicrobial agent.

2. Literature review

2.1. Antimicrobial activities of EOs against foodborne pathogens

Customer acceptability towards plant-derived EOs has increased owing to the adverse effects of traditional sterilization techniques (non-thermal treatment and irradiation) of synthetic antimicrobials, which are responsible for critical health conditions such as hypersensitivity, allergy, asthma, neurological damage, and cancer (Anand & Sati, 2013; Negi, 2012; Rafiq, 2014; Tiwari *et al*., 2009). EOs composed of terpenoid and various volatile compounds are known as alternatives to synthetic antimicrobials, which inhibit gram-negative and gram-positive bacteria, viruses, and organisms, including ticks and pests. These bioactive properties of active compounds contained in EOs have prompted the development and research for determining their bioactivity in cosmetic, pharmaceutical, and food industries owing to their industrial potential.

Himejima *et al.* (1992) identified the antimicrobial compounds in the oleoresin of *Pinus ponderosa* by separating the oleoresin into distillate and residue. The crude oleoresin, distillate, and residue showed different antimicrobial activity patterns against foodborne pathogens. *P. ponderosa* oleoresin (up to 500 μg/mL) exhibited activity against gram-positive bacteria and fungi such as *Bacillus subtilis*, *Brevibacterium ammoniagenes*, and *Candida utilis*, whereas not against gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa*. The distillate contained monoterpenes (*α*-pinene, *β*-pinene, 3-carene, myrcene, limonene, etc.), sesquiterpenes (*α*-longipinene, longifolene, and germacrene D), and a phenolic compound (estragole) and showed inhibitory activity against the fungi and the gram-positive bacteria, but the residue, which consisted of a mixture of diterpene acids, exhibited activity

only against gram-positive bacteria (Himejima *et al.,* 1992). Each compound constituting the distillates had minimum inhibitory concentrations (MICs) of 25 to 100 µg/mL against *Saccharomyces cerevisiae*, and those of 800 µg/mL or less for *C. utilis*, whereas the compounds did not exhibit antifungal activity above 800 µg/mL against the tested fungi.

Chen *et al.* (2016) evaluated the antibacterial activity of EOs extracted from *Alpinia guilinensis*, which was previously used as a spice in China, to improve its applicability as food preservatives. EOs obtained from three different organs of *A. guilinensis* (fruits, leaves, and stems) exhibited distinct antibacterial activity because of a slight difference in the content of compounds. Three EOs showed evident effects on *S. aureus*, and leaf oil exhibited a diameter of inhibition zone of 16.8 mm compared to that of the positive control (streptomycin, 18.3 mm) in the disk diffusion method. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of *E. coli* treated with 1.5 times the MIC of stem oil for 4 h showed that the cells were remarkably damaged and became contorted, pitted, and shriveled. The physical deformity of the cell wall was postulated to have the ability to trigger the expansion of the cell membrane and increase membrane permeability, which interfered with the maintenance of the homeostasis of essential metabolites in bacterial cells (Gao *et al*., 2011).

An antimicrobial assay revealed that the EOs from *Rhanterium suaveolens* Desf., the main components of which are *α*-pinene (25.84%), *β*-pinene (17.57%), 1-octen-3-ol (16.23%), and camphene (12.28%), had antimicrobial activities against foodborne pathogens (*B. subtilis, S. aureus, E. coli,* and *C. albicans*; Salah *et al*., 2019). Further studies have analyzed the correlation of antimicrobial activity among the components. A very strong ($P < 0.01$) correlation was noted between 1-octen-3-ol and pinocarvone amounts and investigated anti-*Listeria ivanovii* and anti-*E. coli* effects.

2.2. Synergistic effects on antimicrobial active terpenoid and other mixtures

The physiological activities of several EOs have been verified, especially in microorganisms, as they showed obvious growth inhibition effects on bacteria and fungi (Burt & Reinders, 2003; Dadalioğlu & Evrendilek, 2004; Popova *et al*., 2009; Sadekuzzaman *et al*., 2018). While the mechanism of the antimicrobial activity induced by EOs is not yet known, studies have revealed the modes of action of single phytochemicals. Terpenoids, one of the phytochemicals, are postulated to have an impact on the disruption of bacterial cell membranes or modulation of bacterial efflux pump, followed by the inhibition of bacterial metabolism and biofilm formation with changes in cell permeability (Barbieri *et al*., 2017). The mechanism of action of terpenoid mixture for the destruction of bacterial cell membranes and determination of the most effective combination of mixtures need to be investigated.

In addition to the identification of antimicrobial activity mechanisms, several studies have shown that a combination of various terpenoids in EOs enhances these microbial inhibitory activities (Barbieri *et al*., 2017; Diniz-Silva *et al*., 2019; Savelev *et al*., 2003). Zengin and Baysal (2014) reported that the combination of α-terpineol and linalool at 1/4MIC + 1/8 MIC; 1/8 MIC + 1/4 MIC, and 1/8 MIC + 1/8 MIC showed synergistic effects against *Salmonella typhimurium, E. coli* O157:H7, and *S. aureus*, respectively.

Several studies attributed the additive and synergistic effects of EO components to the presence of phenolics or alcohols (Table 1). Synergistic effects of terpenoids and phenolics (cinnamaldehyde, thymol, eugenol, and carvacrol) showed higher antimicrobial effect than the sum of individual effects: cinnamaldehyde/eugenol (1:4 or 1:8) and thymol/eugenol (1:4) combinations showed antimicrobial effects against *E. coli* at 50% MIC of each component (Pei *et al*., 2009). Based on these results, the authors postulated that the

synergistic effects of eugenol/thymol were attributed to the ability of thymol to disrupt the outer membrane of *E. coli,* allowing eugenol to interpenetrate the cytoplasm and bind to proteins.

In addition to synergistic effects, additive or antagonism effects might be noted when various phytochemicals are combined, showing antimicrobial interactions against several microorganisms. Lambert *et al.* (2001) reported additive effects when a combination of thymol and carvacrol was inoculated into *S. aureus* and *Pseudomonas aeruginosa* by using the half dilution method. Using the same combination, Gallucci *et al.* (2009) showed antagonism against S. *aureus, B. cereus,* and *E. coli* by applying the checkboard method.

Table 1. Interaction between paired essential oil component combinations against several pathogens.

Pair combinations	**Organism**	**Interaction**	**References**
Thymol/eugenol	*E. coli*	Synergism	Pei *et al.* (2009)
Carvacrol/myrcene	*S. aureus, B. cereus, E. coli*	Antagonism	Gallucci *et al.* (2009)
Cinnamaldehyde/ Eugenol	*Staphylococcus spp., Micrococcus spp., Bacillus spp.,* and *Enterobacter spp.*	Additive	Moleyar and Narasimham (1992)
α-Pinene/limonene *α*-Pinene/Linalool Linalool/ Terpinen-4-ol	*S. cerevisiae*	Synergism, Additive	Tserennadmid *et al*. (2011)

2.3. Pathogen inactivation of EOs derived from juniper species

Juniper species (genus *Cupressus*) are widely distributed throughout the Northern Hemisphere (including Arctic, Tibet, and the Himalayas), forming comparatively high tree lines on earth (Hampe & Petit, 2010). EOs derived from juniper species, which are known to have pharmacological properties, have been studied in various ways.

Majewska *et al.* (2017) reported that juniper species exhibited diverse antimicrobial activities depending on the geographical origin. The mixture of *α*-pinene and sabinene contained in EOs from juniper species showed high antibacterial activities against the following gram-positive bacteria: *B. cereus*, *B. subtilis*, *Micrococcus flavus*, and *S. aureus.* Based on these reports, researchers have been attempting to determine the difference in antimicrobial activities of EOs from juniper species depending on the planting location since the chemical composition of EOs was changed (Bouyahyaoui *et al.,* 2016). El Hajjouji *et al.* (2019) revealed that *α*-pinene, *α*-terpineol, *β*-phellandrene, *δ*-3-carene, *δ*-2-carene, and terpinen-4-ol derived from three different juniper species are effective in suppressing *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa* based on the composition data of juniper species.

Juniperus chinensis belonging to the Cupressaceae family is a coniferous evergreen shrub native to China, Taiwan, Myanmar, Japan, and Korea (Singh *et al*., 2018). *J. chinensis* is known as an ornamental plant, which has more than 100 cultivars and varieties (Lyu *et al*., 2019). Compared to those in other juniper species, the EOs of *J. chinensis* have been rarely investigated (Lyu *et al.*, 2019). Pu and Huang (1999) identified 16 monoterpene and 18 sesquiterpene constituents by employing gas chromatography-mass spectrometry-data system. The main components were sabinene (46.73%), *α*-pinene (17.03%), terpinen-4-ol (5.34%), limonene (4.48%), *γ*-terpinene

(3.88%), and *p*-cymene (2.36%). Conversely, the EO of *J. chinensis* leaf contained the following components: sabinene (19.8%), elemol (18.6%), bornyl acetate (17.5%), and limonene (14.2%; Raina *et al.*, 2005).

Some studies have investigated the physiological characteristics of *J. chinensis* extracts such as antioxidant and antimicrobial activity. Ryu *et al.* (2010) evaluated the antibacterial activities of *J. chinensis* extractives against dermatitis-inducing strains by measuring the inhibition zone diameter at a concentration of 500, 250, and 125 μg/disk: *S. aureus, Staphylococcus epidermidis, Propionibacterium acnes*, and *Malassezia furfur*. In addition to antimicrobial activities, *J. chinensis* extract exerted cytotoxicity against human-derived skin keratinocytes, which was related to antioxidant mechanisms.

Darwish *et al.* (2020) investigated the inter-specific variability effect on the biological activities (antimicrobial and anti-inflammatory activities) of EOs from three *Juniperus* species: *J. communis, J. horizontalis*, and *J. chinensis*. *J. chinensis*, which had the highest percentage of oxygenated monoterpenes (55.03%), the lowest antibacterial activity against *S. aureus* and *E. coli*, but showed high anti-inflammatory activities, including reduction of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and γ-interferon (INF-γ), in lipopolysaccharide-activated white blood cells.

EOs derived from juniper species exhibited excellent antimicrobial activity. The results of these studies suggested that the Sargent juniper, a native species, might have similar effects, whereas research on the physiological properties and its components is insufficient. Therefore, studies on Sargent juniper and the interaction between its sesquiterpenoids, a major component of juniper species, might suggest the method for maximizing its antimicrobial effect and broaden the range of application.

3. Materials and methods

3.1. Materials

Fresh leaves were collected from Sargent juniper (*Juniperus chinensis* var. *sargentii*) provided by the Seoul National University Chilbosan Forest (CF) and National Forest Seed Variety Center in Gangneung Daehwasilsan Forest (NFSV), the Republic of Korea. The EOs of Sargent juniper leaves were isolated using steam distillation at 102 ± 1 °C until their amount remained unchanged and then were dried over anhydrous sodium sulfate (Samchun, 98.5 w/w%, Korea).

Cationic-adjusted Mueller Hinton broth (CAMHB) was purchased from MB Cell (Korea). Mueller Hinton agar (MHA) was purchased from Difco™ BD (Sparks, Maryland).

Listeria monocytogenes (KCTC 3569) and *Salmonella typhimurium* (KCTC 12401) were provided by the Center of Biological Resources of the Korea Research Institute of Bioscience and Biotechnology. *Staphylococcus aureus* (ATCC 33594) and *Escherichia coli* O157:H7 (KCCM 40406) were supplied by The Korea Culture Center of Microorganisms. The bacterial cultures were maintained at 37 °C in different media as shown in Table 2.

Table 2. Culture media of each microorganism.

Microorganisms		**Culture medium**	**Test medium**
Gram-negative	*S. typhimurium*	Nutrient agar	MHA and CAMHB
	E. coli O157:H7	Trypticase soy agar	
Gram-positive	*S. aureus*	Nutrient agar	
	L. monocytogenes	Brain hear infusion agar	

3.2. Chemical analysis of EOs from Sargent juniper

EOs were analyzed using gas chromatography–mass spectrometry (GC-MS) to identify their chemical composition. The GC (Agilent 7890, USA) was equipped with a DB5 column (25 m × 0.32 mm × 0.52 μm). The carrier gas was helium at a flow rate of 2 mL/min. Inlet and MSD transfer line heater temperatures were maintained at 260 °C and 280 °C, respectively. The initial oven temperature was maintained at 50°C for 5 min and then increased to 250 °C at a rate of 5 °C/min and then held for 10 min. Mass spectrometry (Agilent 5973) was used in the EI mode. The samples were diluted at 1:125 (v/v) with ethyl acetate, and 1.0 μL of the sample was injected in the splitless mode. The chemical structure of each constituent was identified by comparison of their retention indices and mass data of their peaks by using standard library data (Willy 7th ed.). The percentage of content was calculated using peak area normalization. Each crude EO and fraction were analyzed using tridecane (Sigma-Aldrich, Korea) as a standard compound.

3.3. The *in vitro* antimicrobial assay of crude EO

3.3.1. Sensitivity test of the crude EO

The agar disk diffusion method with some modifications to the CLSI guidelines was used to determine the sensitivity of the EOs. The solid media plates were swabbed with the respective suspension (0.5 McFarland, 10^8 CFU/mL) and kept in a 9 cm diameter Petri dish by using a sterile cotton swab for 30 min for absorption. Filter paper disks (8.0 mm in diameter) were impregnated with 20 µL EO diluted with dimethylsulfoxide (DMSO; Showa, 99.0%, Japan) at 100 mg/mL and placed on the agar surface. Tetracycline (Sigma-Aldrich, Korea) solution prepared in 20 µL DMSO (100 µg/mL) was used as the positive control, and 20 µL DMSO was used as the negative control. All plates were incubated at 37 °C for 24 h. All experiments were performed in triplicate, and the results are expressed as mean ± standard deviation.

3.3.2. Determination of MIC and MBC of crude EO

The MIC and minimal bactericidal concentration (MBC) were measured using the broth microdilution method, followed by modification according to CLSI guidelines.

All the tests described in this section were performed using CAMHB. The bacterial cell suspensions were adjusted to an optical density of 0.08–0.12 at 625 nm (UV-1601PC spectrophotometer; Shimadzu, Japan); the cell suspension was further diluted to achieve a concentration of 10^5–10^6 CFU/mL.

The samples were dissolved in DMSO at a final concentration of 2% (w/v). Serial doubling dilutions of the oils were prepared in a 96-well microtiter plate at the range of 0.0312% to 1.000% (w/v). Each EO dilution (50 µL) was dispensed into the wells of a microtiter plate. Untreated CAMHB (50 µL) was

used as a negative control, and tetracycline was used as a positive control in parallel experiments. Each well was then inoculated with 150 μL of the suspension. The resulting suspensions were mixed with a micro-pipettor. All microtiter plates against all microorganisms were incubated at 37 °C for 16–24 h. After incubation, the wells were examined for the growth of microorganisms, and the MICs were determined. The MIC was defined as the lowest concentration of EO at which the microorganisms did not show visible growth. The MBCs were confirmed by reinoculating on agar plates with 50 μL of each culture medium from the microplates corresponding to the MIC or higher concentrations. The MBC was defined as the lowest concentration of EO at which the incubated microorganisms were completely killed. Each experiment was replicated three times.

3.3.3. Two-dimensional TLC-direct bioassay of crude EO

Active EOs were subjected to two-dimensional thin layer chromatography (2D TLC) to separate the components. Crude EO (6 μL) was manually spotted onto glass-backed silica gel 60 TLC plates with F254 (MERCK, Germany) by using a capillary pipette. TLC plates were eluted in a mixture of 8:1 (v/v) hexane (Samchun, Korea) and ethyl acetate in a glass TLC developing tank that was saturated with the solvent system before elution. Two-dimensional resolution was achieved following elution in a mixture of 4:1 (v/v) hexane and diethyl ether, and the mobile phase was allowed to evaporate completely before rotating the plate by 90° and eluting along the orthogonal axis. Following elution, separated compounds were visualized using UV light (254 nm) and iodine vapor derivatization, and eluted plates were suspended in a glass chamber containing iodine crystals for 2 min. The retention factor (Rf)

for each spot was calculated as the ratio of the distance of the first spot relative to the total eluted distance (in millimeters).

The 2D TLC-DB was performed to identify the group of active compounds for the active EOs against the screened pathogens. Overnight cultured test bacteria were diluted to approximately 10^6 CFU/mL in sterile distilled water. Pre-eluted 2D TLC plates were placed on the petri dish (90 × 15 mm), and the MHA medium was poured thereon. Next, 200 µL bacterial suspension was transferred to the 2D TLC agar plates, which were left to develop for 24 h at 37 °C. Subsequently, inhibition zones were observed.

3.3.4. GC-MS analysis for the active compounds

Preparative TLC (prep. TLC) was performed under the same conditions as that for TLC-DB. The silica gels (fixed phase) were fractionated according to the active substance group that was absorbed depending on their interaction with silica gel. The active spots were scraped based on the Rf values and the ratio of the moving distance of the group of active compounds. Fractionated silica gel was extracted using 1.2 µL ethyl acetate, centrifuged, filtered once using a hydrophobic syringe filter, and analyzed by using GC-MS to investigate the main components.

3.4. Analysis of synergistic effects on active compounds

3.4.1. MIC test for single compounds

Standard chemicals of the active substance identified using the TLC-direct bioassay were used to determine the MICs of each compound. The standard active substances that could not be purchased were provided by the Seoul National University National Instrumentation Center for Environmental Management and isolated using prep. HPLC (Thermo Dionex, USA). The MIC test for individual compounds was performed using the methods described above by using a concentration ranging from 0.0312% to 0.5%.

3.4.2. The *in vitro* antibacterial activity from the combination effect of active compounds

According to the results of antibacterial assay of crude EO and individual compounds used in this study, the main components of the active fractions were selected, and each isolated compound was acquired from the National Instrumentation Center for Environmental Management, Seoul National University. The antibacterial assays were performed using elemol, γ-eudesmol, and terpinen-4-ol. The antibacterial activity and combinational effects of these components were assessed using the checkerboard method (Chang *et al*., 1995).

The combination effects between each component from the original Sargent juniper compounds were confirmed by measuring the fractional inhibitory concentration index (FICI) depending on the combination of active substances at the concentration from MIC to 1/8 MIC by 2-fold serial dilution.

The inoculum was prepared from colonies that had been grown overnight on CAMHB. The bacterial concentration was adjusted to 0.5

McFarland turbidity (10^8 CFU/mL), following 200 times dilution. In this method, 150 µL of the diluted inoculum was added to a 96-well microtiter plate. Next, 50 µL of active substances diluted with DMSO and CAMHB were added to the plate, in which the final concentration of DMSO was 4%. The MIC was determined after 24 h of incubation at 37 °C. The MIC was defined as the lowest concentration of the compound, alone or in combination with other compounds, that could visibly inhibit the growth of bacteria. The *in vitro* interaction between the active substances was calculated by determining the FIC. The FICI was compared using equation (1), and the synergistic effect was determined using the FICI.

$$\text{FIC index} = FIC_A + FIC_B$$
$$= \left[\frac{\text{MICs of mixed compounds (A)}}{\text{MICs of individual compound (A)}}\right] + \left[\frac{\text{MICs of mixed compounds (B)}}{\text{MICs of individual compound (B)}}\right] \quad \text{(Eq. 1)}$$

FIC_A and FIC_B are the FIC values of compounds A and B, respectively. The FICI obtained was interpreted as follows: ≤ 0.5 denoting synergistic effect; 0.5 < FICI < 0.75 denoting partial synergistic effect; 0.76 < FICI< 1 denoting additive effect; 1–4 and above 4 denoting indifference and antagonism, respectively (Mun *et al*., 2013).

3.4.3. Antibacterial kinetic assay for synergistic combinations

The antibacterial kinetic assay was performed for the combination of active compounds exhibiting synergistic effects, according to the method reported by Bouyahya (2019). The inoculum was prepared from colonies that had been grown overnight on CAMHB. The bacterial concentration was adjusted to 0.5 McFarland turbidity (10^8 CFU/mL), following 200 times dilution.

The inoculum was treated with active compounds at 1/2 MIC and 1/4 MIC of each active compound and their combinations. The control containing only DMSO (4%) was added to the bacterial inoculum. The bacteria were cultivated at 37 °C for 24 h, and the optical density was measured at 10-min intervals by using a UV-vis spectrophotometer (Optizen alpha, Korea). All measurements were performed in triplicate.

3.4.4. Morphological analysis

SEM analysis was performed according to Insuan and Chahomchuen (2020) with some modifications. Overnight cultured bacteria were inoculated in CAMHB and cultured at 37 °C for 4 h. Bacterial cells (0.5 McFarland turbidity standard) were treated with EO and active substances at 1/2 MIC and with DMSO as the negative control; next, the cells were incubated at 37 °C for 3 h, and then harvested using centrifugation for 10 min at 5,000 ×*g* at 4 °C, washed twice with phosphate buffer saline (PBS; pH 7.2, Samchun, Korea), and fixed in 2.5% (v/v) glutaraldehyde for 2 h at 4 °C. Following two rinses with PBS for 10 min, secondary fixation was performed with 1% osmium tetroxide (Sigma Aldrich, Korea) for 4 h. After fixation, cells were briefly washed twice with distilled water, followed by dehydration in 30%, 50%, 70%, 80%, 90%, and 100% ethanol for 10 min each, and dehydrated twice with 100% ethanol. Finally, ethanol was replaced with 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 98%; Samchun, Korea) and dried for 20 min. The cells were filtered through a nylon membrane (0.2 μm, 47 mm; Waters, USA), and a small piece of the membrane was cut into a size of 0.5 × 0.5 cm for the SEM specimen, and then air-dried. The dried specimens were attached to an aluminum stub by using a double-side copper tape and then coated with platinum by using a sputter coater (EM ACE200; Leica, Austria) for 2 min. Subsequently, their morphologies were characterized using SEM (SUPRA 55VP; Carl Zeiss, Germany).

4. Results and discussion

4.1. Chemicals identified in the crude EOs

The EOs extracted from the leaves of Sargent juniper from CF and NFSV were pale-yellow and dark yellow, respectively; the chromatograms are shown in Fig. 3. Each compound of Sargent juniper EO could be analyzed (Table 3). EOs from CF and NFSV were complex mixtures, containing more than 49 and 50 compounds, respectively. The content of constituents was significantly different between the two Sargent juniper EOs. According to GC-MS results, the two samples contained the following components: CF Sargent juniper EO contained bornyl acetate (18.53%), terpinen-4-ol (14.73%), sabinene (10.81%), α-cadinol (8.06%), α-pinene (7.33%), D-limonene (6.66%), and γ-terpinene (5.92); NFSV Sargent juniper EO contained sabinene (23.51%), elemol (19.93%), terpinen-4-ol (7.49%), α-copaen-11-ol (6.26%), γ-terpinene (4.56%), α-cadinol (3.66%), and γ-eudesmol (4.56%). In particular, the contents of elemol, bornyl acetate, sabinene, terpinen-4-ol, and α-copaen-11-ol differed significantly between the Sargent juniper from CF and NFSV.

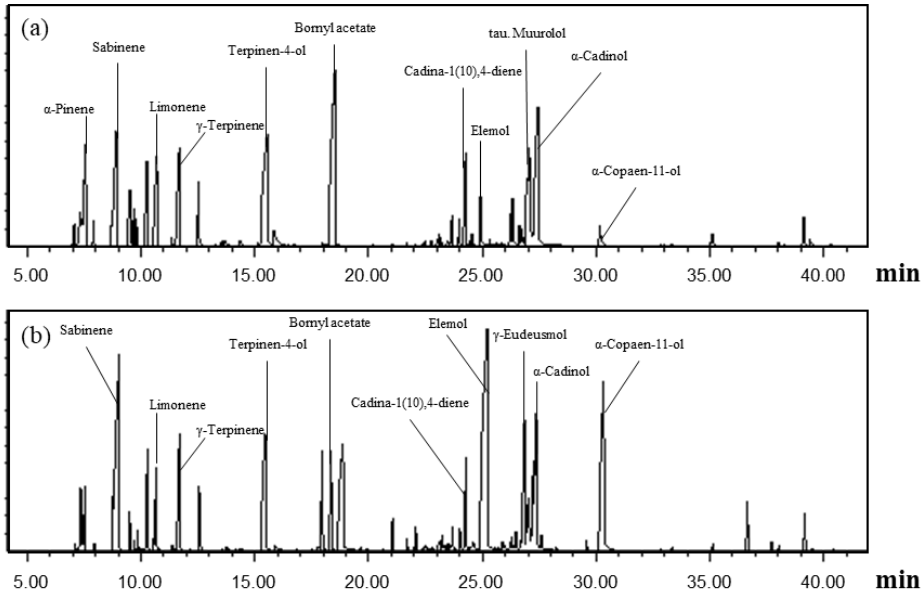


Figure 3. GC-MS chromatograms of Sargent juniper EOs from (a) CF and (b) NFSV.

Table 3. Chemical compositions of Sargent juniper EOs.

		Composition of EOs (%)	
RI	**Compounds**	**CF**	**NFSV**
490	Tricyclene	0.53	0.18
507	2-Thujene	1.38	1.43
519	*α*-Pinene	7.33	1.55
548	Camphene	0.62	0.19
604	*β*-Pinene	-	1.73
623	Sabinene	10.81	23.51
655	*β*-Myrcene	4.25	0.99
669	Terpinolene	0.80	0.21
678	*α*-Phellandrene	0.46	0.38
708	*α*-Terpinene	3.49	2.87
726	o-Cymene	-	0.27
735	Limonene	6.66	2.31
784	*β*-Ocimene	0.28	0.20
805	*γ*-Terpinene	5.92	4.56
865	Terpinolene	2.03	1.52
1064	Terpinen-4-ol	14.73	7.49
1085	*α*-Terpineol	0.38	-
1238	1-Methyl-2-methylene-3,5-divinylcyclohexane	-	2.96
1264	Bornyl acetate	18.53	2.21
1451	*β*-Elemene	0.06	0.48
1495	Caryophyllene	0.07	0.20
1515	*α*-trans-Bergamotenol	0.05	0.10
1521	*γ*-Elemene	0.07	0.38
1548	*β*-Copaene	0.07	0.23
1540	Humulene	0.09	-
1570	cis-Muurola-4(15),5-diene	0.17	0.13
1575	δ-Cadinene	0.08	-
1593	*γ*-Muurolene	0.17	0.34
1587	*β*-Copaene	0.16	-

1600	Germacrene D	-	0.30
1609	*α*-Selinene	-	0.14
1618	isoledene	0.08	0.16
1624	*γ*-Gurjunene	0.07	0.15
1633	*α*-Muurolene	0.56	0.41
1655	*γ*-Cadinene	0.41	0.40
1673	Cadina-1(10),4-diene	2.08	1.78
1686	*α*-Muurolene	0.11	0.13
1693	*α*-Cadinene	0.21	0.43
1738	Elemol	1.07	19.93
1734	*α*-Campholenal	0.09	-
1753	Ledene	0.20	-
1784	cis-Z-*α*-Bisabolene epoxide	0.15	0.17
1791	Longiborneol	-	0.09
1806	*β*-Eudesmol	-	0.13
1795	Isoshyobunone	0.50	-
1810	Aromadendrene oxide-(2)	-	0.27
1802	Verbenyl acetate	0.72	-
1838	Di-epi-1,10-cubenol	0.28	0.16
1851	*γ*-Eudesmol	0.33	3.71
1860	tau.-Cadinol	1.43	1.04
1852	tau.-Muurolol	2.09	-
1855	*δ*-Cadinol	0.83	-
1878	*β*-Eudesmol	-	1.93
1885	*α*-Cadinol	8.06	3.66
1901	Elemol	-	0.24
2089	*α*-Copaen-11-ol	0.57	6.26
2420	Epimanool	0.27	0.16
2525	Butyl 4,7,10,13,16,19-docosahexaenoate	-	0.97
2599	Abietal	0.51	0.81
2680	Abieta-7,13-dien-3-one	0.12	-

4.2. The *in vitro* antibacterial assay for crude EO

4.2.1. Disk diffusion assay for crude EO

A disk diffusion assay was performed to screen the antibacterial activity depending on the difference in the content of the two Sargent juniper EOs (Fig. 4). CF Sargent juniper EO did not exhibit antibacterial activity, whereas Sargent juniper EO from NFSV formed inhibition zones ranging between 12.7 ± 1.2 and 18.5 ± 0.5 mm against the gram-positive bacteria (*L. monocytogenes* and *S. aureus*; Table 4). The maximum zone of inhibition by the NFSV EO was against *L. monocytogenes* (18.5 ± 0.5 mm). The positive control tetracycline (2 μg) had a zone of inhibition against *L. monocytogenes* (18.0 ± 1.0 mm), *S. aureus* (31.9 ± 2.0 mm), *E. coli* O157:H7 (18.1 ± 1.1 mm), and *S. typhimurium* (17.4 ± 1.4 mm). Both EOs from CF and NFSV formed inhibition zones for *L. monocytogenes*. Growth inhibition was exhibited for *L. monocytogenes* by both CF (12.8 ± 0.7 mm) and NFSV (18.5 ± 0.5 mm) EOs, whereas only NFSV EO showed antibacterial activity against *S. aureus* (12.7 ± 1.2 mm) and *E. coli* O157:H7 (10.5 ± 1.2 mm). The results of disk diffusion assay suggested that the EO from NFSV showed antibacterial activity against the tested bacteria except for *S. typhimurium*.

In particular, the NFSV EO exhibited the same growth inhibition zone against *L. monocytogenes* as that of the positive control tetracycline, indicating that the EO had excellent antibacterial activity. In general, gram-negative bacteria are known to be resistant to the EO owing to the lipopolysaccharides constituting their cell wall (Patterson *et al.*, 2019).

Conversely, the EO from CF had low or no antibacterial activity against the tested bacteria. The difference in the antibacterial activity of the EOs from the two species can be attributed to the difference in their composition. The content of *β*-pinene; sabinene; 1-methyl-2-methylene-3,5-

divinylcyclohexane; elemol; γ-eudesmol; β-eudesmol; and α-copaen-11-ol differed by more than 1% for each EO, and these components were higher in NFSV EO. The preceding components may act as the key substances for the antibacterial activity of NFSV EO. The EO from NFSV was selected for further observation of the antibacterial activity by using the MIC assay for quantitative analysis.

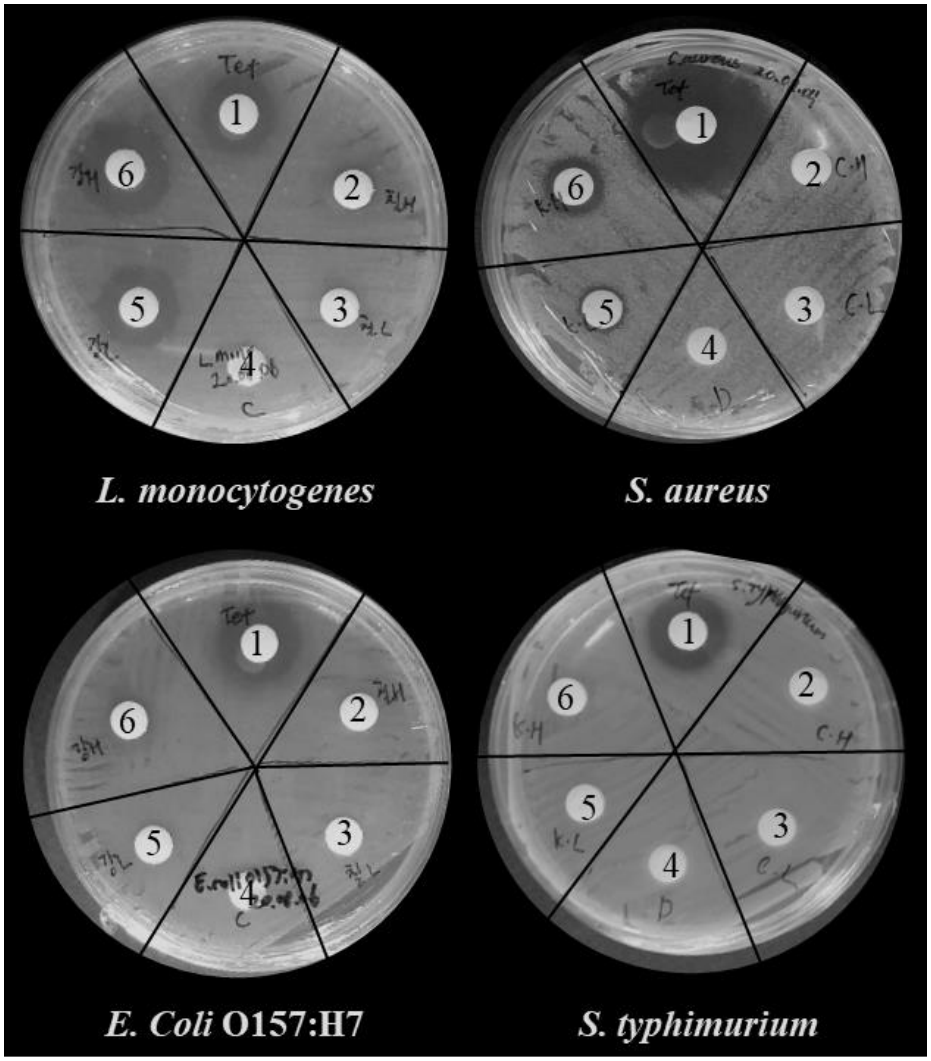


Figure 4. Difference between the inhibition zones caused by the two Sargent juniper EOs from CF and NFSV against *L. monocytogenes, S. aureus, E. coli* O157:H7, and *S. typhimurium*. (1) Positive control (tetracycline, 2 μg/disk), (2) 2 mg/disk of CF EO, (3) 1 mg/disk of CF EO, (4) negative control of 20 μL DMSO, (5) 1 mg/disk of NFSV EO, (6) 2 mg/disk of NFSV EO.

Table 4. Antibacterial activity of Sargent juniper essential oil against different bacterial strains.

Microorganisms	Diameters of inhibition zone (mm)		
	Sargent juniper EO from CF (2 mg/disk)	**Sargent juniper EO from NFSV** (2 mg/disk)	**Tetracycline** (2 μg/disk)
Gram-positive bacteria			
L. monocytogenes	12.8±0.7	18.5±0.5	18.0±1.0
S. aureus	n.d.[*a]	12.7±1.2	31.9±2.0
Gram-negative bacteria			
E. coli O157:H7	n.d.	10.5±1.2	18.1±1.1
S. typhimurium	n.d.	n.d.	17.4±1.4

[*a] Not detected

4.2.2. MIC and MBC of the active EO

The antibacterial activity of the NFSV EO was evaluated by determining the MICs and MBCs against four reference strains (Table. 5). The MIC and MBC values were both 1.25 mg/mL against *L. monocytogenes* 2.50 and 5.00 mg/mL, respectively, against *S. aureus*; 5.00 mg/mL and 10.00 mg/mL, respectively, against *E. coli* O157:H7; and both 2.50 mg/mL against *S. typhimurium*. These results indicated that crude NFSV EO had potential antibacterial activity against the tested strains. The MIC value of crude EO against *E. coli* O157:H7 was lower than that of cypress EO, which had similar compositions as that of NFSV EO, as reported by Park (2008), whereas those against the other bacteria were similar. The corresponding components from crude NFSV EO and cypress EO showed similar antibacterial activity against foodborne pathogens, although quantitative comparison is difficult as they are different species.

The differences in the antibacterial activity with the disk diffusion method and MIC test are attributed to the various diffusion rates of constituents in the EOs. Some researchers have reported that MIC values and disk diffusion assay values do not necessarily correlate because of the different absorption rates for each constituent in EOs on a paper disk (Ncube *et al.*, 2008).

Table 5. Antibacterial activity of Sargent juniper EO from NFSV expressed as minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs).

	Sargent juniper EO from NFSV (mg/mL)	
Microorganism	**MIC**	**MBC**
Gram-positive bacteria		
L. monocytogenes	1.25	1.25
S. aureus	2.50	5.00
Gram-negative bacteria		
E. coli O157:H7	5.00	10.00
S. typhimurium	2.50	2.50

4.3. Screening of active antibacterial fractions

4.3.1. 2D TLC-DB

TLC-DB was performed to ascertain the presence of active phytochemicals that played a large role in the antibacterial activity of NFSV EO. The 2D TLC coupled with direct bioassay is an effective and rapid analytical technique to characterize active groups (compounds with antibacterial activities) from complex mixture. Standard 2D TLC plates developed using NFSV EO are shown in Fig. 5(a) and 5(d), which were the reference index for 2D TLC-DB inoculated with four different strains.

The 2D TLC-DB revealed the presence of 6 antimicrobial fractions in NFSV EO that inhibited the gram-positive bacteria (*L. monocytogenes* and *S. aureus*), but not the gram-negative bacteria (*E. coli* O157: H7 and *S. typhimurium*; Fig. 6). *S. aureus* growth was suppressed in 6 fractions at Rf = 0.14–0.48 (Fig. 5 (c)) on the 2D TLC plate, and *L. monocytogenes* growth was suppressed in 8 fractions at Rf = 0.14–0.59 among the 13 fractions of crude NFSV EOs (Fig. 5 (b)) on the 2D TLC plate.

The growth inhibition was not exhibited for *S. typhimurium* (Fig. 5 (f)) and *E. coli* O157:H7 (Fig. 5 (e)) in the 2D TLC-DB analysis with fractionated EO, whereas crude EO showed inhibition in the MIC analysis against gram-negative bacteria. Growth inhibition was thought to be insignificant for gram-negative bacteria owing to the following two factors. The first factor is the structure of cell walls and membranes of gram-negative bacteria. The antibacterial mechanisms of EOs against bacteria have been suggested to involve (1) the breaking down of the cell wall and enzymes that synthesize cell membrane and (2) increasing of the membrane permeability because of the hydrophobicity of most phytochemicals (Chouhan *et al.*, 2017). Some previous studies have shown that EOs exhibit more activity against gram-positive

bacteria than against gram-negative bacteria. The lipopolysaccharide membrane of gram-negative bacteria limits the diffusion of hydrophobic phytochemicals, whereas the peptidoglycan cell wall of gram-positive bacteria provides less resistance to these compounds (Patterson *et al*., 2019). Therefore, the structural difference in cell walls and cell membranes between gram-positive and gram-negative bacteria might have caused the difference in the antibacterial activity of NFSV EO.

The second factor is the disappearance of the combinational effect between the phytochemicals contained in EO because of the fractionation in the TLC plate. Unlike crude EOs, fractionated EOs did not inhibit the growth of gram-negative bacteria. Bassolé and Juliani (2012) argued that the EO complex mixture showed higher antimicrobial activity than single isolated compounds owing to the synergistic effect of each chemical. Multiple phytochemicals in EOs may strengthen and prolong the antibacterial activity (Patterson *et al.,* 2019). Therefore, the results of 2D TLC-DB against gram-negative bacteria might be evidence of the synergistic effect of the EO mixture.

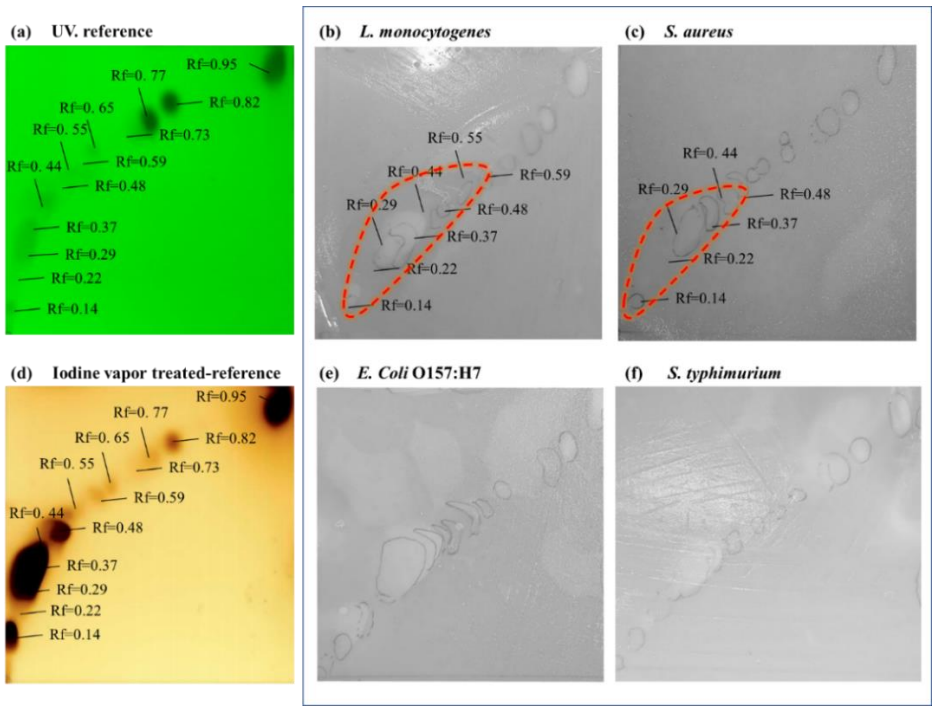


Figure 5. Comparison of 2D TLC-DB results of NFSV EOs against the tested strains. Standard 2D TLC plate under (a) UV 254 nm condition and (d) treated with iodine vapor, TLC-DB of NFSV EOs treated with (b) *L. monocytogenes*, (c) *S. aureus*, (e) *E. coli* O157:H7, and (f) *S. typhimurium*. Images were obtained using a digital smartphone camera.

4.3.2. Morphology (SEM analyses)

The morphology of NFSV EO-treated bacterial cells was observed by using SEM. The morphological changes in *S. aureus* are shown in Fig. 6, and those of *L. monocytogenes* are shown in Fig. 7. The untreated cells had normal cellular morphology with smooth cell walls and intact cell surfaces (Figs. 6A, 6C, 7A, and 7C). In contrast, the NFSV EO-treated cells had an irregular surface and had shrunken. For NFSV EO-treated *L. monocytogenes*, the cells were bent, and both ends burst out. In the case of *S. aureus* (Fig. 7B and 7D), individual cells were separated from each other because the biofilm that maintained the binding between the individuals was not formed (Bazargani *et al*. 2016).

The destruction of bacterial cell wall might further affect the leakage of some intracellular materials, especially extravasation of protoplasm, leading to cell necrosis. The cell wall and cell membranes are important barriers to bacterial strains. When bacteria are attacked by strong antibacterial agents, the bacterial cell membranes are destroyed, leading to the leakage of the internal electrolytes to the outer side of the membrane (Han *et al.*, 2020). Some phytochemicals in plant EOs are known as bacterial growth inhibitors that can break peptidoglycan bonds, which are the main structure of the bacteria cell wall, and finally destroy the cells or cause cell death. In particular, some researchers have argued that phosphor-MurNAc-pentapeptide translocase (MraY), which plays a key role in the formation of peptidoglycans structure, is remarkably influenced by the hydroxyl patterns of antibacterial agents (Dini *et al.*, 2001). Therefore, phytochemicals with hydroxyl substituents in EOs, such as elemol, eudesmol, cadinol, and terpineol, may impact the formation of the cell wall by inhibiting the MraY enzyme.

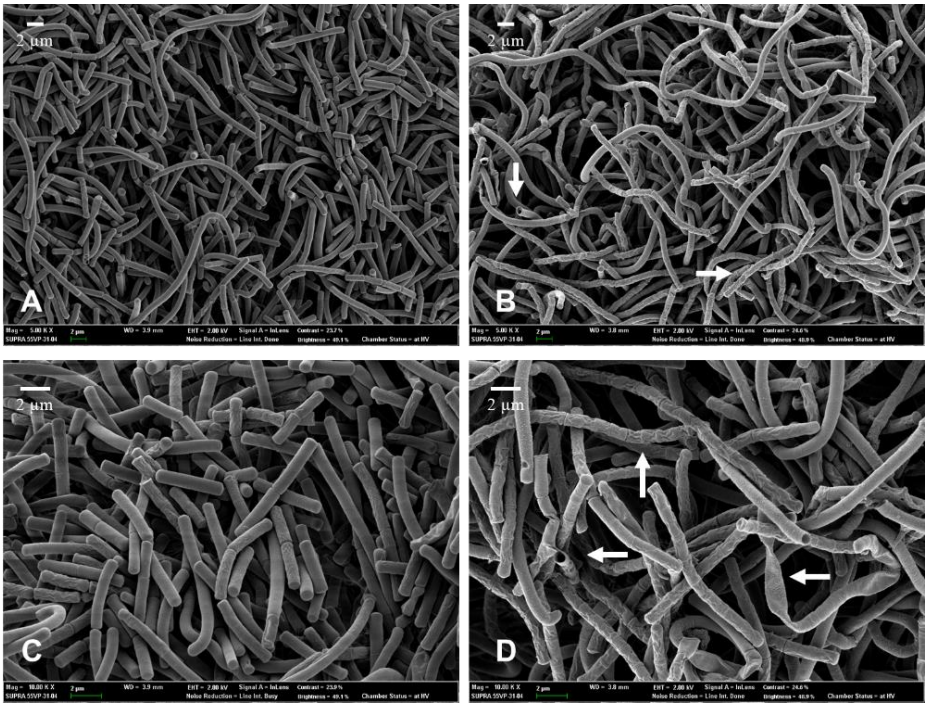


Figure 6. Effects of EO on the morphology of *L. monocytogenes* by using scanning electron microscopy; *L. monocytogenes* without EO (control) (A) 5,000× and (C) 10,000×; *L. monocytogenes* treated with EO at MIC (B) 5,000× and (D) 10,000×.

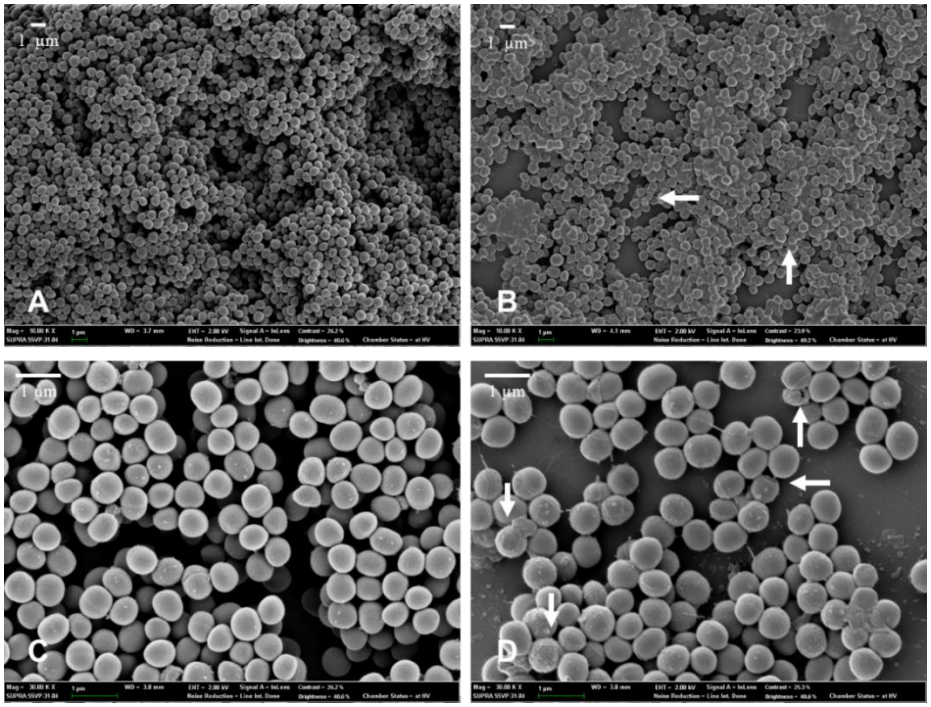


Figure 7. Effects of EO on the morphology of *S. aureus* by using scanning electron microscopy. *S. aureus* without EO (control) (A) 10,000× and (C) 30,000×; *S. aureus* treated with EO at MIC (B) 10,000× and (D) 30,000×.

4.3.3. Identification of active antibacterial fractions by using GC-MS analyses

Antibacterial compounds extracted from TLC-DB plates were characterized using GC-MS except for the trace amount of Rf = 0.00–0.22 fraction. GC-MS analysis revealed that the antibacterial fractions were mixtures of several phytochemicals (Table 6). Elemol was present in all the fractions in the active part of NFSV EO except for fraction F (Rf = 0.59) that consisted of bornyl acetate. Elemol was a major component of fractions A (71.68%), B (78.83%), C (69.65%), and D (73.46%), whereas fraction E mainly consisted of terpinen-4-ol (53.91%). Terpinen-4-ol was also identified in fractions C (12.93%) and D (13.82%) as a minor component. Another minor component, γ-eudesmol, was widely distributed in fractions A–E and was contained from 8.28% to 19.06% in the fractions shown in Table 6. Sesquiterpenoid such as elemol, α-eudesmol, β-eudesmol, γ-eudesmol, and α-cadinol accounted for more than 80% of the fraction at Rf = 0.29–0.48, whereas terpinen-4-ol and bornyl acetate were dominant at Rf = 0.55–0.59. In addition, β-eudesmol and α-eudesmol were also identified in fractions A and B as minor components having concentrations of below 5%. In fraction A, α-cadinol was detected at 14.15%. Except for terpinen-4-ol and bornyl acetate, all detected chemicals are sesquiterpenoid alcohols. Although the mechanism of antibacterial action of phytochemicals remains unknown, Zengin and Baysal (2014) reported that lipophilicity and hydrophobicity in the presence of hydroxyl substituents in terpenoids are one of the determinants for their antibacterial mode. Therefore, elemol, eudesmol isomers, cadinol, and terpinen-4-ol, which have a hydroxyl group in the hydrocarbon backbone, are thought to act as the determining elements of antibacterial activities.

Two types of sesquiterpenoid, elemol (ELE), and γ-eudesmol (γ-EUD), and a monoterpenoid, terpinen-4-ol (TER), were the main components found

in three or more fractions. TER, which was contained in 53% of the fraction E, is a substance contained in several medicinal crops and known for its antifungal and antibacterial activities. In general, sesquiterpenoid have been long recognized to have antimicrobial activity. In particular, farnesol and xanthorrhizol have been reported to exhibit growth inhibitory effects in bacterial growth such as *S. aureus* (Mahizan *et al.*, 2019). However, most studies have been conducted on fractions consisting of terpenoid mixture, but not on the antimicrobial activity of single compounds, particularly ELE and eudesmol isomers. In this study, evaluation of antibacterial activity for each single phytochemical was evaluated, followed by an investigation of the synergistic effect of the main components of antibacterial active fractions, ELE, γ-EUD, and TER.

Table 6. Antibacterial components identified using 2D TLC-DB, followed by GC-MS.

	Composition (Area[*a], %)					
Fraction (Mean Rf value)	**A** (0.29)	**B** (0.37)	**C** (0.44)	**D** (0.48)	**E** (0.55)	**F** (0.59)
Compounds						
Terpinen-4-ol	-[*b]	-	12.93	13.82	53.91	-
Bornyl acetate	-	-	-	-	-	100
Elemol	71.68	78.83	69.65	73.46	27.03	-
***γ*-Eudesmol**	8.28	11.26	17.42	12.72	19.06	-
β-Eudesmol	5.87	5.82	-	-	-	-
α-Eudesmol	-	4.09	-	-	-	-
α-Cadinol	14.15	-	-	-	-	-
Others	0.02	-	-	-	-	-

[*a] Percentage for peak area is the ratio of each peak area to the total peak area on the basis of FID values of GC-MS

[*b] Not detected

4.4. The *in vitro* antibacterial assay for the major active phytochemicals

4.4.1. Disk diffusion assay for active phytochemicals

In this study, 2D TLC-DB of NFSV EO showed the presence of three antibacterial compounds (Rf = 0.29 - 0.59). Antibacterial activities of the active compounds, ELE, TER, and γ-EUD, were evaluated for two gram-positive bacteria (*L. monocytogenes* and *S. aureus*).

All the compounds showed higher antibacterial activity than crude EO from NFSV (Fig. 8 and Table 7). For *L. monocytogenes*, γ-EUD exhibited maximum inhibition zone (16.3 ± 4.9 mm) among the active compounds. The inhibition zone of *L. monocytogenes* treated with other active compounds ranged from 13.2 to 15.5 mm, whereas that treated with crude EO was 10.6 ± 1.2 mm in diameter. For *S. aureus*, ELE and γ-EUD showed a large inhibition zone of 10.5 ± 0.5 and 10.3 ± 0.7 mm, respectively. TER exhibited higher antibacterial activity against *S. aureus* than crude EO, although they showed relatively small activity (8.1 ± 0.0 mm). According to Park (2008), for antibacterial activity against foodborne pathogens of TER, TER showed similar activity against *L. monocytogenes*, but not consistently against *S. aureus*. Since the trends of the two strains were the same in previous studies, the reason for the difference in results in this study is attributed to the difference in the concentration of the substance and the strains.

According to the results, both bacteria showed relatively high sensitivity to the three active compounds compared to the same amount of EO. This result can be attributed to the fact that the amount of each compound in the EO was less than the amount applied to the actual disk.

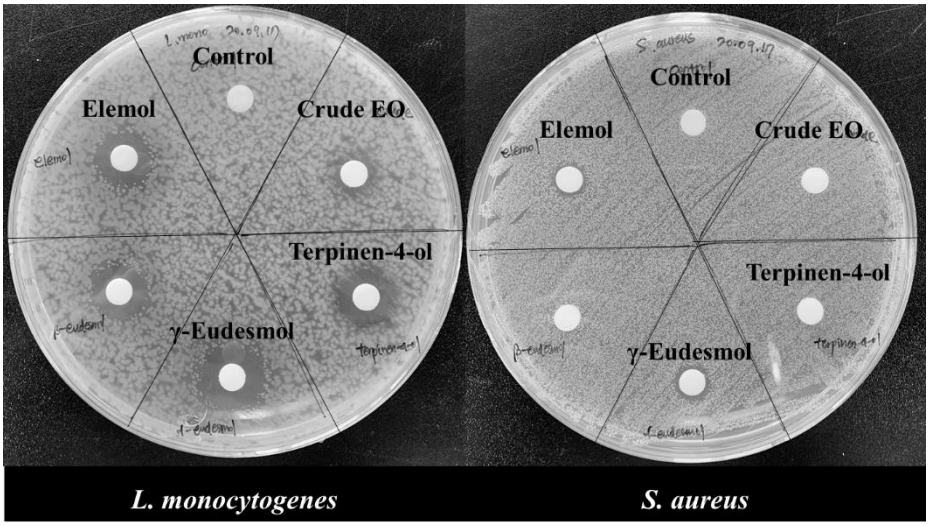


Figure 8. Difference between inhibition zones caused by single active phytochemicals consisting of NFSV EO against *L. monocytogenes* and *S. aureus*.

Table 7. Antibacterial activities of active phytochemicals consisting of NFSV essential oil and positive control against *L. monocytogenes* and *S. aureus*.

Microorganism	Diameters of inhibition zone (mm)				
	Crude EO	ELE	γ-EUD	TER	Tetra -cycline
L. monocytogenes	10.6 ±1.2	15.5 ±2.0	16.3 ±4.9	13.2 ±5.4	18.0 ±1.0
S. aureus	0.0 ±0.0	10.5 ±0.5	10.3 ±0.7	8.1 ±0.0	31.9 ±2.0

* All the samples and positive control were placed on the disk at 2 mg/disk and 2 μg/disk, respectively.

4.4.2. MICs and MBCs for active phytochemicals

The antibacterial activity of the individual active compounds was evaluated by determining the MICs and MBCs to two gram-positive bacteria (Table 8). The MIC values of ELE and γ-EUD against *L. monocytogenes* were both 312.5 μg/mL, whereas the MBC values were above 625 μg/mL. TER had relatively high MIC values (1,250 μg/mL) against *L. monocytogenes* compared to other compounds. *S. aureus* also showed sensitivity to active compounds. ELE had the highest MICs and MBCs against *S. aureus*, both were 312.5 μg/mL whereas TER showed lower activity against *S. aureus* than crude EO. The MIC and MBC values of γ-EUD were 1,250 and 2,500 μg/mL, respectively, which showed the same activity as that of crude EO. In particular, for *L. monocytogenes*, the MIC values of ELE and γ-EUD were 100 times and those TER were 400 times different from that of the positive control (tetracycline). Conversely, for *S. aureus*, the antibacterial activity of each phytochemical was found to be 8,900 times lower than that of the positive control.

These results indicated that ELE and γ-EUD had potential antibacterial activity as a single substance. Moreover, TER was detected in a large amount in the bacteria-active fraction, but showed relatively lower activity than ELE and γ-EUD, indicating that TER does not directly exhibit antibacterial activity.

Previous studies confirmed that oxygenated terpenes such as terpineol, farnesol, and citronellol exhibit better antimicrobial activity than compounds that have hydrocarbon structures such as limonene, terpinene, and α-pinene (Guimarães *et al.*, 2019). Furthermore, previous studies suggested that sesquiterpene alcohols such as ELE and eudesmol isomer in *Phebalium squamulosum* EO exhibited moderate to high antimicrobial activities, whereas relatively low activities were reported in the EOs consisting of monoterpenes (Ayaz *et al*., 2017). In addition to the results of these previous studies, ELE and γ-EUD are thought to be the key compounds with antibacterial activity.

Table 8. Antibacterial activities of active phytochemicals consisting of NFSV EO expressed as MICs and MBCs.

Microorganism		Antibacterial active concentration (μg/mL)				
		Crude EO	ELE	γ-EUD	TER	Tetracy-cline
L. monocytogenes	MIC	1,250	312.5	312.5	1,250	3.125
	MBC	2,500	1,250	625	5,000<	12.5
S. aureus	MIC	1,250	312.5	1,250	2,500	0.035
	MBC	2,500	312.5	2,500	2,500	0.035

4.4.3. Combinational effects for antibacterial activities of active phytochemicals

The MIC values of the three active compounds (ELE, γ-EUD, and TER) showing antibacterial activity were higher than that of each compound contained in the actual crude EO. The results of the previous GC-MS chemical analysis suggested that the content of each compound in 1,250 μg/mL crude EO was 249 μg/mL of ELE, 42 μg/mL of γ-EUD, and 94 μg/mL of TER, which was less than the MIC value of each phytochemical. The checkerboard method was used to investigate whether the combination of ELE, γ-EUD, and TER produced higher inhibition via a synergistic interaction.

The checkerboard method and isobologram for the three active compounds yielded combinational profiles, additive and synergistic effects (Fig. 9 and Table 9). The combination study was performed by depicting an isobologram for EC_{90} (effective concentration for 90% inhibition) and EC_{50} (effective concentration for 50% inhibition) against *S. aureus* and *L. monocytogenes* (Fig. 9). The value of the area under the straight line in each graph means the concentration that shows the synergistic effect, which was output based on the FICI.

For the results for *L. monocytogenes*, all three combinations of active compounds showed synergistic (FICI < 1) and additive effects (FICI = 1), whereas synergistic effects for *S. aureus* were only observed in the TER/ELE combination. The combined MICs showing synergistic effects against *L. monocytogenes* were ELE at 39.1 μg/mL with TER of 312.5 μg/mL (FICI = 0.375), ELE at 78.1 μg/mL with TER of 156.3 μg/mL (FICI = 0.375), 39.1 μg/mL of ELE and γ-EUD (FICI = 0.25), and γ-EUD at 39.1 μg/mL with TER of 156.3 μg/mL (FICI = 0.25). An outstanding synergy was observed against *S. aureus* when 39.1 μg/mL of ELE was combined with 1,250 μg/mL of TER. All other dose combinations showed additive effects. For both bacteria at all

concentrations tested, neither indifferent nor antagonistic effects appeared (Table 9).

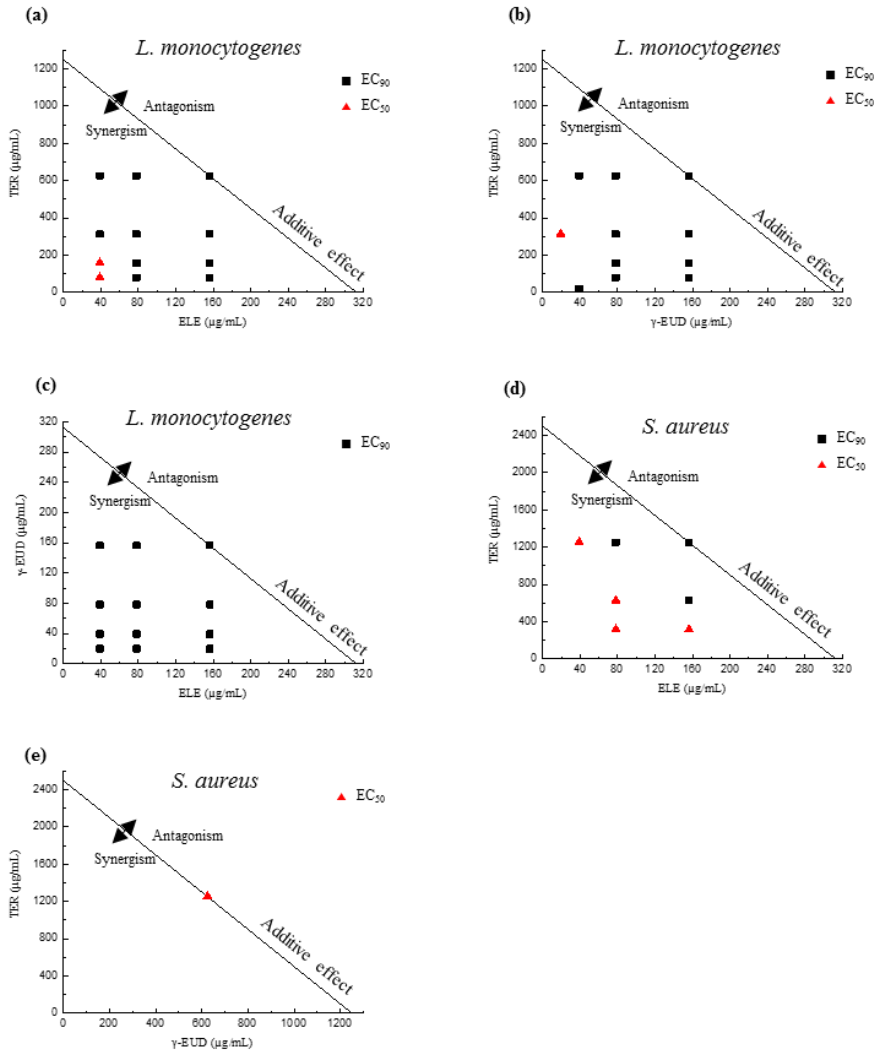


Figure 9. Isobologram depicting the combinational effects of phytochemicals against (a, b, c) *L. monocytogenes* and (d, e) *S. aureus*. EC_{50} (▲) and EC_{90} (■) were depicted for (a) TER/ELE, (b) TER/γ-EUD, and (c) γ-EUD/ELE against *L. monocytogenes*, and (d) TER/ELE and (e) TER/γ-EUD against *S. aureus*.

Table 9. Combination results of active phytochemicals against *L. monocytogenes* and *S. aureus* by using the checkerboard titration method.

Bacterium	Components		MIC (μg/mL)		Combined MIC (μg/mL)		FICI	Effect
	A	B	A	B	A	B		
L. monocytogenes	ELE	γ-EUD	312.5	312.5	39.1	39.1	0.25	Synergistic
	ELE	TER	312.5	1,250	39.1	312.5	0.375	Synergistic
					78.1	156.3	0.375	Synergistic
	γ-EUD	TER	312.5	1,250	39.1	156.3	0.25	Synergistic
S. aureus	ELE	γ-EUD	312.5	1,250	156.2	625	1	Additive
	ELE	TER	312.5	2,500	39.1	1,250	0.625	Synergistic
	γ-EUD	TER	1,250	2,500	625	1,250	1	Additive

4.4.4. Bacterial growth kinetics for synergistic combinations

The growth kinetics of two bacteria on ELE/TER combination having synergistic activity for both strains are shown in Fig. 10, in which the y-axis is the result of converting the measured optical density value to a log scale based on the initial cell concentration. Through the change of the bacterial concentration over time, the ELE/TER combination remarkably reduced the bacterial concentration in comparison with that of their individual effects at 24h (Fig. 10). Each combination of ELE/TER exhibited the same trends for both the bacteria. ELE at 1/2 MIC plus TER at 1/2 MIC combination showed the highest growth inhibition activity, whereas ELE at 1/4 MIC plus TER at 1/2 MIC exhibited the lowest inhibitory activity among the conditions of the combination.

The TER-infused inoculum grew in a similar pattern to that of the control. Conversely, when ELE was at 1/2 MIC (156.3 μg/mL) and TER was at 1/4 MIC (312.5 μg/mL), significant growth inhibition was noted (Fig. 10). The synergistic effect of ELE/TER was noted because ELE at 1/2 MIC showed a remarkable synergistic effect, even though the concentration of TER was 1/4 MIC. These findings confirmed the synergistic effect of the phytochemicals, i.e., ELE/TER combination, in the above experiment. The antibacterial activity of each EO was amplified by the addition of 312.5 μg/mL TER and 157.3 μg/mL ELE.

Friedman *et al.* (2004) divided the antibacterial agents into two types: fast- and slow-acting compounds. In this study, the standard time for fast- and slow-acting compounds was set to 2 h. ELE was considered a fast-acting compound since it inactivated both the strains (*L. monocytogenes* and *S. aureus*) immediately after treatment (Fig. 10). In contrast, TER did not exhibit growth inhibition at 1/2 MIC, ELE at 1/2 MIC with TER at 1/2 MIC and ELE at 1/4

MIC with TER at 1/2 MIC showed fast action than the single compound of ELE. In addition to the growth inhibition rate, ELE and TER might exert a synergistic effect, as revealed by previous studies, since the final cell counts of the strains treated with ELE at 1/2 MIC plus TER at 1/2 MIC were maintained at the lowest when combined. Previous studies suggested that the compounds exhibiting antibacterial activity in both MICs and time-kill kinetics had polar substituents and low molecular weight. These characteristics might increase the antibacterial capacity by increasing the penetration of substances through the cell membrane (Guimarães *et al.*, 2019). That is, ELE exhibiting fast action against strains might penetrate a part of the cell wall made of peptidoglycan to destroy the structure, and TER further penetrates the destroyed cell wall, thereby completely inhibiting cell capacity.

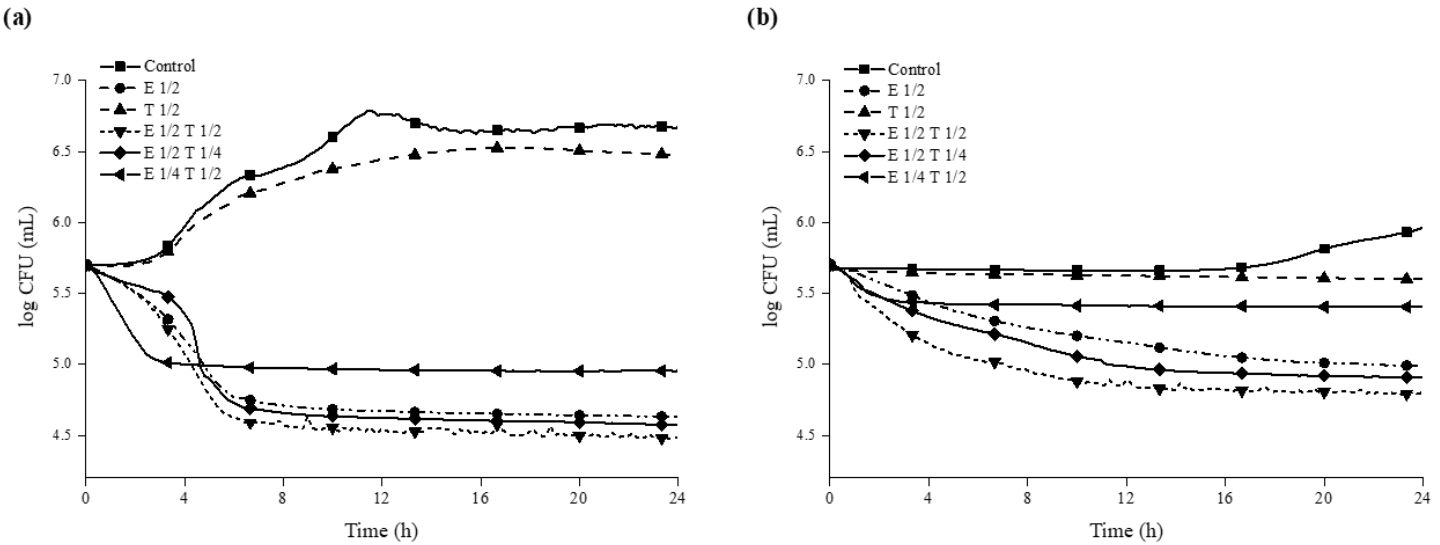


Figure 10. The growth curve tracking for the synergistic combination of ELE and TER against (a) *L. monocytogenes* and (b) *S. aureus*. Control (-■-), ELE at 1/2 MIC (-●-), TER at 1/2 MIC (-▲-), ELE at 1/2 MIC plus TER at 1/2 MIC (-▼-), ELE at 1/2 MIC plus TER at 1/4 MIC (-◆-), and ELE at 1/4 MIC plus TER at 1/2 MIC (-◀-).

5. Conclusion

In this study, the antibacterial sesquiterpenoid in Sargent juniper essential oils (EOs) was investigated by using direct bioassay techniques such as the disk diffusion method and MIC analysis against foodborne pathogens (*L.monocytogenes, S. aureus, S. typhimurium,* and *E. coli* O157:H7). The antibacterial compounds depending on the chemical compositions were identified using a 2D TLC-DB. The 2D TLC-DB results were used to evaluate the combined effect of the isolated substances contained in the active fraction by applying checkerboard methods and analyzing the growth curve.

The antibacterial activities of EOs from two Sargent junipers harvested from Chilbosan Forest (CF) and National Forest Seed Variety Center (NFSV) exhibited a remarkable difference. The antibacterial activity was higher in NFSV EO with higher contents of elemol, bornyl acetate, sabinene, terpinen-4-ol, and α-copaen-11-ol. The lowest MIC value of 1,250 μg/mL was noted for NFSV EO against *L. monocytogenes*, and the maximum MIC value of 5,000 μg/mL was noted against *E. coli* O157:H7.

The NFSV EO showed a relatively higher sensitivity to the gram-positive bacteria. The 2D TLC-DB analysis results for the NFSV EO showed that the growth of gram-positive bacteria (*L. monocytogenes* and *S. aureus*) was suppressed in the range of Rf = 0.14–0.59, whereas that of gram-negative bacteria was not inhibited in any region. The fraction in which the growth of gram-positive bacteria was inhibited contained terpinen-4-ol, bornyl acetate, elemol, γ-eudesmol, β-eudesmol, α-eudesmol, and α-cadinol. The chemical structures of each single phytochemical are all in the form of terpenoid and had a hydroxyl group in common. In particular, the content of elemol, terpinen-4-ol, and γ-eudesmol was higher in the range from Rf = 0.29 to Rf = 0.55 where the growth of bacteria was completely suppressed. In the SEM images of gram-

positive bacteria at the boundary of the inhibition zone, the bacterial cell wall was destroyed or warped, whereas that of the unaffected zone had a smooth surface and destroyed. Certain components in the EO, such as elemol, terpinen-4-ol, and γ-eudesmol, were considered to cause morphological changes in the strains.

The antibacterial activities were evaluated for elemol, γ-eudesmol, and terpinen-4-ol, which are expected to be the key phytochemicals in the destruction of cell wall of gram-positive bacteria. Elemol showed the highest activity with MIC values of 312.5 μg/mL against both gram-positive bacteria, *L. monocytogenes* and *S. aureus*. γ-Eudesmol and terpinen-4-ol suppressed bacterial growth at higher concentrations than those of elemol. The concentration of active phytochemicals exhibiting antibacterial activity was greater than that of the EO, which is considered to show activity even at lower concentrations owing to the synergistic effect between the phytochemicals. Therefore, the interactions between each phytochemical depending on the combined ratio were evaluated.

Synergistic effects were observed in all combinations of elemol, γ-eudesmol, and terpinen-4-ol against *L. monocytogenes* by using the checkerboard method. For *S. aureus*, the elemol/terpinen-4-ol combination showed a synergistic effect, and the other combinations showed only the additive effect. In particular, terpinen-4-ol, which did not exhibit significant bacterial growth inhibitory activity with an isolated compound, showed considerable antibacterial activity when a small amount of elemol was added because of the synergistic effect. According to the growth curve for elemol and terpinen-4-ol against both gram-positive bacteria, growth inhibition was faster when terpinen-4-ol was added with elemol than when the single compound, elemol, was added to the inoculum. Terpinen-4-ol could promote elemol to inhibit bacterial growth.

These results indicate that the addition of a trace amount of elemol to terpinen-4-ol-based natural antibiotics that are abundant in nature can improve the potential for producing highly effective antimicrobial agents. In addition, elucidation of the efficacy of new natural resources-derived sesquiterpenoid against foodborne pathogens might increase the industrial potential of elemol and terpinen-4-ol as synergistic phytochemicals.

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초 록

식중독균 활성 저해를 위한 시너지적 파이토케미컬로서의 눈향나무 정유 기반 세스퀴터페노이드의 활용

이다송

환경재료과학전공

농림생물자원학부

서울대학교 대학원

항생제 내성 균주의 출현으로 현재 다양한 식중독 유발 균주에 즉각적으로 대응할 수 있는 새로운 항생제의 개발이 절실해지고 있다. 이에 대한 대응책의 일환으로, 최근 식물 정유에서 유래된 파이토케미컬이 천연 항미생물제로 주목받고 있다. 정유 그 자체로도 뛰어난 항미생물 활성을 보이는 것으로 밝혀졌으나, 정유 내 파이토케미컬의 구성 성분과 이들 간의 상호작용에 대한 연구는 미미한 실정이다. 따라서, 식물 유래 파이토케미컬 중 특히 단리가 어려운 세스퀴터페노이드의 경우 항미생물 활성과 상호작용에 대한 연구가 지속적으로 요구되어 왔다.

본 연구에서는 식물 유래 세스퀴터페노이드의 활성을 박막 크로마토그래피-생리활성 평가법(TLC-DB)과 분취용 박막

크로마토그래피(prep. TLC)를 통해 다각도로 구명하고자 한다. 대상 정유는 고도에 따라 채취한 국내의 눈향나무 2 종의 잎에서 추출하였다. 디스크 확산법을 통해 그람양성균과 그람음성균에 대한 두 눈향나무 정유의 항박테리아 활성을 확인하고, 활성 정유에 대하여 1 차 스크리닝을 진행하였다. 이후 최소억제농도(MIC)와 최소살균농도(MBC)를 측정함으로써 두 종류의 눈향나무 정유 중 항박테리아 활성을 나타냈던 정유의 미생물 생장 억제 농도를 정량하였다. TLC 평가로 정유 내 활성물질을 구명하였으며, prep. TLC 를 통해 해당 활성 분획을 획득하였다. 또한 주사전자현미경을 통해 활성 분획이 처리된 균주의 세포벽 파괴 현상을 관찰하였다. 활성 분획 내 단일 파이토케미컬의 항박테리아 활성을 측정하였으며, 이들 간의 상호작용은 체커보드 방법과 성장곡선 추적 법을 통해 확인하였다.

본 연구를 통해, sabinene, elemol, terpinen-4-ol, α-copaen-11-ol 및 γ-terpinene 을 다량 함유한 눈향나무 정유(NFSV 정유)가 식중독 균주에 대해 더 높은 활성을 가진다는 것을 밝혔다. 항균 평가 결과, 고활성을 띄었던 NFSV 정유는 시험 그람양성균에 대해 높은 활성을 나타낸 반면, 그람음성균의 경우 상대적으로 낮은 활성을 보였다. TLC-DB 유도 스크리닝을 통해, 그람양성균은 NFSV 정유가 분획 된 TLC 판 상에서 생장이 억제되었으나, 그람음성균의 경우 분획 된 TLC 판 상에서는 생장억제가 발생하지 않았다. 해당 생장억제 영역의 주사전자현미경 이미지에서는, 균주 세포벽을 손상 및 파괴가 관찰되었고, 이를 통해 활성물질에 의한 균주의 세포벽 파괴 및 생장 억제를 입증하였다. 특히 2D TLC-DB 결과, NFSV 정유 내의 항박테리아

활성물질 중 elemol, γ-eudesmol, terpinen-4-ol 이 다량 검출되었다. 앞선 결과를 바탕으로 세가지 주요 화합물의 그람양성균에 대한 조합효과를 구명하였다. Elemol, γ-eudesmol 은 *L. monocytogenes* 와 *S. aureus* 에 대하여 모두 뛰어난 생장억제 효과를 나타냈다. Elemol 과 γ-eudesmol 은 균주 생장억제에 직접적으로 영향을 미치는 반면, terpinen-4-ol 은 상대적으로 낮은 활성을 나타내며 간접적으로 균주 생장억제에 영향을 미칠 것으로 사료된다. 체커보드법을 통한 isobologram 결과, *L. monocytogenes* 에 대하여 terpinen-4-ol 이 elemol 혹은 γ-eudesmol 과 함께 투입되었을 때 해당 조합이 시너지 효과를 보임을 밝혔다. 체커보드 분석을 통해 평가한 두 그람양성균인 *L. monocytogenes* 과 *S. aureus* 에 대하여 elemol 과 terpinen-4-ol 의 조합에서 MIC 값을 감소됨을 확인하였고, 이들 균주의 생장곡선 추적 결과 elemol/terpinen-4-ol 조합이 시너지 효과를 나타냄을 입증하였다.

자연에서 쉽게 획득할 수 있는 모노터페노이드인 terpinen-4-ol 에 미량의 elemol 성분이 첨가됨으로써 항박테리아 활성을 크게 증폭시켰다. 이러한 시너지 효과는 식음료 산업에서 천연 항균제로의 잠재성을 향상시키며, 자연 유래 세스퀴터페노이드 기반 파이토케미컬의 산업적 활용가능성을 증진시킬 수 있을 것으로 기대된다.

주요어: 눈향나무, 세스퀴터페노이드, 식중독균, 항박테리아활성, 시너지효과, 엘레몰
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