A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Insecticidal, tick-repellent and anti-inflammatory activities and safety properties of supercritical fluid extract from *Chamaecyparis obtusa*

초임계유체추출을 이용한 편백 성분의 살충, 기피, 항염효과 및 안전성 평가

By

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Major in Entomology

Department of Agricultural Biotechnology

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UNDER THE DIRECTION OF ADVISER YOUNG-JOON AHN
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Insecticidal, tick-repellent and anti-inflammatory activities and safety properties of supercritical fluid extract from *Chamaecyparis obtusa*

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Abstract

Supercritical fluid extraction (SFE) is a rapid, selective and convenient method for sample preparation prior to the analysis of compounds in the volatile product of plant matrices. In this study, inscticidal and repellency describes a laboratory study to examine the active constituent isolated from the *C. obtusa* and supercritical fluid extract from *C. obtusa* against house dust mites, hard tick and German cockroach. Also, effects of the insecticidal phytochemicals on AChE inhibition and action on a model aminergic receptor system of German cockroach were investigated.

The acaricidal constituent of *C. obtusa* branch oil was identified as *β*-thujaplicin against adult *D. farinae* and *D. pteronyssunus*. The active constituent of the *C. obtusa* branch was identified as *a*-thujaplicin ($C_{10}H_{12}O_2$) by spectroscopic analyses. The toxicity of *C. obtusa* oil and *β*-thujaplicin and the acaricides to adult *D. farinae* was examined by the direct contact bioassay. As judged by 24 h LD_{50} values, acaricidal activity of *C. obtusa* oil (3.42 μg/cm^2) and *β*-thujaplicin (4.02 μg/cm^2) used was high than that of benzy

benzoate (4.31 µg/cm^2), dibutyl phthalate (31.62 µg/cm^2) and DEET (78.34 µg/cm^2). Also, The toxicity of *C. obtusa* oil and *β*-thujaplicin and the acaricides to adult *D. pteronyssinus* were examined by the direct contact bioassay. As judged by 24 h LD_{50} values, acaricidal activity of *C. obtusa* oil (4.12 µg/cm^2) and *β*-thujaplicin (3.84 µg/cm^2) used was high than that of benzy benzoate (4.21 µg/cm^2), dibutyl phthalate (30.72 µg/cm^2) and DEET (75.64, µg/cm^2). These results indicate that acaricidal activity of *C. obtusa* likely results from by *a*-thujaplicin. *a*-Thujaplicin merits further study as potential house dust mite control agents or lead compounds.

The repellency of *C. obtusa* oil was evaluated in comparison with the two commercial repellency (Icaridin and DEET) against *H. longicornis*. In filter-paper diffusion method, *C. obtusa* oil (3.2 µg/cm^2) gave 100% repellency and icaridine (3.2 µg/cm^2) gave 96.1 % repellency, respectively, at 120 min. The repellency of the 31 compounds against *H. longicornis* was compared with that of DEET at 3.2 mg/cm^2. Responses varied according to the test compound and exposure time. *α*-Terpinene, terpinene-4-ol and α-terpineol gave 100% repellency 30 min post-treatment but 85.5, 56.4 and 68.9% repellency at 120 min. *C. obtusa* oil, *α*-terpinene, terpinene-4-ol and *α*-terpineol described merit further study as potential fumigants or leads for the control of *H. longicornis*.

The toxicity of *C. obtusa* oil and 31 compounds against adult females of the German cockroach, *Blattella germanica* L., was examined using direct contact and vapor phase toxicity bioassays and compared with those of deltamethrin, dichlorvos, permethrin, and propoxur, four commonly used insecticides. As judged by the 24 h LC_{50} values, *C. obutsa* oil (2.77 mg/cm^2) was less effective than propoxur (0.18 mg/cm^2), dichlorvos (0.007 mg/cm^2), deltamethrin (0.013 mg/cm^2), or permethrin (0.05 mg/cm^2). The insecticidal

activity of 31 compounds used against adult female *B. germanica* As judged by the 24 h LC_{50} values, the adulticidal activity of *a*-thujone (0.09 mg/cm^2) was comparable to that of permethrin (0.05 mg/cm^2). The toxicity of linalool and *a*-terpineol ranging from 0.10 to 0.12 mg/cm^2, were higher than that of propoxur (0.18 mg/cm^2). In vapor phase toxicity tests, *a*-terpineol (21.89 mg/L of air) was the most toxic fumigant followed by linalool (26.20 mg/L of air) and *a*-thujone (36.22 mg/L of air) On the basis of 24 h LC_{50} values. *a*-Terpineol described merit further study as potential fumigants or leads for the control of *B. germanica*. The German cockroach AChE inhibitory activity of 31 test compounds were tested. Base on IC_{50} values, α-Pinene (IC_{50}, 0.38 mM) exhibited the strongest inhibiton.

In the study, the constituent compounds of *C. obutsa* oil utilizing supercritical flulid extraction, examined their anti-inflammatory effect *in vitro* and *in vivo*. *In vitro*, Anti-inflammatory effects were assessed by measuring the levels of secretory proteins and mRNA of TNF-α, IL-4 and IL-6 production. The results showed that β-pinene induced nitric oxide production (LPS stimulated RAW 264.7 cells), down-regulates *m*RNA expression of inflammatory genes and pro-inflammatory cytokines, such as iNOS and IL-6. Also, β-thujaplicin suppressed the DNP-BSA induced β-hexosaminidase secretion in IgE-sensitized RBL-2H3 cells, down-regulates mRNA expression of inflammatory genes and pro-inflammatory cytokines, such as TNF-α and IL-4. Furthermore, an in *vivo* anti-inflammatory study was performed using a TPA-induced skin inflammation mouse model, and the results showed that β-pienen and β-thujaplicin reduced TPA-induced inflammation and attenuated the expression of iNOS in TPA-induced mouse skin tissue. Thus, β-pienen and β-thujaplicin demonstrated anti-inflammatory activity both in LPS-induced RAW 264.7 cells and TPA-stimulated mouse skin and may therefore serve as a potential anti-inflammatory agent.

The safty evaluation was designed to determine the safety profile of *C. obtusa* oil utilizing supercritical flulid extraction, based on acute oral toxicity, primary dermal irritation and primary eye irritation studies. The acute oral LD_{50} of *C. obtusa* oil is greater than 5,000 mg/kg of body weight in rats. Also, the single-dose acute dermal LD_{50} of *C. obtusa* oil is greater than 5,000 mg/kg of body weight in both male and female rats. The primary dermal irritation index (PDII) of *C. obtusa* oil was calculated to be 1.8, thus classifying *C. obtusa* oil to be slightly irritating to the skin. In primary eye irritation studies, the maximum mean total score (MMTS) of *C. obtusa* oil was observed to be 0.33 and classifying *C. obtusa* oil to be minimally irritating to the eye.

In conclusion, *C. obtusa* derived preparations containing active constituents described herein merit further study as potential insecticides for the control of acaricide against house dust mites, repellency aganist hard tick and cockroach for protecting humans from bites and nuisance caused by vectors. Also, presented that possible applications of essential oil from *C. obtusa* as a useful anti-inflammatory agent. Moreover, the anti-inflammatory effects of the major pharmacological components present in the essential oil of *C. obtusa* might accelerate the development of new drugs for various inflammatory diseases.

Key words: Supercritical fluid extraction, *Chamaecyparis obtusa*, house dust mite, hard tick, cockroach, acetylcholinesterase inhibition, anti-inflammation, safety evaluation.

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Contents

Abbreviation

AChE, acetylcholinesterase

DEPT, distortionless enhanced by polarization transfer

EI-MS, electron impact-mass spectrometry

ELISA, enzyme-linked immunosorbent assay

EPA, environmental protection agency

GC, gas chromatography

GC-MS, gas chromatography-mass spectrometry

HPLC, high presser liquid chromatography

IL-4, interleukin-4

IL-6, interleukin-6

iNOS, inducible nitric oxide

LDL, low density lipoprotein

LPS, lipopolysaccharide

NMR, nuclear magnetic resonance

RAW 264.7, Murine macrophage leukemia

RH, relative humidity

RT, reverse transcription

SFE, supercritical fluid extraction

TNF- *a*, tumer necrosis factor-*a*

WHO, world health organization

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Introduction

The development of new separation techniques for the chemical industries has received a lot of attention due to the environmental restrictions, the need for minimizing the energy costs, and human health regulations (Coelho L.A.F. *et al.,* 1996). Since the last decade supercritical fluid extraction is regarded as an alternative to the classical liquid-solid extraction techniques such as maceration, percolation, lixiviation, microwave assisted extraction, etc. which are characterised by a number of inconvenients, particularly the solvent toxicity, its cost and impact on the environment, contrarily to the supercritical fluid extraction, a clean process which enables to achieve high extraction yields and hence important degrees of purity of the desired compounds (Zermane *et al.,* 2012).

In industrialized countries most individuals spend over 95% of their time within closed environments, where the air may contain pollutants and contaminants at higher concentrationsthan those found in the open air (Mumcuoglu *et al.,* 1999). For this reason the quality of the air in closed environments is presently considered to be at least as important as that of the open air for health in general and for atopic dermatitis, bronchial asthma, rhinitis, and conjunctivitis in particular (Mumcuoglu *et al.,* 1999). It has been suggested that this increase is in response to provocative factors, such as house dust mites (Rothe and Grant-Kels, 1996). Toward the development of diagnostics and atherapeutic vaccine, important house dust mite allergens have been explored and now classified as major house dust mite antigens (Tovey, 1981; Lind, 1985; Platts-Mills, 1989). The most important pyroglyphid mites are *Dermatophagoides pteronyssinus* (Trouessart) and

Dermatophagoides farinae (Hughes) for the following three reasons: (1) their cosmopolitan occurrence and abundance; (2) they are a major source of multiple potent allergens; (3) their causal association with sudden infant death syndrome (Arlian, 1989; Fain *et al*., 1990; Helson, 1971). Living environmental changes (such as a rise in the number of apartment households with central heating, space heating, tighter windows, and wallto-wall carpeting) have improved conditions for the growth of dust mites (Van bronswijk *et al.*, 1971). Control of these mite populations has been principally through the use of chemicals such as benzyl benzoate and *N*,*N*-diethyl-m-toluamide (DEET) (Van bronswijk *et al.*, 1971). Although effective, their repeated use has sometimes resulted in the widespread development of resistance (Van bronswijk *et al.*, 1971; Lee *et al.*, 2001), undesirable effects on nontarget organisms, and fostered environmental and human health concerns (Lee *et al*., 2001, Hayes *et al.*, 1991). These problems have highlighted the need for the development of new strategies for selective control of dust mites.

The hard tick, *Haemaphysalis longicornis*., which is widely distributed in East Asia and Oceania (Fujisaki *et al.*, 1994; Silva *et al.*, 2005; Tanaka *et al.*, 2012), can act as a vector for viruses, chlamydia, rickettsia, bacteria, and protozoa (Fujisaki *et al.*, 1994; Guan *et al.*, 2002; Jongejan and Uilenberg, 2004; Li *et al.*, 2009). In South Korea, this species is the most abundant tick and infests humans and domestic animals, with infestation rates peaking during the summer. At present, common anti-tick measures include the spraying of synthetic drugs, regular medicated bathing of livestock, using smoke agents in forests as acaricidal drugs, and applying synthetic acaricides both in the environment and to animals (Regassa, 2000; Iori *et al.*, 2005; Patarroyo *et al.*, 2009). The use of anti-tick drugs usually includes chemical agents containing synthetic pyrethroids,

organophosphates, and amitraz (Gazim *et al.*, 2011). Although these acaricides are beneficial when properly used, misuse has led to poisoning of humans and animals (FAO, 1998; Zorloni *et al.*, 2010). It also leads to the problems of the agents being only partially successful, of the parasites developing serious resistance, of drug residue, and environmental hazards (Fernandes and Freitas, 2007; Ribeiro *et al.*, 2007; Nong *et al.*, 2012, 2013).

The German cockroach, *Blattella germanica* L., is the most important primary medical insect pests because of its even cosmopolitan occurrence and abundance in homes and other buildings as potential carriers of fecal pathogens and a major source of allergens (Wirtz, 1984; Schal and Hamilton, 1990; Rozendaal, 1997; Arlian, 2002). Additionally, cockroach exuviae are found to support large populations of *Dermatophagoides pteronyssinus* Trouessart, resulting in exacerbated cases of bronchial asthma (Wirtz, 1984; Schal and Hamilton, 1990; Rozendaal, 1997). Control of cockroach populations worldwide is largely dependent on continued applications of residual insecticides such as chlorpyrifos, dichlorvos (DDVP), propoxur, pyrethrin, and pyrethroids, stomach poisons such as hydramethylnon and sulfluamid, and insect growth regulators such as flufenoxuron (Schal and Hamilton, 1990; Rozendaal, 1997). Their repeated use has disrupted natural biological control systems and led to resurgences of the cockroach (Schal and Hamilton, 1990) and has often resulted in the development of resistance (Schal and Hamilton, 1990; Cochran, 1989). Increasing levels of resistance to the most commonly used insecticides have caused multiple and overdosed treatments, fostering serious human health concerns (Schal and Hamilton, 1990). These problems have highlighted the need for the development of selective control alternatives for *B.*

germanica, particularly with fumigant action because many insecticides are repellent to them and are therefore avoided (Schal and Hamilton, 1990; Wooster and Ross, 1989) and it is difficult to reach deep, insecticide-free harborages and to apply insecticides to sensitive environments such as computer facilities, food industrial facilities, and hospitals (Schal and Hamilton, 1990).

Plant essential oils have been suggested as an alternative source of materials for insect control because some of them are selective to certain pests, often biodegrade to nontoxic products, and have little or no harmful effects on nontarget organisms (Isman, 2001; Mumcuoglu *et al.*, 2002). They can be applied to the resting and hiding places in the same way as other conventional insecticides. They also provide useful information on resistance management because certain plant extracts or phytochemicals can be highly effective against insecticide-resistant insect pests (Lindquist *et al.*, 1990; Ahn *et al.*, 1997). In addition, some plant essential oils or their constituents have been proposed as an alternative to the commonly used synthetic insecticides because they were exempted for minimum risk pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requirements (U.S. EPA, 1996). Furthermore, plant essential oils are widely available, and some are relatively inexpensive compared with plant extracts (Isman, 2001). Because of this, much effort has been focused on plant essential oils or their constituents as potential sources of commercial control agents.

In chapter I, inscticidal and repellency describes a laboratory study to examine the active constituent isolated from the *Chamaecyparis obtusa* branch and supercritical fluid extract from *C. obtusa* against house dust mites, hard tick and German cockroach. The inscticidal and repellency activities were compared with those of eight currently available

insecticides and repellents such as benzyl-benzoate, DEET, deltamethrin, dibutyl-phthalate, dichlorvos, icaridine, permethrin and propoxur to assess their use as future commercial pesticide and repellent. Also, effects of the insecticidal phytochemicals on AChE inhibition and action on a model aminergic receptor system of German cockroach were investigated.

In chapter II, the constituent compounds of *C. obutsa* oil utilizing supercritical flulid extraction, examined their anti-inflammatory effect *in vitro* and *in vivo*. *In vitro*, Anti-inflammatory effects were assessed by measuring the levels of secretory proteins and mRNA of TNF-α, IL-4 and IL-6 production. Furthermore, an *in vivo* anti-inflammatory study was performed using a TPA-induced skin inflammation mouse model. The safty evaluation was designed to determine the safety profile of *C. obtusa* oil utilizing supercritical flulid extraction, based on acute oral toxicity, primary dermal irritation and primary eye irritation studies.

Literature Review

1. Supercritical fluid extraction

Supercriticality a strange and intriguing state in which solids can dissolve in gases, and liquids can alternate between reflectivity and transparency (Poliakoff, 2001; Erika, 2005). The discovery of the supercritical state is attributed to Baron Cagniard da la Tour in 1822, who heated alcohol in a sealed gun barrel and listened to the musket ball rolling about. This acoustic effect caused by the fluctuation when the fluid passed through the critical point. He also observed that the boundary between a gas and a liquid disappeared for certain substances when the temperature was increased in a sealed glass container. Subsequent work by Hannay and Hogarth in 1879 demonstrated the solvating power of supercritical ethanol (Tc = 243 ℃; pc = 63 bar) by studying the solubility of cobalt (II) chloride, iron (III) chloride, potassium bromide and potassium iodide. They noticed that the solubility of the chlorides was much higher than would be expected by their vapor pressure. In addition, they also noted that increasing pressure caused the metal chlorides to dissolve while decreasing the pressure caused precipitation. Despite this early promise, however, the utility of supercritical fluids for extraction was left dormant for many years. The pioneering work of Francis (1954) on liquefied gases is noteworthy. In this work, Francis compiled an extensive list of solubility for 261 compounds in near-critical carbon dioxide. A major development in the use of supercritical carbon dioxide was the filing of various patents between 1964 and 1976 on extraction of coffee, tea, hops and spices. This development has led to the growth of applications in the engineering field of supercritical

fluid technology. A major development was the installation by Kraft General Foods of a decaffeination plant for their Maxwell House Coffee Division in 1987, which uses an extraction cell with a height of 25 m. This utilization of supercritical fluids for pilot and full-scale plant operations has continued to develop and diversify. In these situations supercritical fluids are being used for a wide diversity of applications. It is thus not suprising to find that supercritical fluids encompass a multidisciplinary field that includes engineers, chemists, food scientists, material scientists and workers in biotechnology, agriculture and environmental control (Poliakoff, 2001; McHugh and Krukonis, 1994; Mukhopadhyay, 2000; Dean, 1998; Tomasko, 1999; Pellerin, 1991; Erika, 2005).

The supercritical fluid extraction (SFE) technology has advanced tremendously since its inception and is a method of choice in many prcessing industries. Over the last two decades, SFE has been well received as a clean and environmentally frandly "green" processing technique and in some cases, and alternative organic solvent-based extraction of natural products (Mohammed *et al.,* 2012). Since the end of the 1970s, supercritical fluids has been used to isolate natural products, but for a long time the applications concentrated on only few products. Now the development of processes and equipment is beginning to pay off and industries are getting more and more interested in supercritical techniques (Reverchon and Taddeo, 1995).

Supercritical fluid solvents are of interest in chemical processes both for their involvement in chemical reactions as well as their solvent effects, that are influenced by pressure and temperature. Supercritical fluid (SCF) solvents such as SC-CO_2 are intermediates between liquid and gases and considered important in the separation processes based on the physicochemical characteristics including density, viscosity,

diffusivity and dielectric constant which are easily manipulated by pressure and temperature. A fluid that exists at a state above the critical temperature (Tc) and the critical pressure (Pc) is in a supercritical condition and the uniqueness of a SCF is that its density is pressure dependent. The density can be attuned from liquid to vapor condition with continuity (Mohammed *et al.*, 2012).

In a SCF extraction process, the most important regions in the pressure-temperature-composition space are those of (i) 2-phase, liquid-vapour (LV), solid-vapor (SV), or liquid-liquid (LL) equilibrium; (ii) 3-phase, liquid-liquid-vapor (LLV), solid-liquid-vapour (SLV), solid-solid-vapor (SSV) equilibria, and sometimes; (iii) 4-phase equilibria: solid-SCF mixtures. As an extractive solvent, SCF can break up a multi component mixture based on the different volatile capacities of each component (Mohammed *et al.*, 2012).

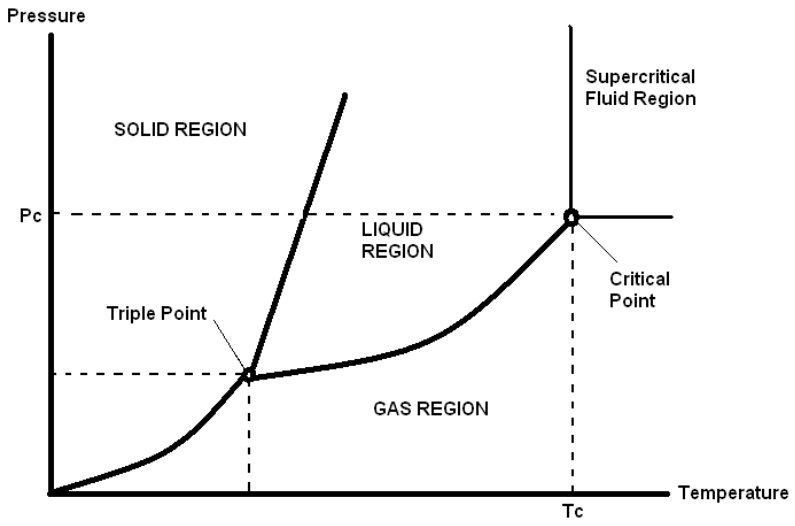


Fig. 1. Pressure-temperature phase diagram of supercritical fluid.

Supercritical fluid extraction facilitates the detachment of the extract from the supercritical fluid solvent by simple expansion. An added benefit is derived from the liquid like densities of the supercritical fluids with superior mass transfer distinctiveness that enables the easy release of solutes, compared to other liquid solvents. This uniqueness is owed to the high diffusion and very low surface tension of the supercritical fluid that enables easy infiltration into the permeable make-up of the solid matrix to reach the solute (Akgerman *et al.*, 1991; Erkey *et al.*, 1993; Salgin *et al.*, 2007).

Since the early 1980s, the use of SC-CO_2 in the extraction of oil or lipid from various sources, both plants (Ixtaina, 2010; Nik, 2009; Zaidul, 2006) and animals (Létisse, 2006; Sahena, 2010) has been studied extensively. In addition, the application of SC-CO_2 in the extraction of minor constituents from various plant sources has also been widely studied (Chuang and Brunner, 2006; Ibáñez *et al.*, 2000). Recently, Pourmortazavi et al. (Pourmortazavi, 2007) reported that carbon dioxide is used in more than 90% of all analytical supercritical fluid extractions. The low critical temperature of carbon dioxide (31.1 ℃) makes it attractive for thermally labile food products. Other solvents including ethane and propane are also used as supercritical fluids for the extraction of natural compounds. These solvents have high solvating power enabling higher solubility of lipid components compared to SC-CO_2. The main demerits of ethane and propane are their flammability and high cost. Organic solvents such as hexane are used widely in lipid extraction and fractionation operations that can achieve almost complete recovery of oil from a sample matrix. In many countries, health and safety regulations are getting stricter in addressing environmental problems created by the use of organic solvents and these issues are forcing the industries to search for alternative processing methods. The solvent

is unsafe to handle and unacceptable as it is harmful to human health and the environment, restricting its use in the food, cosmetic and pharmaceutical industries (Liu *et al.*, 2009). Furthermore, the major drawback of the solvent extracted products is the high level of residues left in the final products that must be desolventized before consumption. Therefore, SC-CO_2 is seen as a more favorable alternative to organic solvents in the extraction of fats and oils, and meets the growing consumer demand for safe natural fats and oils of excellent quality (Temelli, 2009).

Table 1. Comparison of physical and transport properties of gases, liquids and SCFs

	Gas	Supercritical fluid	Liquid
Density (kg/cm^3)	1	200-700	1,000
Diffusivity (cm^2/s)	10^{-1}	10^{-3} -10^{-4}	10^{-5}
Viscosity (cm^2/s)	10^{-5}	10^{-4}	10^{-3}

Pressure, temperature, particle size and sample pre-treatment are most important factors in oils as well as high value bioactive desired compounds extraction from the natural sources using supercritical fluid, because of the influence they have on the quality of the extracts. Generally, the solubility of the solute in the supercritical fluid solvent depends on the choice of SFE operating pressure and temperature (Bocevska and Sovova, 2007). These extraction parameters are in fact, directly responsible for the extract composition and component functionalities (Diaz-Maroto *et al.*, 2002). The performance of SFE or the quality of extracts can also be influenced by other factors such as bed geometry, the number of extraction and separation vessels and the solvent flow rate (Reverchon and Marrone, 2001).

The application of carbon dioxide as a supercritical fluid has been extensively studied over the past three decades, especially in food processing. Supercritical fluid (SCF) at its critical temperature and pressure shows unique properties different from those of either gasses or liquids under standard conditions. Carbon dioxide can easily penetrate through the solid matrix and dissolve the desired extract due to its dual gaseous and liquid-like properties. SFE exploits the ability of chemicals to function as outstanding solvents for certain desired components under a suitable set of pressure and temperature conditions. The final products obtained by supercritical carbon dioxide (SC-CO_2) extraction retain their quality and the stability of thermally labile natural components is assured without changing the bioactivity of natural molecules. SFE has been shown as a technically feasible alternative to both extraction and refining processes, especially for natural oils and bioactive compounds (Gracia *et al.*, 2009). It is fairly rapid because of the low viscosity and high diffusivity associated with SCF. Extraction selectivity can be achieved by changing the temperature, pressure and co-solvent and the extracted material is easily recovered by simply depressurizing, allowing the supercritical CO_2 to return to gaseous state and evaporate leaving little or no traces of solvent (Mohamed *et al.*, 2002). The natural fats and oils obtained by SC-CO_2 extraction are of excellent quality and are comparable to those obtained by organic solvent extraction methods (Gómez *et al.*, 1996). The solvent power of SC-CO_2 is good since it dissolves non-polar to slightly polar compounds. The addition, of small quantities of polar organic solvent as modifiers can improve the extraction of polar compound by increasing the solubility of the analyte in CO_2, or by reducing its interaction with the sample matrix or both (Rial-Otero *et al.*, 2007). Examples of the substances used thus far as supercritical solvents and their critical

temperature and pressure are given in Table 2 (Rizvi *et al.,* 1994). Among them, CO_2 is the most common supercritical fluid solvent, and has been extensively studied for its potential applications in many different fields, including the food processing industries. Due to the low critical temperature and pressure, low cost, wide availability, non-flammability and environmentally friendliness, supercritical CO_2 is the most acceptable supercritical solvent in food applications as well as in other applications without any declaration (Zaidul *et al.,* 2007).

Table 2. Properties of some solvents as mobile phase at the critical point.

Solvent	Critical Temperature (℃)	Critical Pressure (bar)
Carbon dioxide (CO_2)	31.1	72
Nitrous oxide (N_2O)	36.5	70.6
Ammonia (NH_3)	132.5	109.8
Ethane (C_2H_6)	32.3	47.6
n-Butane (C_4H_{10})	152	70.6
Diethyl ether (Et_2O)	193.6	63.8
Tetrahydrofuran (THF, $C4H_8O$)	267	50.5
Dichlorodifluoromethane (CCl_2F_2)	111.7	109.8

2. House dust mite

The house dust mite evolved 23 million years ago as a scavenger living in birds nests or similar habitats. Approximately 10,000 years ago this mite found its way into the human environment (Colloff *et al.,* 1992). The house dust mite is about half the size of a dot or period on a newspaper. The mite has no eyes, no organised breathing system,

cannot drink and lives for approximately 3-4 months. Twenty droppings a day may be produced by the mite, which means approximately 2,000 during its lifetime. The house dust mite can get nourishment from its own droppings and may eat them up to three times over. The females can lay from 60-100 eggs depending upon living conditions, which ideally, are warm dark and damp for breeding mites (Arlian, 1989).

Mite allergens are considered one of the most potent allergens in house dust. The most common genus of mites found in house dust in north America and Europe is Dermatophagoides, of which there are two species, *Dermatophagoides pteronyssunus* (European house dust mite) and *Dermatophagoides farinae* (American house dust mite). Their body parts and fecal excreta are initially 10 to 50 ㎛ in diameter but break down into smaller fragments that become airborne when dust is disturbed (Sukumar *et al.*, 1991). According to one study, more than half the weight of mite allergens within a home were found to be less than 5 ㎛ in length. Because of their small size, fecal materials are the primary health concern due to the fact that they can be inhaled into the lower airways of the lungs. If quantities of mite allergens are siginicant, IgE antibodies can form, leading to allergic reactions in the susceptible portion of the population. (Arnason *et al.*, 1989; Hostettmann and Potterat, 1997).

Along with respiratory symptoms, high levels of dust mite allergens have also been correlated with atopic dermatitis (AD), charaterized by itchy and irritated skin. In gemal, these studies suggest that those also likely to develop skin sensitization if exposed to high concentrations of mite allergens (Arnason *et al.*, 1989). As one might expect, most mite allergens are formed by adult mite during their active phase. The survival of active adult

mites (both male and female) is limited to 4 to 11 days at humidities below 50% relative humidity (RH) at 25℃. The protonymph, however, which is one of the dormant larval forms, can survive for months at low humidities and then evolve to the more active forms when optimal conditions return. These protonymphs are particularly difficult to remove with normal vacuuming since they can bury themselves within surfaces (Arlian, 1977, 1992).

D. farinae and *D. pteronyssunus* is confined to the domestic house dust environment, particularly to the microecosystems formed by mattresses, carpets, and plush toys； these are generally considered to be a subsitute for its original habitat, which was probably the nests of birds and small mammals (Wharton, 1976). Mite were found in 97% of the dust samples. The average number of mites ranged from 84 to 2,053 mite/g of dust. The most prevalent species of mites were *D. farinae* and *D. pteronyssunus*, which were found in 85.6% and 71.3% of the samples, respectively. The house dust mites (*D. farinae* and *D. pteronyssunus*, and *Euroglyphus rnaynei*) consituted 95% of the mites. Most of the mites were isolated from the carpets and sofas (37.0 % and 33.7 %, respectively) and less from the beds (29.3 %) (Mumcuoglu *et al.*, 1999). An estimated number of approximately 30,000 described species of Acari, ditributed among moer than 1,700 genera, was given by Radford (1950) but the number of undescribed species could exceed this total by up to twenty-fold (Kim, 2002).

Control of house dust mite populations includes environmental, physical and chemical methods. In relation to physical control, it may be possible to control mite levels in homes by maintaining the appropriate temperature and relative humidity in their

microhabitat because temperature and fluctuations in relative humidity in mite microhabitats directly influence the survival and population dynamics of house dust mite allergens in indoor environments (Arlian, 1977, 1992；Kwon and Ahn, 2002). Changes in living environments such as a rise in the number of apartment households with centrally installed heating, space heating, tighter windows and fitted carpets have improved conditions for mite growth, which resulted in difficulty of the mite control (Pollart et al, 1987; Kwon and Ahn, 2002).

Environmental control has been considered as one of the useful for controlling house dust mite populations. Wahing of bedding is only effective in killing mites at temperatures greater than 70 ℃ and that vacuuming of capets, although helpful in removing surface dust including mite fecal pelltes, should not be considered equivalent to replacing carpets with vinyl inadequate suction or leaking bags can exacerbate the problem by increasing the quantities of allergen that become airborne (Susan *et al*, 1987). The effect on mite allergen concentrations cannot be comparable to the effect of removing carpets, mattresses, and hot washing of bedding. But these ways are too labor-intensive to do by onself at home. It is hard to think as ultimate controlling mite method (Kwon and Ahn, 2002; Kim, 2002).

The use of liquid insecticides such as organophophates and pyrerhoids, insect growth regulators such as methoprene and repellents such as benzyl benzoate, dubutyl phthalate and DEET are still the most effective for house dust mite removal and prevention of reinfestation. Mitchell et al. (1985) alreday reported that pirimiphos methyl reduced mite populations by 60% in carpets and by more than 50% in chairs in Great Britain and acaricidal effects of the chemical persisted at least 3 weeks after application.

Pirmiphos-methyl has low mammalian toxicity and appears to be a nonirritant to asthmatic lungs. Benzyl bezoate is also very effective but irksome in application (Schober et al, 1987). It has been also reported that the juvenile hormone micics methoprene and hydroprene are effective at suppressing *D. farinae* population (Dowing *et al*., 1993). Tannic acid cannot kill mites directly but has an eliminating effect of allergen. A non-toxic fungicidal agent natamycin was found to not only inhibit the development of D. pteronyssunus on mattress-dust substrate, but reduce also the percentage of gravid females and in the average number of eggs laid per female (de Saint Gerges-Gridelet, 1987; Kwon and Ahn, 2002; Kim, 2002).

A number of recent studies in Europe have focused on reducing fungal levels in the dust (Bronswijk *et al.*, 1971; de Saint Georges-Gridelet, 1987) because fungi are a necessary component of house dust mite food chain. The bactericidal and fungicidal disinfectant spray Paragerm AK, an acaricide consisting mainly of balsmic essences, is a typical hospital disinfectant mixture and used in houses in France because of the repeated observation that hospitals lack dust mites and its safety to use around asthmatics (Penaud *et al.*, 1977). However, trials using Paragerm AK in houses were limited by its smell, and it is not as effective at killing mites as are some other chemical (Penaud *et al.*, 1977). Nipagin diminishes the number of xerophilic fungi needed for mite nutrition (Penaud *et al.*, 1977). Household disinfectants combined with benzyl benzoate were also found to be effective against house dust mites in the Netherlands (Schober *et al.*, 1987).

3. Hard tick

Ticks were the first arthropods to be established as vectors of pathogens and currently they are recognized, along with mosquitoes, as the main arthropod vectors of disease agents to humans and domestic animals globally (Jongejan and Uilenberg, 2004; Colwell, 2001). Moreover, the incidence of tick-borne diseases (TBDs) is increasing worldwide (Nicholson *et al.*, 2010; Dantas-Torres, 2010). For instance, more than 250,000 human cases of Lyme borreliosis were reported from 2000 to 2010 in the United States (http://www.cdc.gov/lyme/stats/chartstables/reportedcases_statelocality.html), and the disease is also increasing in Europe, where over 50,000 cases are reported each year in humans (Piesman and Eisen, 2008).

Haemaphysalis is a genus of hard ticks found in Europe, Asia, Africa and Australia. They affect cattle, sheep, goats, horses, camels, dromedaries, dogs, cats, and humans, but also a large range of wild mammals, birds and reptiles. There are about 150 Haemaphysalis species worldwide, with species-specific distribution and prevalence. Most species occur in Southeast Asia. The most relevant species for livestock and pests are the following: *Haemaphysalis concinna* is found in Central Europe, East and Southeast Asia, China, Japan. It occurs in humid scrubby forests as well as in meadows and peatlands. It avoids dense forests. *Haemaphysalis inermis* is found around the Mediterranean, in East Europe, Near and Middle East. Prefers deciduous and mixed forests as well as grasslands. *Haemaphysalis leachi*, the yellow dog tick, the African dog tick. Occurs in Tropical and Southern Africa. *Haemaphysalis longicornis*, the bush tick, the scrub tick. Is found in Australia, New Zealand, China, Japan, Western Pacific Rim, several Pacific Islands (e.g. Hawaii). Prefers mild and humid climates. *Haemaphysalis*

punctata, the red sheep tick; Europe, Middle East, North Africa and Japan. It prefers moderate Atlantic climate but supports also mild continental climate. Usually it does not appear in large numbers. (http://parasitipedia.net/index.php?option=com_content&view=article&id=2549&Itemid= 2826)

As all hard ticks, *Haemaphysalis* ticks are obligate parasites: they cannot survive without feeding blood of their hosts. *Haemaphysalis* ticks are of a small to medium size, i.e. engorged adult females reach about 1 cm length. They have short mouthparts and are of a yellowish to dark brownish color. Most *Haemaphysalis* species are three-host ticks, i.e. larvae, nymphs and adults can be found free-living in the environment waiting for a suitable host to pass by. Larvae and nymphs usually infect small mammals (e.g. rodents, rabbits, moles, but also reptiles and birds), whereas adults prefer larger ones, often dogs, but also livestock, wildlife and humans. The life cycle lasts 1 to 3 years depending on the species and on ecological and climatic conditions. (http://parasitipedia.net/index.php?option=com_content&view=article&id=2549&Itemid= 2826)

As for all ticks, *Haemaphysalis* bites cause stress and blood loss to the hosts. A few ticks are usually well tolerated by livestock and pets, but infestations with dozens or hundreds of ticks can significantly weaken affected animals and cause weight loss, reduced fertility, decreased milk production. *Haemaphysalis* ticks are all vectors of several tick-borne diseases of livestock, pets and humans. *Haemaphysalis concinna* can transmit tularaemia, various Rickettsia species and several encephalitis viruses. Haemaphysalis inermis can cause tick paralysis. Haemaphysalis leachi leachi can transmit canine and feline babesiosis, Mediterranean spotted fever, Q-fever, Boutonneuse fever. *Haemaphysalis longicornis* can transmit several species of Rickettsia, Ehrlichia,

Anaplasma, and Theileria as well as Q-fever. Haemaphysalis punctata can cause tick paralysis and transmits several microbial pathogens such as of Babesia, Anaplasma, Brucella, Theileria, Borrelia, tularaemia, Q-fever, tick-borne encephalitis virus. (http:// parasitipedia.net/index.php?option=com_content&view=article&id=2549&Itemid= 2826)

Biological control of *Haemaphysalis* and other ticks using their natural enemies remains a research topic has not yet delivered really effective and sustainable solutions for tick population control. Tick predators such as insects (e.g. ants, wasps), small rodents or birds (e.g. cattle egrets, guinea fowls, oxpeckers, etc.) do in fact consume significant amounts of ticks, but will not eliminate tick populations. One reason is that they are not specific tick predators, but feed on whatever is available. The most promising results have been obtained so far with so called entomopathogenic fungi, i.e. fungi that are pathogenic for ticks and other insects (e.g. *Beauveria bassiana*, *Metarhizium anisopliae*, etc.). In certain countries there are commercial products for crop protection based on such fungi, and there are reports that they can effectively control ticks on cattle too. However, a systematic commercial approach to tick control using such fungi is still missing. In addition, such biological control methods target tick population control and not protection of single animals or knocking down already attached ticks on an infected animal. For the reasons previously mentioned population control of *Haemaphysalis* ticks is virtually impossible in most endemic regions (http://parasitipedia.net/index.php?option=com_ content&view=article&id=2549&Itemid= 2826).

There are no specific vaccines against *Haemaphysalis* ticks. A few reports have shown efficacy of the Boophilus tick vaccine against other tick species, but little is know about its effect against *Haemaphysalis* ticks. There are no repellents, chemical or natural,

that effectively prevent *Haemaphysalis* ticks from attaching to livestock or pets, or that cause already attached ticks to detach. There are no traps that effectively reduce tick populations in the pastures: whatever domestic or wild animals are much more attractive for ticks than any possible trap. So far there are no herbal remedies commercially available really effective for protecting livestock from Haemaphysalis ticks in endemic regions (http://parasitipedia.net/index.php?option=com_content&view=article&id=2549 &Itemid= 2826).

Chemical control and prevention of Haemaphysalis ticks on livestock is based on the same parasiticides used for the control of other tick species. Most products approved for tick control are based on veteran contact parasiticides of various chemical classes such as: Organophosphates (e.g. chlorfenvinphos, chlorpyrifos, coumaphos, diazinon, ethion, etc.), Amidines (mainly amitraz, also cymiazole), Synthetic pyrethroids (e.g. cypermethrin, deltamethrin, flumethrin, permethrin). All are for on animal use. In most countries there are currently no tickicides approved for pasture treatment against cattle ticks. The major reason is that to effectively control the ticks on pasture the dose will be lethal for almost any invertebrate fauna in the pastures and for most of the vertebrates that feed on them (birds, reptiles, rodents, etc.). In addition, pastures would be also contaminated with chemicals, which could be toxic for livestock or leave illegal residues in meat and/or milk (http://parasitipedia.net/index.php?option=com_content&view=article&id=2549&Itemid= 2826).

4. Cockroach

Approximately 4,000 species of cockroaches have been described from most parts of the world, although the suborder Blattaria is primarily tropical and subtropical. Most

species live in forests where some are semi-aquatic, others burrow, bore into wood, or live in caves; a few even live commensally with other insects, such as ants (Roth and willis, 1957). They are predominalntly nocturnal and are either omnivorous or vegetarian. While most cockroaches have little contact with man, about a dozen species have become adapted to a peridomestic environment and have some degree of medical importance. In temperate regions they have colonized and flourished in man-made pseudo-tropical situations. The three most important species are *Blattella germanica*, *Blatta oriental* is and *Periplaneta americana* (Jung, 2006).

There are two main areas of concern with regard to cockroaches and their potential for causing disease in humans: the allergic reactions, including lung and skin reactions, and the vector potential of cockroaches for a variety of organisms. Cockroaches can provoke clinical reactions as a contactant, injectant, ingestant, and inhalant (Jung, 2006).

Cockroaches are common in human dwellings worldwide and are the sources of potent allergens. Allergy to cockroaches was first reported in 1964 by Bemton and Brown, who showed that 40% of their asthmatic patients were sensitive to cockroaches. Since that time, many studies have shown that among exposed populations in the United States, China, Costa Rica, Hong Kong, Malaysia, Taiwan, Japan, and Europe, the sensitivity to cockroaches ranges from 23-60%. Among patients with atopic rhinitis, atopic dermatitis, and asthma, the prevalence of sensitivity to cockroach allergens in Italy, France, and Brazil is reported to be 12.5, 24.5, and 55%, respectively (Birnbaum, 1995; Peruzzi, 1999; Santos, 1999). Studies of inner-city areas of the United States reported that 36.8% of the children were allergic to cockroach allergens (Rosenstreich, 1997). Cockroach species known to be the sources of allergens include the Asian cockroach, *Blattella*

asahinai; *B. germanica*; *P. americana*; and Oriental cockroach, Blatta orientalis (Helm, 1990; Kang, 1996). The brown-banded cockroach, *S. longipalpis*, commonly inhabits homes and is a potential source of allergens that has not been investigated. The cockroaches, *B. germanica* and *P. americana*, commonly found in human dwellings and schools worldwide, are the sources of many potent characterized allergens (Mendoza, 1970; Schulaner, 1970; Pollart, 1991; Chapman, 1996; Eggleston, 1996; van Wijnen, 1997). Cockroach allergens are associated with fecal material, saliva, secretions, exoskeletons, and dead bodies (Arruda, 2001). Extracts prepared from wash of bodies, dead bodies, exoskeletons, and feces all contain allergens (Lehrer, 1991; Menon, 1991). Through study for allergens from *B. germanica* and *P. americana*, the allergens Bla g 1, Bla g 1 (BD 90k), Bla g 2, Bla g 4, Bla g 5, Bla g 6, Per a 1, Per a 3 and Per a 7 have been partially characterized (Schou, 1990; Pollart, 1991; Arruda, 1997).

Aside from the reports on allergies, there is also strong evidence that cockroaches are also involved in spreading infectious diseases. Cockroaches are capable of carrying disease organisms and bacteria on their bodies and in their fecal material. Most of the literature on this topic reports on one of two models of study: either (i) roaches were collected from a specific location and compared with control roaches from another area, or (ii) roaches were inoculated or fed with pathogenic organisms. In both models, the esults were determined by testing for the external and/or internal presence of the pathogenic organism (Cloarec *et al.*, 1992; Fotedar *et al*, 1991).

There are the following categories of pathogens detected in roaches; bacteria, viruses, fungi, protozoa, and helminths. These pathogens can cause diseases naturally or experimently. The diseases caused by the pathogenic bacteria which have been found

occurring naturally in or on cockroaches include both general and specific infections such as bubonic plague (*Pasteurella pestis*), dysentery (*Shigella alkalescens*), diarrhea (*Shigella paradysenteriae*), urinary tract infection (*Pseudomonas aeruginosa*), abscesses (*Staphylocaccus anrenns*), food poisonings (*Clostridium perfringens, Escherichia coli, Streptococcus fae*cal and *P. aeruginosa*), gastroenteritid (*Salmonella schottmuelleri, S. bredeney* and *S. oranienburg*), typhoid fever (*Salmonella typhosa*), leprosy (*Mycobacterium leprae*) and nocardiosis (Actinomyces spp.). The diseases caused by pathogenic bacteria experimentally introduced either into or onto cockroaches include Asisatic cholera, pneumonia, diphtheria (*Cotynebacterium diphtheriae*), glanders (*Pseudomonas mallei*), anthrax (*Bacillus anthracis*), black leg (*Clostridium chauvoei*), tetranus (*Clostridium tetani*) and tuberculosis (Mycobacterium spp.). The diseases are occurred mainly by bacteria, but it becomes known that viruses, fungi, protozoa, and helminthes can almost set up various diseases (Jung, 2006).

To suppress cockroach populations in the urban environment, physical, biological, and chemical methods all have been used. Physical methods mean mainly exclusion and sanitation. As for sanitation, cockroaches need largely three factor; moisture, harborage, and food resource. The single most important factor in determining cockroach survival is availability of water. Especially, *B. germanica* live less than two weeks when there is no supply of free water even if food is abundant. Therefore it is important to eliminate all source of moisture that contributes to cockroach survival. They prefer dark, warm crack and crevices. Excess clutter provides numerous locations suitable for cockroach habitation. The elimination of these harborages is important in controlling infestations. However, physical controls have limits of prevention of cockroach, because there is a

great deal of inconvenience for people to do these methods (Jung, 2006).

Biological controls mean using natural enemies. Natural cockroach enemies include parssitic wasp, nematodes, spiders, toads, etc. However, natural enemies have not played an important role in managing cockroach population (Jung, 2006).

Chemical control means mainly management using synthetic insecticides. Until recently, efforts to control cockroaches have relied almost exclusively on repeated applications of synthetic pesticides. Surveys have shown that more than 1/3 of all the pesticides used in developed country are applied in the urban environments and most of these pesticides are applied in the home. However, the chemical approach to cockroach control has become increasingly less popular. This is primarily due to the development of multiple chemical resistance among cockroach populations and increased public concern about insecticide exposure in theit living environment. These two issues have greatly emphasized the need for a more holistic and less toxic approach to cockroach management (Jung, 2006).

5. Essential oil

Essential oils extracted from a wide variety of plants and herbs have been traditionally employed in the manufacture of foodstuffs, cosmetics, cleaning products, fragrances, herbicides and insecticides. Further, several of these plants have been used in traditional medicine since ancient times as digestives, diuretics, expectorants, sedatives, etc., and are actually available in the market as infusions, tablets and/or extracts. Essential oils are also popular nowadays due to aromatherapy, a branch of alternative medicine that claims that essential oils and other aromatic compounds have curative effects. Moreover,

in the last decades, scientific studies have related many biological properties (antioxidant, anti-inflammatory, antiviral, antibacterial, stimulators of central nervous system, etc.) of several plants and herbs to some of the compounds present in the essential oil of the vegetal cells (Chao *et al.*, 2005; Jirovetz *et al.*, 2006; Mimica-Dukic *et al.*, 2004; Tuberoso *et al.*, 2005).

Plant essential oils are produced commercially from several botanical sources, many of which are members of the mint family (Lamiaceae) (Isman, 1999). The oils are generally composed of complex mixtures of monoterpenes, biogenetically related phenols, and sesquiterpenes (Isman, 1999). Examples include 1,8-cineole, the major constituent of oils from rosemary (*Rosmarinus officinale*) and eucalyptus (*Eucalyptus globus*); eugenol from clove oil (*Syzygium aromaticum*); thymol from garden thyme (*Thymus vulgaris*); and menthol from various species of mint (Mentha species) (Isman, 1999). A number of the source plants have been traditionally used for protection of stored commodities, especially in the Mediterranean region and in southern Asia, but interest in the oils was renewed with emerging demonstration of their fumigant and contact insecticidal activities to a wide range of pests in the 1990s (Isman, 2000). The rapid action against some pests is indicative of a neurotoxic mode of action, and there is evidence for interference with the neuromodulator octopamine (Enan, 2001; Kostyukovsky, 2002) by some oils and with GABA-gated chloride channels by others (Priestley, 2003).

Some of the purified terpenoid constituents of essential oils are moderately toxic to mammals, but, with few exceptions, the oils themselves or products based on oils are mostly nontoxic to mammals, birds, and fish (Isman, 2000; Stroh, 1998). However, as broad-spectrum insecticides, both pollinators and natural enemies are vulnerable to

poisoning by products based on essential oils (Isman, 2006). Owing to their volatility, essential oils have limited persistence under field conditions; therefore, although natural enemies are susceptible via direct contact, predators and parasitoids reinvading a treated crop one or more days after treatment are unlikely to be poisoned by residue contact as often occurs with conventional insecticides (Isman, 2006).

6. Perspective

Because of insecticide resistance, environmental concern, and health safety, the use of many conventional insecticides such as OP and carbamate insecticides will be restricted by recent pesticide regulation policies in the USA (US EPA, 2010), EU (Anonymous, 2005), and South Korea (KLRI, 2007). Until 2003, the US government banned or severely restricted 64 insecticides belonging to the categories of UN PIC (Prior Informed Consent), UN Severely Hazardous Pesticide Formulations (SHPF), and US PIC lists such as chJordinieform and ethyl parathion (US EPA, 2003). In spite of the wide spread public concerns for long-term health and environmental effects of conventionally used insecticides, plant-based natural insecticides have not yet occupied much portion in the insecticide marketplace (Chang, 2013). Due to the improved conditions for mosquito growth by changes in living environments and global warming, increase in the mosquito population and their vector diseases is forecasted (Chang, 2013). Accordingly, consumer's demands for new minimum-risk mosquitocides will be increasing. Considering these situations, botanical mosquitocides might replace some synthetic ones in the near future (Chang, 2013).

Many researches on the plant source such as plant extracts, plant essential oils, and

their constituents to use as an insecticide are being carried out Until now, any natural products able to exceed the effect of conventional insecticide were not developed (Chang, 2013). Considering the actual state, natural product-based biocides will play a role as alternatives in specific situations or parts, not in all parts that synthetic chemicals are used (Chang, 2013). In addition, some plant-derived constituents gave high toxicity to some aquatic organisms (Chang, 2013). Therefore, these insecticidal compounds may be treated in a limited area, such as closed septic tank or the serious insecticide-resistance location where the mosquito population has been increased rapidly (Chang, 2013).

Many of these compounds have rat oral acute LD_{50} values in the 2-3 g/kg range, whereas commercial insecticides consisting of mixtures of essential oil compounds produce <50 % mortality in rat (often no mortality) at 5 g/kg, the upper limit required for acute toxicity tests by most pesticide regulatory agencies (Isman, 2001). Until now, mosquitoes have developed resistance to various insecticides, but resistance mechanism associated with octopamine receptors has not been reported with mosquitoes (Isman, 2001). Continued application of pyrethroid, carbamate, organophosphates, and insect growth regulators are frequently used for the control of mosquitoes. Dependence on these insecticides to manage the mosquito-borne diseases is considerable success, but also caused harmful effect on other organisms in the ecosystems (Isman, 2001). Due to the toxicity as well as growing incidence of insecticide resistance, the need for novel insecticides and for naturally occurring insecticides has been increased (Isman, 2001). This societal need for the development of more selective and more new strategies against mosquito resulted in the detailed studies on plant essentia] oil or extracts (Siddiqui *et al.*, 2004; Rasheed *et al.*, 2005; Amer and Mehlhom, 2006 a, b; Sharma *et al.*, 2006).

Chapter I. Insecticidal and tick-repellent activities of supercritical fluid extract from *Chamaecyparis obtusa*

Introduction

Toward the development of diagnostics and atherapeutic vaccine, important house dust mite allergens have been explored and now classified as major house dust mite antigens (Tovey, 1981; Lind, 1985; Platts-Mills, 1989). The most important pyroglyphid mites are *Dermatophagoides pteronyssinus* (Trouessart) and *Dermatophagoides farinae* (Hughes) for the following three reasons: (1) Their cosmopolitan occurrence and abundance; (2) They are a major source of multiple potent allergens; (3) Their causal association with sudden infant death syndrome (Arlian, 1989; Fain *et al.,* 1990; Helson, 1971). Control of these mite populations has been principally through the use of chemicals such as benzyl benzoate and *N,N*-diethyl-m-toluamide (DEET) (Van bronswijk *et al.,* 1971). Although effective, their repeated use has sometimes resulted in the widespread development of resistance (Van bronswijk *et al.,* 1971; Lee *et al.,* 2001), undesirable effects on nontarget organisms, and fostered environmental and human health concerns (Lee *et al.,* 2001, Hayes *et al.,* 1991). These problems have highlighted the need for the development of new strategies for selective control of dust mites.

The hard tick, *Haemaphysalis longicornis*., which is widely distributed n East Asia and Oceania (Fujisaki *et al.,* 1994; Silva *et al.,* 2005; Tanaka *et al.,* 2012), can act as a vector for viruses, chlamydia, rickettsia, bacteria, and protozoa (Fujisaki *et al.,* 1994; Guan *et al.,* 2002; Jongejan and Uilenberg, 2004; Li *et al.,* 2009). In South Korea, this

species is the most abundant tick and infests humans and domestic animals, with infestation rates peaking during the summer. At present, common anti-tick measures include the spraying of synthetic drugs, regular medicated bathing of livestock, using smoke agents in forests as acaricidal drugs, and applying synthetic acaricides both in the environment and to animals (Regassa, 2000; Iori *et al.,* 2005; Patarroyo *et al.,* 2009). The use of anti-tick drugs usually includes chemical agents containing synthetic pyrethroids, organophosphates, and amitraz (Gazim *et al.*, 2011). Although these acaricides are beneficial when properly used, misuse has led to poisoning of humans and animals (FAO, 1998; Zorloni *et al.,* 2010). It also leads to the problems of the agents being only partially successful, of the parasites developing serious resistance, of drug residue, and environmental hazards (Fernandes and Freitas, 2007; Ribeiro *et al.,* 2007; Nong *et al.*, 2012, 2013).

The German cockroach, *Blattella germanica* L., is the most important primary medical insect pests because of its even cosmopolitan occurrence and abundance in homes and other buildings as potential carriers of fecal pathogens and a major source of allergens (Wirtz, 1984; Schal and Hamilton, 1990; Rozendaal, 1997; Arlian, 2002). Control of cockroach populations worldwide is largely dependent on continued applications of residual insecticides such as chlorpyrifos, dichlorvos (DDVP), propoxur, pyrethrin, and pyrethroids, stomach poisons such as hydramethylnon and sulfluamid, and insect growth regulators such as flufenoxuron (Schal and Hamilton, 1990; Rozendaal, 1997). Their repeated use has disrupted natural biological control systems and led to resurgences of the cockroach (Schal and Hamilton, 1990) and has often resulted in the development of resistance (Schal and Hamilton, 1990; Cochran, 1989).

Plant essential oils have been suggested as an alternative source of materials for insect control because some of them are selective to certain pests, often biodegrade to nontoxic products, and have little or no harmful effects on nontarget organisms (Isman, 2001; Mumcuoglu *et al.*, 2002). They can be applied to the resting and hiding places in the same way as other conventional insecticides. They also provide useful information on resistance management because certain plant extracts or phytochemicals can be highly effective against insecticide-resistant insect pests (Lindquist *et al.*, 1990; Ahn *et al.*, 1997). In addition, some plant essential oils or their constituents have been proposed as an alternative to the commonly used synthetic insecticides because they were exempted for minimum risk pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requirements (U.S. EPA, 1996). Furthermore, plant essential oils are widely available, and some are relatively inexpensive compared with plant extracts (Isman, 2001). Because of this, much effort has been focused on plant essential oils or their constituents as potential sources of commercial control agents (Isman, 2001).

In this chapter, Inscticidal and repellency describes a laboratory study to examine the active constituent isolated from the *C. obtusa* branch and supercritical fluid extract from *C. obtusa* against house dust mite, hard tick and German cockroach. The inscticidal and repellency activities were compared with those of eight currently available insecticide and repellent, benzyl-benzoate, DEET, deltamethrin, dibutyl-phthalate, dichlorvos, icaridine, permethrin and propoxur to assess their use as future commercial pesticide and repellent. Also, effects of the insecticidal phytochemicals on AChE inhibition and action on a model aminergic receptor system of German cockroach were investigated.

Materials and Methods

1.1. Apparatus

^{1}H and ^{13}C NMR spectra were recorded in DMSO with a Bruker NMR at 600 MHz (TMS as an internal standard), respectively, and chemical shifts are given in δ (parts per million). UV spectra were obtained in methanol with a Jasco V-550 spectrometer and EI-MS spectra on a JEOL GSX 400 spectrometer. Silica gel (0.063 - 0.2 mm. Merck, Darmstadt, Germany) was used for column chromatography. Pre-coated silica plates (Kieselgel 60 F_{254}, Merck) were used for analytical thin-layer chromatography (TLC). A high performance liquid chromatography (HPLC; 2535Q Preparative System, Waters) was used for isolation of active priciples.

1.2. Chemicals

Thirty-one compounds used in this study were as follows: *a*-cedrene, *β*-chmigrene, *a*-humulene, *cis*-nerolidol and *a*-terpinene were purchased from Aldrich (Milwaukee, WI); *a*-pinene, camphene, sabinene, *β*-pinene, myrcene, phellandrene, 4-cymene, limonene, *γ*-terpienen, linalool, lisobornyl acetate, terpinene-4-ol, *a*-terpineol, *a*-fenchyl acetate, linalyl acetate, *a*-thujone, terpinyl acetate, longpinene, trans-caryophyllene, *β*-caryophyllene, neryl isobutyrae, *a*-cedrol, *trans*-nerolidol and terpinolene from ChromaDex (California, United States); *β*-eudesmol was supplied by Sigma (St. Louis, MO); *β*-thujaplicin was supplied by Tokyo Kasei (Tokyo, Japan). Deltamethrin and dichlorvos were obtained from the Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon,

Korea. Icaridine, Permethrin and propoxur were obtained from the National Institute of Health, Korea Center for Disease Control and Prevention, Seoul, Korea. Benzyl benzoate, and *N*,*N*-diethyl-*m*-toluamide (DEET) were purchased from Aldrich (Milwaukee, WI). Dibutyl phthalate was supplied by Sigma (St. Louis, MO). All other chemicals were of reagent grade.

1.3. Isolation of active constituents from *C. obutsa*

The dried *C. obtusa* branch (5 kg), purchased as commercially available product, were finely powdered, extracted twice with methanol (10 L) at room temperature, and filtered(Tokyo filter paper No. 2, Tokyo Roshi, Japan). The combined filtrate was concentrated in vacuo at 35 ℃ to yield approximately 2.8%. The extract (140 g) was sequentially partitioned into *n*-hexane (39.9 g), chloroform (9.1 g), ethyl-acetate (13.3 g), butanol (37.1 g) and water-soluble (40.6 g) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation (EYELA autojack NAJ-100 Japan) at 35 ℃, and the water portion was freeze dried. The hexane (15 g) portion was chromatographed on a silica gel column (Merck 70-230 mesh, 600 g, 5.5 i.d. × 68 cm) and successively eluted with a gradient step of *n*-hexane: EtOAc (5:1 to 0:1, v/v), giving seven fractions (H1-H7). The bioactive H2 (4.2 g) fraction was rechromatographed on a silica gel column and successively eluted with *n*-hexane:EtOAc (8:2, v/v). The column fractions were analyzed by TLC, and fractions with similar TLC patterns were pooled. In this step the six fractions (H21-H26) were obtained. The active H22 fraction (1.1 g) was purified by preparative HPLC (2535Q Preparative System, Waters) for separation of the biologically active constituent. The column was Shim pack

VP-ODS (250L × 4.6 mm, Shimadzu), using *n*-hexane : EtOAc (80:20) at a flow rate of 1 mL/min and detection at 240 nm. In this step the five fractions (H221-H225) were obtained. The active H221 fraction (157 mg) was rechromatographed under the same condition. Finally, an active compound (87 mg) with a retention time of 3.25 min was isolated. The structure of the active isolate was determined by instrumental analysis. ^{1}H and ^{13}C NMR spectra were recorded in DMSO with a Bruker NMR at 600 MHz (TMS as an internal standard), respectively, and chemical shifts are given in δ (parts per million). UV spectra were obtained in methanol with a Jasco V-550 spectrometer and EI-MS spectra on a JEOL GSX 400 spectrometer.

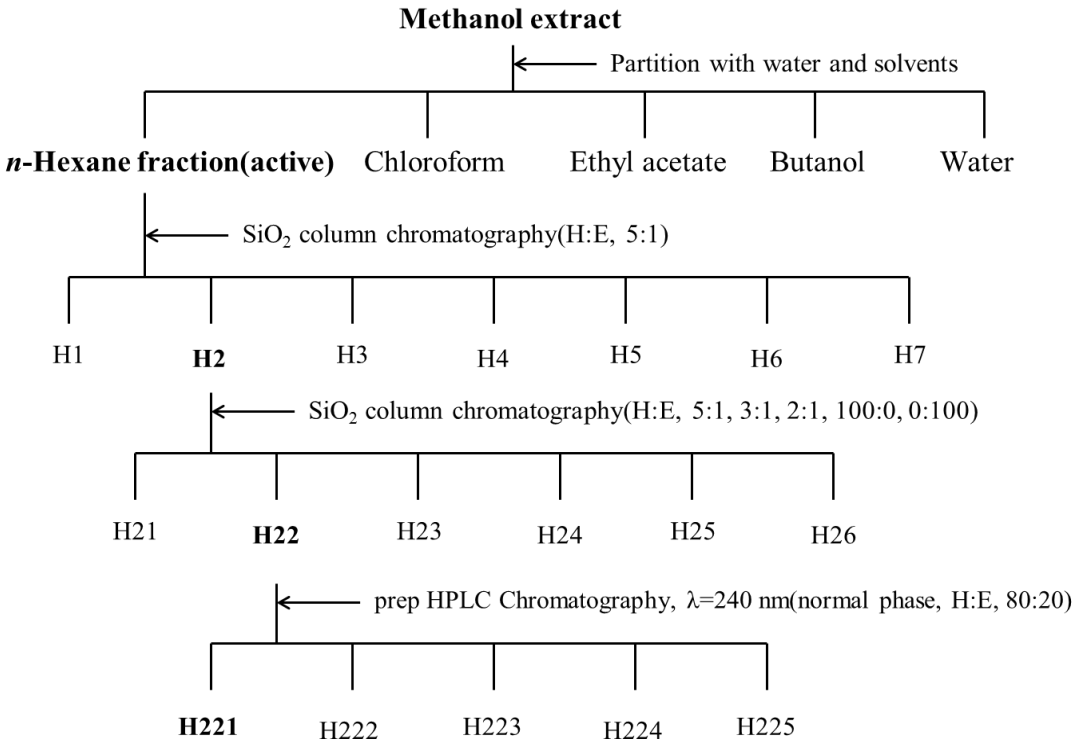


Fig. 2. Isolation procedures of repellent principles from *C. obutsa* branch.

1.4. Supercritical fluid extraction of *C. obtusa*

The experiments were carried out in the dynamic extraction unit shown in Fig. 3, which mainly consists of three parts: a CO_2 reservoir, an extractor vessel and three separator vessels in series, accompanied by a thermostatic bath, a metering pump, a cryostat, the necessary instrumentation to control the pressures, temperatures, mass flow rates and valves for the extract collection. The operating temperature and pressure can reach up to 60 ℃ and 500 bar, respectively, with a maximum gas mass flow rate of 0.70 ml/min. As a pretreatment, *C. obtusa* leaves were dried and finely ground. A mass of 100 g was weighed in a precise Scaltec instrument balance (precision of ±0.1mg) and the mean particle diameter of the resulting powder was determined at around 1 mm.

The sample was then allowed to reach the constant extraction temperature before charging CO_2 into the high-pressure pump from the storage cylinder. The CO_2 gas was further compressed to the desired pressure of the pump. After 1 hr, a time corresponding to the static extraction, the extractor valve was opened and the intermediate valves between the separators were continuously adjusted in order to regulate the pressure and, hence, to keep a constant flow rate. Samples were taken every 15 min, by means of the valves placed at the bottom of the separators, and weighed to obtain the mass of the essential oil. The dynamic extraction was pursued for 3.5 hr, after which it was noted that the extracted mass was very low. Finally, the glass containing the extracted essential oil was kept in a freezer, ready for chromatographic analysis. The operating conditions such as pressure, temperature, particle diameter and supercritical fluid flow rate were fixed.

The extractions were performed at temperatures in the range of 30 – 60 ℃, particle size 0.5 - 2 mm and at pressures between 100 and 500 bar. These ranges are the most

frequently adopted for similar system types where the CO_2 is at its supercritical state. Therefore, the temperature was controlled thermostatically and the pressure was regulated by means of a membrane pump, which enabled to reach the extraction pressure. The maximum mass flow rate was 0.70 ml/min through the pump, which was also connected to a cryostat in order to liquefy the CO_2. The flow rate of the latter was measured by means of a Coriolis force flow meter and, hence, indicated the amount of CO_2 used during the extraction. The separation (CO_2/essential oil) was carried out at 10 ℃ in the first separator and at 30 ℃ in the two others. The temperature of the first separator was set at 10 ℃ in order to enable the recovery of certain secondary components such as waxes, resins, fatty acids etc. present in the solid matrix and, hence, a The CO_2 used in this study was 99.5 % (w/w) pure and supplied by Hanguk-gas-well coporation. Also, the extracted essential oil could be recovered from the separators and CO_2 vented to the atmosphere.

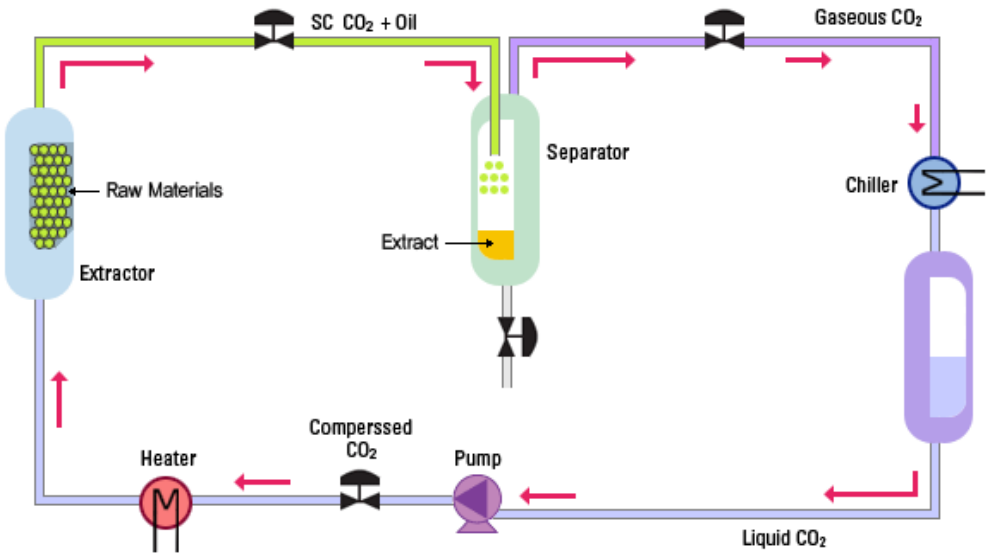


Fig. 3. Diagram of supercritical fluid.

1.5. Test insects

1.5.1. Dust Mites

Cultures of *D. farinae* and *D. pteronyssinus* were maintained in the laboratory for 5 years without exposure to any known acaricide. They were reared in plastic containers (15 cm × 12 cm × 6 cm) containing 30 g of sterilized diet (fry feed No. 1/dried yeast, 1:1 by weight) at 25 ± 1 ℃ and 75% relative humidity in the dark. The fry feed (Miropa) was purchased from Korea Special Feed Meal Co. Ltd., South Korea.

1.5.2. Hard tick

H. longicornis was collected from naturally infested goats in Jeju, South Koera. After collection, *H. longicornis* were maintained in the laboratory without exposure to any known acaricide. They were reared in plastic containers (15cm × 12cm × 6 cm) at 25 ± 1 ℃ and 75% relative humidity in the dark.

1.5. 3. Cockroaches

Cultures of *B. germanica* were maintained in the laboratory for 9 years without exposure to any known insecticide. They were reared with calf chow pellets (Samyang, Seoul) in glass jars (30 cm × 30 cm) at 27 ± 1 ℃ and 55 ± 5% relative humidity (RH) under a 12:12 h light/dark cycle.

1.6. GC/MS analysis

The *n*-hexane fraction of *C. obtusa* branch and supercritical fluid extract of *C.*

obtusa was analyzed on a gas chromatograph-mass spectrometer (GCMS-QP2010, Shimazu). The GC column was a 30 m × 0.25 mm i.d. VF-5MS (0.25 ㎛) fused silica capillary column (J&W Scientific, Folsom, CA). The GC conditions were as follows: injector temperature, 230 ℃; column temperature, isothermal at 50 ℃ for 5 min, then programmed to 280°C at 3 ℃/min, and held at this temperature for 19 min; ion source temperature, 200 ℃. Helium was used as the carrier gas at a rate of 1.0 mL/min. The effluent of the GC column was introduced directly into the source of the MS. Spectra were obtained in the EI mode with 70 eV of ionization energy. The sector mass analyzer was set to scan from 50 to 800 amu for 2 s. Compounds were identified by comparison with retention times, and the mass spectra were obtained with authentic standards on the GC-MS system used for analysis. When an authentic sample was not available, identification was carried out by comparison of mass spectra with those in the mass spectra library (The Wiley Registry of Mass Spectral Data, 8th ed.).

1.7. Bioassay for 3 species medical insects

1.7.1. Dust mite

A contact bioassay was used to evaluate the toxicities of *C. obtusa* oil, 31 test compounds, *C. obtusa* derived material and three insecticides applied to cotton fabric, to *D. farine* and *D. putrescentiae* (7-10 days old). Four to six concentrations of each test materials in 20 ㎕ of ethanol were applied to circular pieces of black cotton fabric (5 cm diameter). Control fabric circle received 20 ㎕ of ethanol. After drying in a fume hood for 20 sec, each fabric circle was placed on the bottom of a petri dish (5 × 1 cm).

Groups of 25 adults mites were individually placed on the fabric and each petri dish was covered. Benzyl benzoate, DEET, and dibutyl-phthalate served as standards for comparison in contact toxicity test.

Treated and control (ethanol only) mites were held under the same conditions as used for colony maintenance. Evaluation of acaricidal activity was made at 25 hr after treatment under a binocular microscope (×20). Mites were considered to be dead if appendages did not move when they were prodded with dowel. All treatments were three times.

1.7.2. Tick

A filter-paper repellent bioassay was used to evaluate the toxicities of *C. obtusa* oil, 31 test compounds, *C. obtusa* derived material and two repellents, DEET and Icaridine, to *H. longicornis*. A volume of 1 ml of the concentrations was added to Petri dishes (10 cm diameter, 2 cm height) containing filter paper disks to absorb the liquid. 10 larval ticks were placed on the filter paper, sprayed twice with the extract to be tested, at 25 - 27 ℃ and 70 - 80 % relative humidity, with each treatment consisting of three replicates.

1.7.3. Cockroach

1.7.3.1. Contact Toxicity Bioassay

A filter-paper contact toxicity bioassay was used to evaluate the toxicities of *C. obtusa* oil, 31 test compounds, *C. obtusa* derived material and four insecticides, deltamethrin, dichlorvos, permethrin, and propoxur, to adult female *B. germanica*. Cockroaches were exposed to appropriate amounts of materials, each of which were

dissolved in 50 $\mu\ell$ of methanol or acetone and applied to filter papers (Whatman no. 2; 5 cm diameter). Control filter papers received 50 $\mu\ell$ of methanol or acetone. After drying in a fume hood for 2 min, each filter paper was placed on the bottom of a polyvinyl chloride (PVC) container (120 mL). Groups of 10 females (7 - 8 days old) were separately placed on each container containing calf chow pellets and covered with a lid. Treated and control (methanol or acetone only) females were held at the same conditions used for colony maintenance. Adult mortalities were determined 24 h after treatment. Adults were considered to be dead if appendages did not move when they were prodded with a wooden dowel. All treatments were replicated three times. The LC_{50} values were calculated by probit analysis (SAS Institute, 2004). The toxicity was considered to be significantly different when 95% confidence limit levels of the LC_{50} values failed to overlap.

1.7.3.2. Vapor Phase Toxicity Bioassay.

Fumigant toxicity of *C. obtusa* oil, 31 test compounds, *C. obtusa* derived material and four insecticides against adult female *B. germanica* was investigated using the vapor phase toxicity bioassay as above. Groups of 10 females (7-8 days old) were separately placed on the bottom of a PVC container (120 mL). The container was then covered with gauze. Appropriate amounts (0.63-96 mg) of each tested material in 50 $\mu\ell$ of methanol or acetone were applied to filter papers (Whatman no. 2; 4.25 cm diameter), which is equivalent to 5.25-800 mg/L of air. Dichlorvos served as a standard for comparison in fumigant toxicity tests. After drying in a fume hood for 2 min, each treated paper was attached to the inner side of a lid with a small amount of solid glue and the container was

covered with the lid. Control filter papers received 50 ㎕ of methanol or acetone. Treated and control (methanol or acetone) females were held at the same conditions used for colony maintenance. Evaluation of adulticidal activity was made 24 h after treatment. All treatments were replicated three times.

1.8. AChE inhibiton

The AChE assay was performed by the method of Ellman *et al.* (1961). *B. germanica* were rapidly frozen and stroed -80℃until use. Experiments involving exposure to test compounds and subsequent dialysis, portions of five cockroaches were homogenized twice for 10s each in ground glass homogenizers containing 1 mL of ice-cold 0.09% NaCl, 0.1 M sodium phosphate at pH 7.4 and 0.1% BSA. *B. germanica* sections containing the thorax and head were detached and homogenized. Centrifuge: 15,000 g, 4℃, 30 min Filtration Ellmans reaction mixture was made from a combination of 0.5 mM AChE and 1 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) in a 50 mM sodium phosphate buffer (pH 8.0). The rates of hydrolysis by AChE were monitored spectrophotomatically using a 96-well microtiter plate reader. Each Compounds, 10 ㎕ and 50 mM sodium phosphate buffer (30 ㎕) were mixed with the enzyme solution (10 ㎕). Reaction mixture (50 ㎕) was further added to give a final volume of 100 ㎕, and the mixture was incubated at 37℃ for 30 min. Absorbance at 417 nm was measured immediately after adding the Ellmans reaction mixture. Measurement was repeated for 30 min at 2 min intervals to verify that the reaction occurred linearly. Blank reaction was measured by substituting saline for the enzyme.

1.9. Data analysis

1.9.1. House dust mite

Mortality percentages were determined and transformed to arcsine square root values for analysis of variance (ANOVA). The Scheffé multiple comparison test was used to test for significant differences among the test compounds and acaricides (SAS Institute, 2004). LD_{50} values were calculated by probit analysis using SAS (SAS Institute, 2004). Acaricidal activity was considered to be significantly different when 95% confidence limit levels of the LD_{50} values failed to overlap. Relative toxicity (RT) was determined calculated as the ratio of benzyl benzoate LD_{50}/the other compound LD_{50}.

1.9.2. Hard tick

The repellent index was calculated according to the formula (Schreck *et al.*, 1977): % repellency = $[(Ta - Tb)/Ta] \times 100$, where *Ta* is the number of hard ticks in control and *Tb* is the number of hard ticks in the treated. The percentages of repellency were transformed to arcsine square-root values for analysis of variance (ANOVA). Treatment means were compared and separated by the Scheffé test or Bonferroni multiple-comparison method at $P = 0.05$. Means ± SE of untransformed data are reported (SAS Institute, 2004).

1.9.3. German cockroach

Data were corrected for control mortality using Abbott's formula (1925). Mortality percentages were transformed to arcsine square root values for analysis of variance

(ANOVA). The Bonferroni multiple-comparison method was used to test for significant differences among the treatments (SAS Institute, 2004). A *t*-test was used to test for significant differences between two treatment methods (SAS Institute, 2004). Means ± SE of untransformed data are reported. Concentration- or dose-mortality data were subjected to probit analysis (SAS Institute, 2004).

Results

1. Supercritical extract of *C. obtusa* leaf

To evaluate the influence of the level of each of the influencing factors, namely, pressure, temperature and extraction time, on the extraction yield, the variation in the oil yield as a function of the changes in the different levels of the factors was depicted in Fig. 4. The effect of particle size on extraction rate is shown in Fig. 4. In this figure, one can see that the extracting rate decreased when paticle size increased because the intrapaticle diffusion resistance is smaller for smaller particle size due to the shorter diffusion path. In the case of the particle (0.5 - 2 mm), intraparticle diffusion resistance offers As expected, the extraction yield was enhanced significantly with increasing pressure. This is because raising the extraction pressure at a constant temperature led to a higher fluid density thus increased the solubility of the oil. Temperature affects the density of the fluid, the volatility of the extract components and desorption of the extracts from the matrix. It can be seen from Fig. 2b that increasing temperature from 40 ℃ to 60 ℃ resulted in a significant increase in the oil extraction yield, while further increasing temperature from 60 ℃ resulted in only a small increase in the yield.

This can be explained as that at a higher temperature, although the fluid density decreases, the solubility of the oil still increases. Therefore, the extraction yield under different temperatures depends on a complex balance between the supercritical CO_2 density and the volatility of the extracted components under a given condition. The extraction time had a positive influence on the extraction yield. The extraction yield

gradually increased with increasing time, and reached a maximum at 3 hr within the tested range.

(a) Particle size : 0.5 mm (b) Particle size : 2 mm

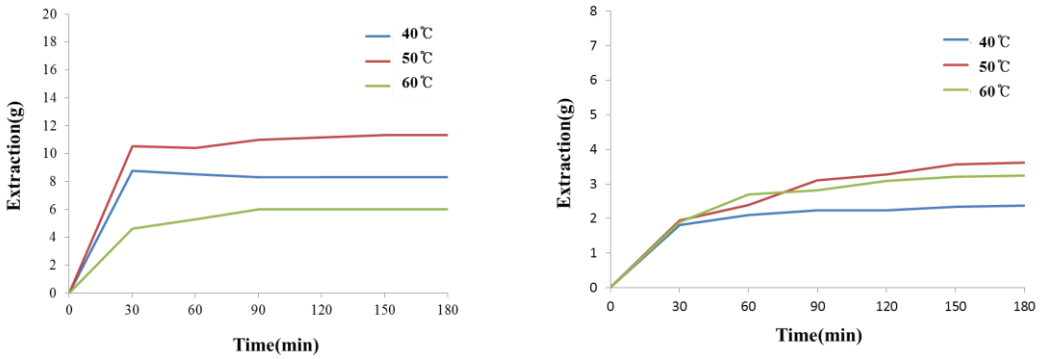


Fig. 4. Effect of particle size, temperature and extraction time on yield of extract from *C. obtusa*

Table 3. Effect of particle size, temperature and extraction time on yield of extract from *C. obtusa*.

Particle size (mm)	Pressure (bar)	Temperature (℃)	Extraction (g)	Yield (%)
0.5 mm	150	40	8.2	8.2
0.5 mm	150	50	11.34	11.34
0.5 mm	150	60	6	6
2 mm	150	40	2.4	2.4
2 mm	150	50	3.6	3.6
2 mm	150	60	3.24	3.24

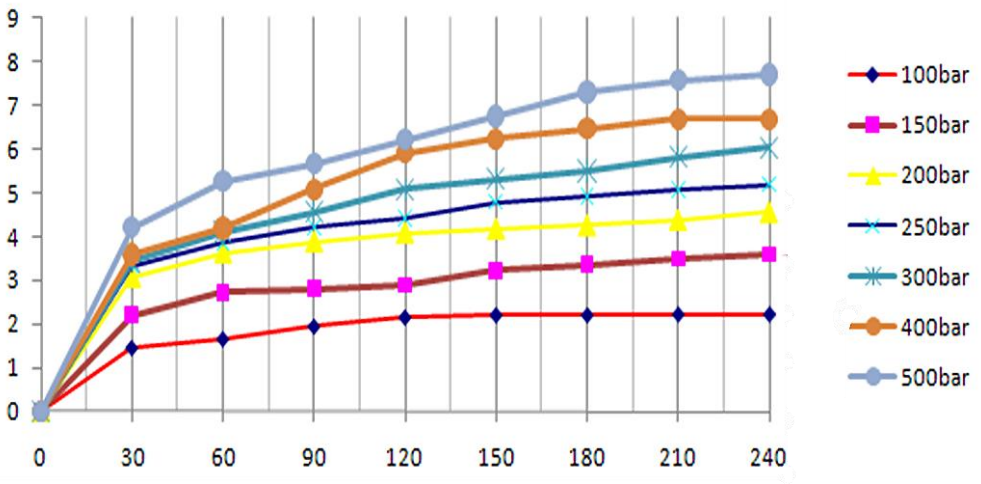
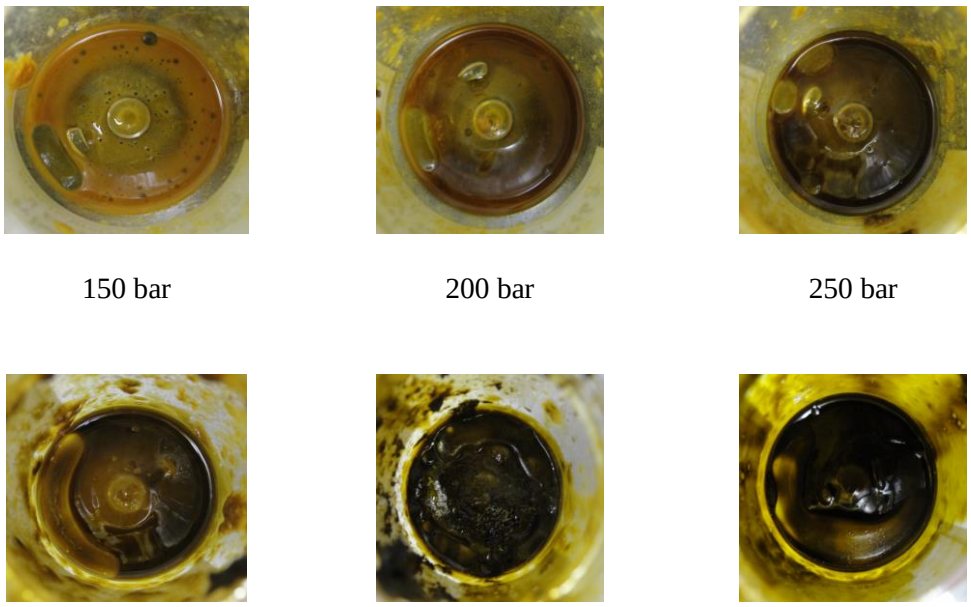


Fig. 5. Effect of pressure and extraction time on yield of *C. obtusa*



150 bar 200 bar 250 bar

300 bar 400 bar 500 bar

Fig. 6. Shape by the pressure of *C. obtusa* using supercritical fluid extraction

Table 4. Effect of pressure and extraction time on yield of extract from *C. obtusa.*

Pressure(bar)	Temperature(℃)	Extraction(g)	Yield(%)
100	50	2.23	2.23
150	50	3.6	3.6
200	50	4.56	4.56
250	50	5.19	5.19
300	50	6.05	6.05
400	50	6.69	6.69
500	50	7.71	7.71

The compositions of *C. obtusa* leaf oil extracted with the supercritical CO_2 extraction. It can be seen that 31 compounds were identified in the *C. obtusa* leaf oil. In general, terpinyl acetate (36.89 %), sabinene (15.33 %) and bonyl-acetate (11.82%) were the major components in the *C. obtusa* leaf oil in the range pressure (150 bar), temperature (50℃) and particle size (0.5 mm) (Table 5).

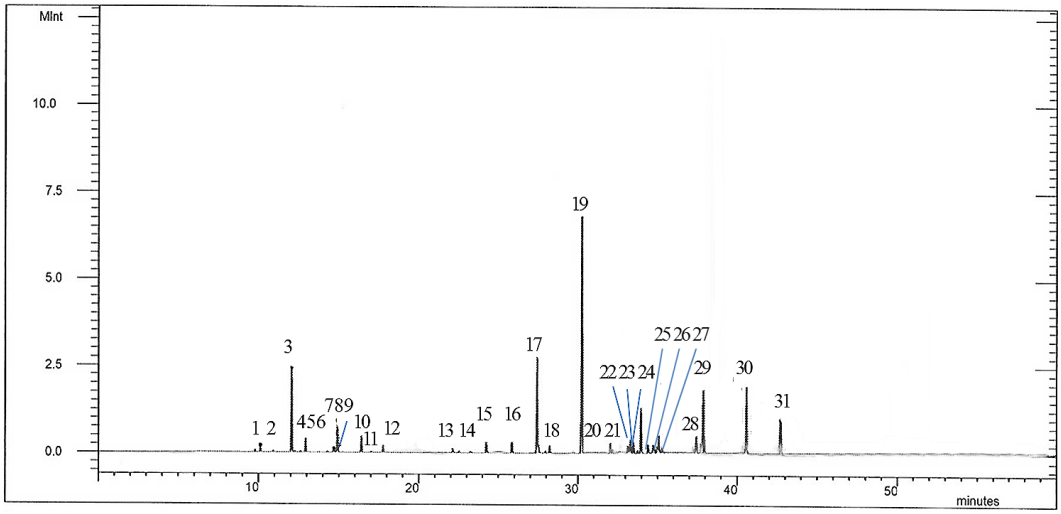


Fig. 7. Gas chromatogram of supercritical extraction at 150 bar, 50℃ and 0.5 mm from *C. obtusa*.

Table 5. GC-MS determined chemical components of *C. obtusa* leaf utilizing supercritical fluid extraction at 150bar, 50℃ and 0.5 mm

Peak	Compound Name	Retention Time	Area	Area(%)
1	α-Pinene	10.115	1969878	1.40
2	Camphene	10.906	27452	0.20
3	Sabinene	12.064	2157E6	15.33
4	β-Pinene	12.276	22499	0.16
5	Myrcene	12.934	394634	2.80
6	Phellandrene	13.736	7385	0.05

Table 5. (continued)

7	*α*-Terpinene	14.283	16287	0.12
8	4-Cymene	14.683	155516	1.11
9	Limonene	14.910	432788	3.08
10	*γ*-Terpinene	16.408	400197	2.84
11	Terpinolene	17.776	132482	0.94
12	Linalool	18.582	10586	0.08
13	Terpinene-4-ol	22.523	33670	0.24
14	*α*-Terpineol	23.248	23651	0.17
15	*α*-Fenchyl acetate	24.245	70211	0.50
16	Linalyl acetate	25.832	120102	0.85
17	Bornyl acetate	27.425	1.664E6	11.82
18	*α*-Thujone	27.527	54451	0.39
19	Terpinyl acetate	30.250	5.191E6	36.89
20	Longpinene	30.255	93955	0.67
21	Isolongifolene	32.174	35853	0.24
22	*α*-(-)-Cedrene	33.156	124892	0.89
23	trans-Caryophyllene	33.308	292547	2.08
24	*β*-Caryophyllene	33.308	292547	2.08
25	*α*-Humulene	35.304	194827	1.38
26	*β*-Chmigrene	35.886	47909	0.34
27	Neryl isobutyrate	35.878	716316	5.09
28	cis-Nerolidol	37.875	235849	1.68
29	trans-Nerolidol	38.988	184748	1.31
30	*α*-Cedrol	40.950	377507	2.68
31	*β*-Eudesmol	42.689	364813	2.59

Terpinyl acetate (34.86 %), sabinene (15.21 %) and bonyl-acetate (11.09%) were the major components in the *C. obtusa* leaf oil in the range pressure (200 bar), temperature (50 ℃) and particle size (0.5 mm) (Table 6).

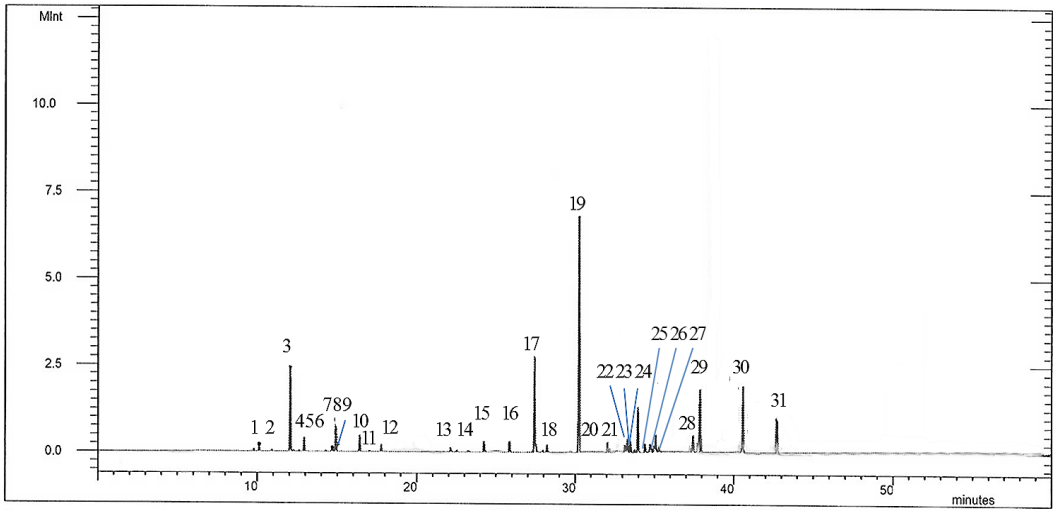


Fig. 8. Gas chromatogram of supercritical extraction at 200 bar, 50℃ and 0.5 mm from *C. obtusa*.

Table 6. GC-MS determined chemical components of *C. obtusa* leaf utilizing supercritical fluid extraction at 200 bar, 50℃ and 0.5 mm.

Peak	Compound Name	Retention Time	Area	Area(%)
1	*α*-Pinene	10.115	189453	1.35
2	Camphene	10.906	28079	0.20
3	Sabinene	12.064	2.126E6	15.21
4	*β*-Pinene	12.276	201453	0.00
5	Myrcene	12.934	371045	2.65
6	Phellandrene	13.736	6917	0.05
7	*α*-Terpinene	14.283	17849	0.13
8	4-Cymene	14.683	157305	1.13
9	Limonene	14.910	408319	2.92
10	*γ*-Terpinene	16.408	380224	2.72
11	Terpinolene	17.776	131771	0.94

Table 6. (continued)

12	Linalool	18.582	10362	0.07
13	Terpinene-4-ol	22.523	38312	0.27
14	*α*-Terpineol	23.248	22637	0.16
15	*α*-Fenchyl acetate	24.245	104860	0.75
16	Linalyl acetate	25.832	259252	1.85
17	Bornyl acetate	27.425	1.551E6	11.09
18	*α*-Thujone	27.527	51180	0.37
19	Terpinyl acetate	30.250	4.874E6	34.86
20	Longpinene	30.255	248282	1.78
21	Isolongifolene	32.174	33612	0.24
22	*α*-(-)-Cedrene	33.156	120499	0.89
23	trans-Caryophyllene	33.308	280172	2.00
24	*β*-Caryophyllene	33.308	280223	2.00
25	*α*-Humulene	35.304	21011	0.15
26	*β*-Chmigrene	35.886	80232	0.57
27	Neryl isobutyrate	35.878	638007	4.56
28	cis-Nerolidol	37.875	484956	3.47
29	trans-Nerolidol	38.988	503983	3.60
30	*α*-Cedrol	40.950	378033	2.70
31	*β*-Eudesmol	42.689	184638	1.32

Terpinyl acetate (36.90 %), sabinene (12.15 %) and bonyl-acetate (10.72 %) were the major components in the *C. obtusa* leaf oil in the range pressure (150 bar), temperture (50 ℃) and particle size (2 mm) (Table 7).

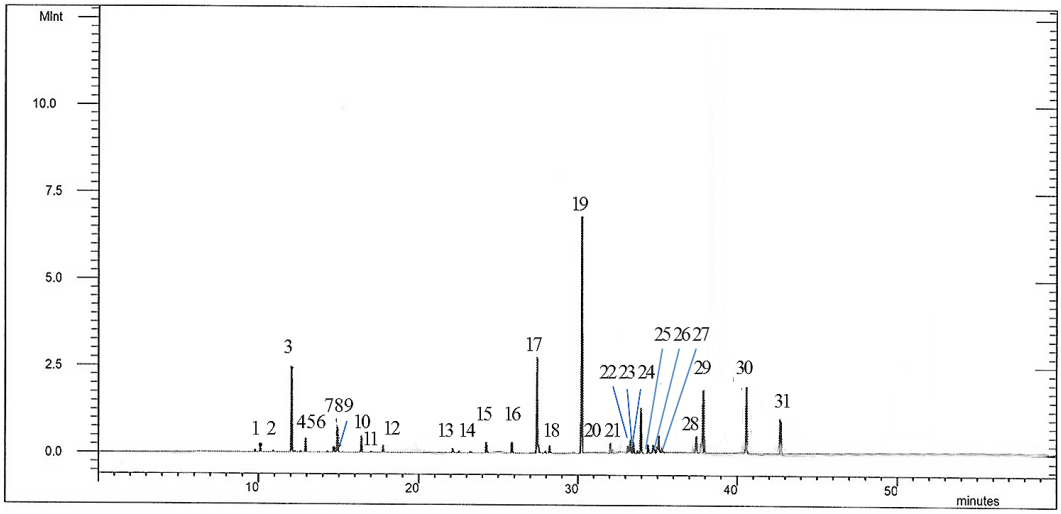


Fig. 9. Gas chromatogram of supercritical extraction at 150 bar, 50℃ and 2 mm from *C. obtusa*.

Table 7. GC-MS determined chemical components of *C. obtusa* leaf utilizing supercritical fluid extraction at 150 bar, 50℃ and 2 mm.

Peak	Compound Name	Retention Time	Area	Area(%)
1	*α*-Pinene	10.115	128552	1.07
2	Camphene	10.906	18496	0.15
3	Sabinene	12.064	1.460E6	12.15
4	*β*-Pinene	12.276	19566	0.16
5	Myrcene	12.934	263830	2.20
6	Phellandrene	13.736	6635	0.06
7	*α*-Terpinene	14.283	20865	0.17
8	4-Cymene	14.683	143986	1.20
9	Limonene	14.910	315746	2.63
10	*γ*-Terpinene	16.408	281983	2.32
11	Terpinolene	17.776	98040	0.82

Table 7. (continued)

12	Linalool	18.582	10521	0.09
13	Terpinene-4-ol	22.523	52488	0.44
14	*α*-Terpineol	23.248	25084	0.21
15	*α*-Fenchyl acetate	24.245	63370	0.53
16	Linalyl acetate	25.832	185063	1.54
17	Bornyl acetate	27.425	1.289E6	10.72
18	*α*-Thujone	27.527	42920	0.36
19	Terpinyl acetate	30.250	4.434E6	36.90
20	Longpinene	30.255	181186	1.51
21	Isolongifolene	32.174	26742	0.22
22	*α*-(-)-Cedrene	33.156	99776	0.83
23	trans-Caryophyllene	33.308	249992	2.08
24	*β*-Caryophyllene	33.308	248966	2.07
25	*α*-Humulene	35.304	39799	0.33
26	*β*-Chmigrene	35.886	21690	0.18
27	Neryl isobutyrate	35.878	561189	4.67
28	cis-Nerolidol	37.875	440223	3.66
29	trans-Nerolidol	38.988	228461	1.90
30	*α*-Cedrol	40.950	642552	5.35
31	*β*-Eudesmol	42.689	4177332	3.48

Terpinyl acetate (33.22 %), sabinene (18.30 %) and bonyl-acetate (10.05 %) were the major components in the *C. obtusa* leaf oil in the range pressure (200 bar), temperature (50 ℃) and particle size (2 mm) (Table 8).

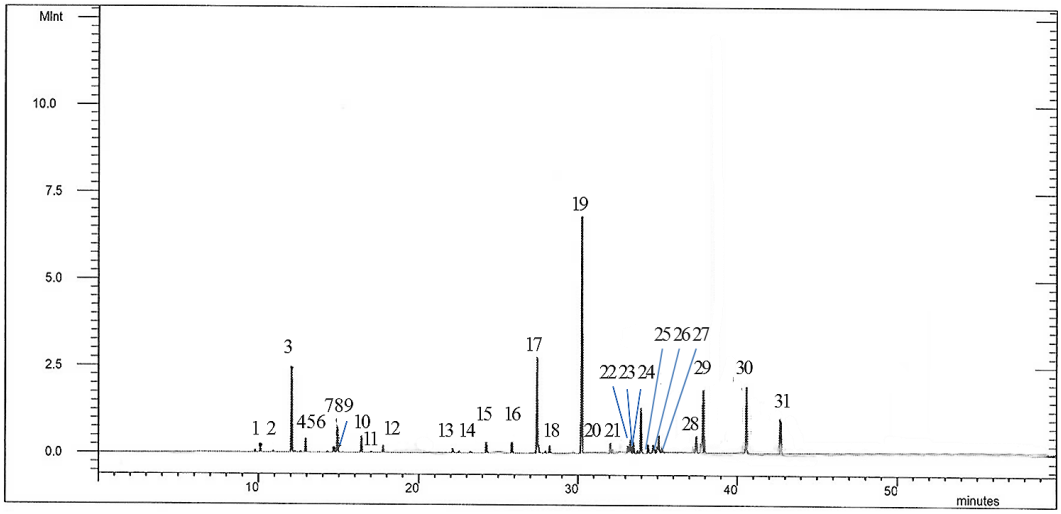


Fig. 10. Gas chromatogram of supercritical extraction at 200 bar, 50℃ and 2 mm from *C. obtusa*.

Table 8. GC-MS determined chemical components of *C. obtusa* leaf utilizing supercritical fluid extraction at 200 bar, 50℃ and 2 mm.

Peak	Compound Name	Retention Time	Area	Area(%)
1	*α*-Pinene	10.115	198614	1.72
2	Camphene	10.906	26665	0.23
3	Sabinene	12.064	2.109E6	18.30
4	*β*-Pinene	12.276	13060	0.11
5	Myrcene	12.934	338200	2.93
6	Phellandrene	13.736	5800	0.05
7	*α*-Terpinene	14.283	18507	0.16
8	4-Cymene	14.683	221424	1.95
9	Limonene	14.910	372182	3.23
10	*γ*-Terpinene	16.408	290178	2.52

Table 8. (continued)

11	Terpinolene	17.776	103223	0.90
12	Linalool	18.582	12285	0.11
13	Terpinene-4-ol	22.523	40908	0.35
14	α-Terpineol	23.248	22989	0.20
15	α-Fenchyl acetate	24.245	64995	0.56
16	Linalyl acetate	25.832	184466	1.60
17	Bornyl acetate	27.425	1.159E6	10.05
18	α-Thujone	27.527	38404	0.33
19	Terpinyl acetate	30.250	3.829E6	33.22
20	Longpinene	30.255	79177	0.69
21	Isolongifolene	32.174	25436	0.22
22	α-(-)-Cedrene	33.156	91713	0.80
23	trans-Caryophyllene	33.308	205698	1.78
24	β-Caryophyllene	33.308	205698	1.78
25	α-Humulene	35.304	16062	0.14
26	β-Chmigrene	35.886	19625	0.17
27	Neryl isobutyrate	35.878	450780	3.91
28	cis-Nerolidol	37.875	376137	3.26
29	trans-Nerolidol	38.988	320951	2.78
30	α-Cedrol	40.950	576615	5.00
31	β-Eudesmol	42.689	109482	0.95

2. Acaricidal activity of *C. obtusa* against house dust mites

2.1. Solvent extraction of *C. obtusa*

When the methanolic extracts of *C. obtusa* leaves, branch, fruit and seed were bioassayed by the fabic diffusion method. Acaricidal activity was observed against *D. farinae* and *D. pteronyssinus* (Table 9). The dust mite species were equally susceptible, and the extracts of *C. obtusa* leaves, branch, fruit and seed showed a clear dose-response relationship for both species. *C. obtusa* leaves extract exhibited 100% motailty against two dust mite species at 24 h post-treatment at a concentration 1 mg/cm^2, whereas *C. obtusa* branch (87.2 %), fruit (10.5 %) and seed (0 %) against *D. farinae.* respectively. Also, *C. obtusa* leaves extract exhibited 100% motailty against two dust mite species at 24 h post-treatment at a concentration 1 mg/cm^2, whereas *C. obtusa* branch (82.3 %), fruit (23.7 %) and seed (0 %) against *D. pteronyssinus.* respectively.

Table 9. Acaricidal activties of methanol extract from *C. obtusa* against *D. farinae* and *D. pteronyssinus*.

Sample	Mortality (mean ± SE, %)[a]			
	D. farinae		*D. pteronyssinus*	
	2 mg/cm^2	1 mg/cm^2	2 mg/cm^2	1 mg/cm^2
Leaf	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a
Branch	87.2 ± 2.7bc	84.5 ± 1.8bc	92.7 ± 5.4bc	82.3 ± 7.5ab
Fruit	25.2 ± 3.4c	10.5 ± 1.0c	25.2 ± 5.2c	23.7 ± 1.8c
Seed	0 ± 0.0d	0 ± 0.0d	0 ± 0.0d	0 ± 0.0d

[a] Means with a column followed by the same letter are not significantly different (P = 0.05, Scheffe's test) (25 adults per replicate; 3 replicates per treatment; *n* = 75). Mortalities were transformed to arcsine square-root before ANOBA. Means (± SE) of untransformed data are reported.

Fractions obtained from the methanol extract of *C. obtusa* branch were bioassayed by the fabic diffusion method (Table 10). Significant difference in acaricidal acitivy in fractions of the extract was observed, and they were used to identify peak activity fractions for the next step in the purification. At a concentration 1 mg/cm^2, the *n*-hexane soluble fration from methanol extract from *C. obtusa* branch exhibited 100% mortality against two species dust mites at 24 h post-treatment.

Table 10. Acaricidal activties of each solvent fraction from *C. obtusa* branch against *D. farinae* and *D. pteronyssinus*.

Sample	Mortality (mean ± SE, %)[a]	
	D. farinae	*D. pteronyssinus*
	1 mg/cm^2	1 mg/cm^2
n-Hexane	100 ± 0.0a	100 ± 0.0a
Chloroform	5.1 ± 1.2b	3.3 ± 2.2b
Ethyl acetate	0 ± 0.0b	0 ± 0.0b
Butanol	0 ± 0.0b	0 ± 0.0b
Water	0 ± 0.0b	0 ± 0.0b

[a] Means with a column followed by the same letter are not significantly different (P = 0.05, Scheffe's test) (25 adults per replicate; 3 replicates per treatment; *n* = 75). Mortalities were transformed to arcsine square-root before ANOBA. Means (± SE) of untransformed data are reported.

The acaricidal activities of each subfraction derived form the *n*-hexane fraction are given in Table 11.

Table 11. Acaricidal activity of various fraction obtained from the extract of *C. obtusa* branch against *D. farinae* and *D. pteronyssinus*

Fraction	Rate, $\mu g/cm^3$	Mortality (mean ± SE, %)[a]	
		D. *farinae*	*D. pteronyssinus*
H1	1	0±0.0c	0±0.0c
H2	1	100 ± 0.0a	100 ± 0.0a
H3	1	2.2±1.0bc	7±0.8bc
H4	1	0±0.0c	0±0.0c
H5	1	0±0.0c	0±0.0c
H6	1	0±0.0c	0±0.0c
H7	1	0±0.0c	0±0.0c
H21	1	0±0.0c	0±0.0c
H22	1	100 ± 0.0a	100 ± 0.0a
H23	1	21±1.1bc	25±3.2bc
H24	1	0±0.0c	0±0.0c
H25	1	0±0.0c	0±0.0c
H26	1	0±0.0c	0±0.0c
H221	1	100 ± 0.0a	100 ± 0.0a
H222	1	0±0.0c	0±0.0c
H223	1	0±0.0c	0±0.0c
H224	1	0±0.0c	0±0.0c
H225	1	0±0.0c	0±0.0c

[a] Means with a column followed by the same letter are not significantly different (P = 0.05, Scheffe's test) (25 adults per replicate; 3 replicates per treatment; *n* = 75). Mortalities were transformed to arcsine square-root before ANOBA. Means (± SE) of untransformed data are reported.

Because of the strong activity of the *n*-hexane fraction, isolation of the biologically active component was pursued. Dry film method-guided fractionation of *C. obtusa* afforded an active constituent identified by spectroscopic analyses, including MS and NMR, and by direct comparison with authentic compound. The biologically active constituent was characterized as *β*-thujaplicin (Figure 16). This compound was identified on the basis of the following evidence. *β*-Thujaplicin (2-hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one): ($C_{10}H_{12}O_2$, MW=164.2); EI-MS (70 eV) *m/z* (% relative intensity) M^+ 164 (76), 149 (4), 136 (12), 121 (100), 103 (11), 91 (15), 77 (17), 65 (8), 51 (5); ^{1}H NMR (CD_3OD) δ 7.1 (1H, *t*, *J* = 11.0 Hz), 6.44 (1H, *d*, *J* = 10.2 Hz; 0.6 Hz), 6.44 (1H, *t*, *J* = 10.2 Hz), 2.52 (1H, *m*, *J* = 6.84 Hz; 1.8 Hz), 1.06 (3H, *d*, *J* = 1.8Hz); ^{13}C NMR (CD_3OD) δ 172.6, 171.7, 121.8, 131.6, 128.7, 157.0, 134.2, 37.1, 21.6.

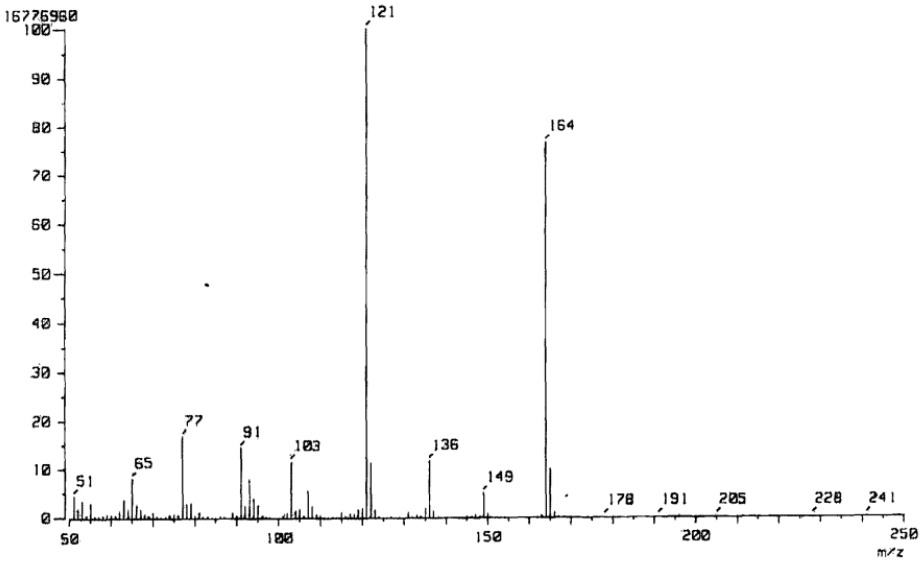


Fig. 11.EI-MS spectrum of compound

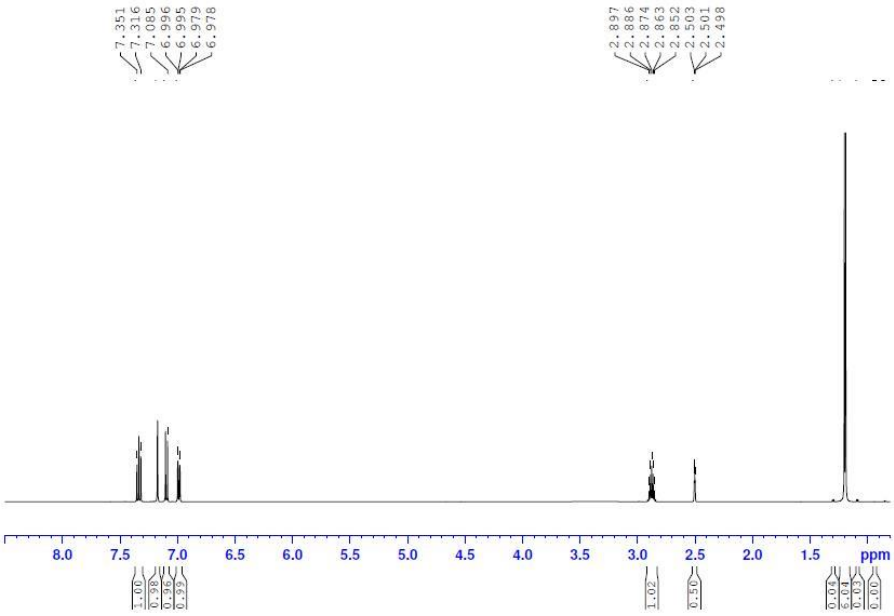


Fig. 12.^{1}H-NMR spectrum of compound

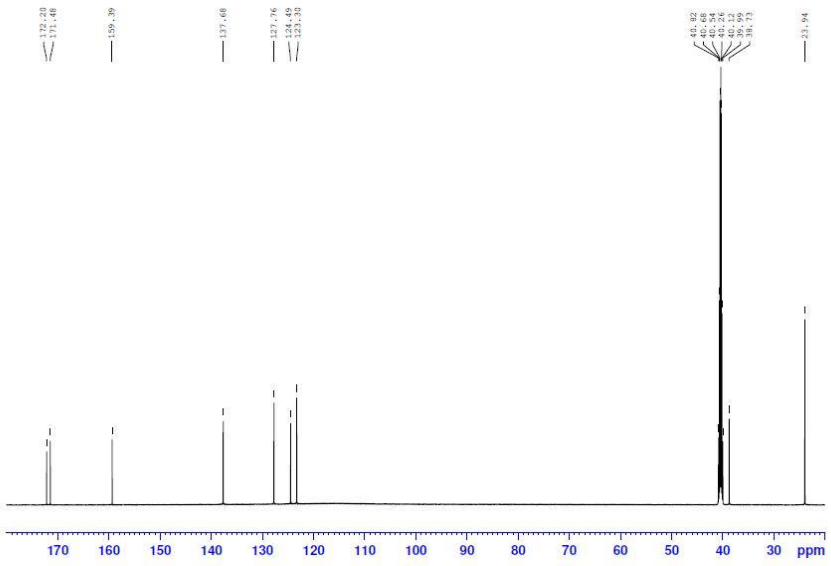


Fig. 13. ^{13}C-NMR spectrum of compound

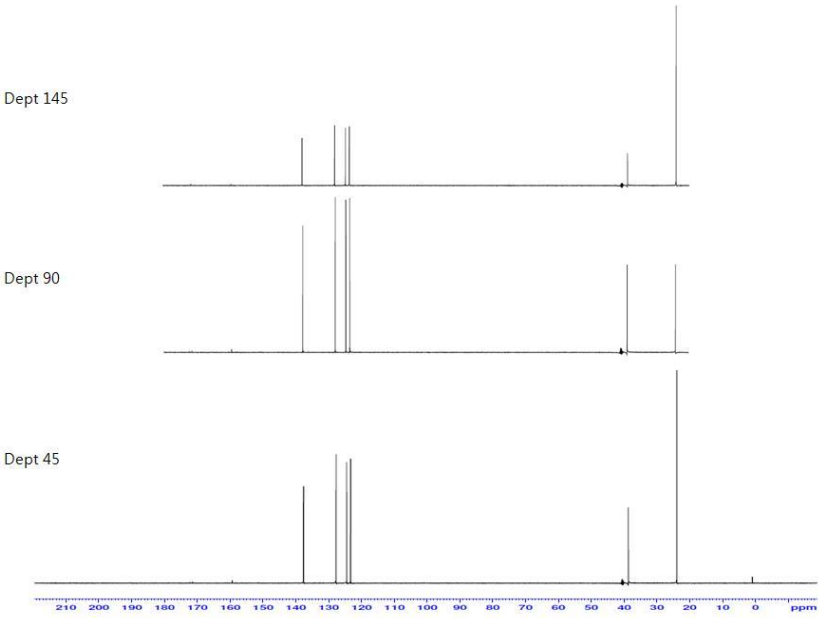


Fig. 14.DEPT spectrum of compound

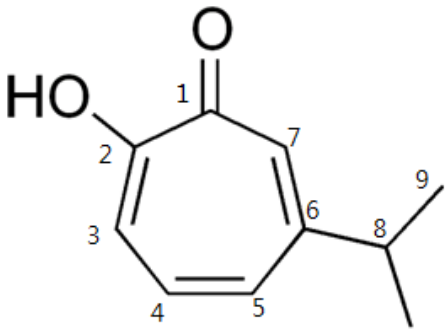


Fig. 15. Structure of *β*-Thujaplicin

Table 12. ^{1}H NMR(600MHz) and ^{13}C NMR(600MHz) spectral data of compound.

Carbon	Partial Structure	δ_C(ppm)	δ_H(ppm)	600MHz reference
1		172.6	-	-
2		171.7	-	-
3	CH	121.8	7.1	d (*J*=11.0Hz)
4	CH	131.6	6.44	dd (*J*=10.2Hz, *J*= 0.6Hz)
5	CH	128.7	6.44	d (*J*=10.2Hz)
6	C	157.0	-	-
7	CH	134.2	6.40	S
8	CH	37.1	2.52	dq (*J*=6.84Hz, *J*= 1.8Hz)
9	CH_3	21.6	1.06	d (J=1.8Hz)
10	OH	-	7.33	S

The toxicity of *C. obtusa* oil and *β*-thujaplicin and the acaricides to adult *D. farinae* were examined by the direct contact bioassay (Table 13). As judged by 24 h LD_{50} values, acaricidal activity of *C. obtusa* oil (LD_{50}, 3.42 $\mu g/cm^2$) and *β*-thujaplicin (LD_{50}, 4.02 $\mu g/cm^2$) used was high than that of benzy benzoate (LD_{50}, 4.31 $\mu g/cm^2$), dibutyl phthalate (LD_{50}, 31.62 $\mu g/cm^2$) and DEET (LD_{50}, 78.34 $\mu g/cm^2$). Also, The toxi city of *C. obtusa* oil and *β*-thujaplicin and the acaricides to adult *D. pteronyssinus* we re examined by the direct contact bioassay (Table 13). As judged by 24 h LD_{50} valu es, acaricidal activity of *C. obtusa* oil (LD_{50}, 4.12 $\mu g/cm^2$) and *β*-thujaplicin (LD_{50}, 3.84 $\mu g/cm^2$) used was high than that of benzy benzoate (LD_{50}, 4.21 $\mu g/cm^2$), dibutyl phthalate (LD_{50}, 30.72 $\mu g/cm^2$) and DEET (LD_{50}, 75.64, $\mu g/cm^2$).

Table 13. Toxicity of *C. obtusa* oil utilizing supercritical flulid extraction against *D. farinae* and *D. pteronyssinus* using the direct toxicity bioassay during 24hr exposure

Compound[a]	Mite species	slope(±SE)	LD_{50}(μg/cm^2)	95% cl[b]	RT[c]
C. obtusa oil	*D. farinae*	5.72±0.62	3.42	2.87~4.02	1.26
	D. pteronyssinus	3.67±0.32	4.12	3.52~4.87	1.02
β-Thujaplicin	*D. farinae*	3.62±5.3	4.02	3.88~4.31	1.07
	D. pteronyssinus	5.32±7.4	3.84	3.68~3.97	1.10
Benzyl benzoate	*D. farinae*	1.56±0.22	4.31	2.98~5.32	1
	D. pteronyssinus	3.88±0.38	4.21	3.67~4.84	1
DEET	*D. farinae*	5.36±0.36	78.34	75.59~81.32	0.06
	D. pteronyssinus	1.72±0.34	75.64	73.32~78.18	0.06
Dibutyl phthalate	*D. farinae*	3.98±6.32	31.62	29.46~34.01	0.14
	D. pteronyssinus	2.63±0.26	30.72	27.72~33.56	0.14

[a] Exposed for 24 hr.

[b] CL denotes confidence.

[c] Relative toxicity = LD_{50} value of benzyl benzoate/ LD_{50} vaule of each chemical.

The acaricidal activity of 31 compounds and three acaricides (benzyl benzoate, DEET and dibutyl phthalate) against adult *D. farinae* was examined by using direct contact toxicity bioassay (Table 14). As judged by 24 h LD_{50} values, *α*-cedrol (LD_{50}, 10.59 $\mu g/cm^2$) was the most toxic acaricide but was lower than that of benzy benzoate (LD_{50}, 4.31 $\mu g/cm^2$), against adult *D. farinae*. The toxicity of *α*-terpineol and bornyl acetate (LD_{50}, 24.32 - 28.64 $\mu g/cm^2$) were more toxic than dibutyl phthalate (31.62 $\mu g/cm^2$). Also, linalool, terpinyl acetate, linalyl aceatate, terpinene-4-ol, and isobornyl acetate (LD_{50}, 34.52-68.72 $\mu g/cm^2$) were more toxic than DEET (LD_{50}, 75.64, $\mu g/cm^2$). Little or no acaricidal activity (>203.6 $\mu g/cm^2$) was observed with the other 22 compounds against adult *D. farinae*. There was no mortality in the untreated controls.

Table 14. Toxicity of *C. obtusa* oil compounds against *D. farinae* using the direct toxicity bioassay during 24hr exposure

Compound[a]	slope(±SE)	LD_{50}(μg/cm^2)	95% cl[b]	RT[c]
α-Pinene	3.4±7.8	153.34	148.78~157.98	0.03
Camphene		>203.6		
Sabinene		>203.6		
β-pinene	5.3±7.2	187.54	183.88~191.03	0.02
Myrcene		>203.6		
Phellandrene		>203.6		
α-Terpinene		>203.6		
4-Cymene		>203.6		
Limonene	2.6±0.28	150.34	147.54~154.01	0.03
γ-Terpinene	6.1±0.68	180.62	177.42~183.02	0.02
Terpinolene	2.35±0.28	120.57	115.51~124.59	0.04

Table 14. (continued)

Linalool	2.28±0.21	34.52	31.25~36.87	0.12
Terpinene-4-ol	7.18±0.98	48.75	44.78~51.05	0.09
α-Terpineol	4.40±2.11	24.32	22.16~26.87	0.18
α-Fenchyl acetate		>203.6		
Linalyl acetate	4.92±1.59	40.56	37.65~42.85	0.11
Bornyl acetate	6.07±1.42	28.64	25.35~30.86	0.15
α-Thujone		>203.6		
Terpinyl acetate	7.51±0.82	40.52	37.68~43.58	0.11
Longpinene		>203.6		
Isolongifolene		>203.6		
α-Cedrene		>203.6		
trans-Caryophyllene		>203.6		
β-Caryophyllene		>203.6		
α-Humulene	2.34±5.6	112.6	109.2~115.1	0.04
β-Chmigrene		>203.6		
Neryl isobutyrate		>203.6		
cis-Nerolidol		>203.6		
trans-Nerolidol		>203.6		
α-Cedrol	5.68±3.5	10.59	8.98~12.35	0.41
β-Eudesmol		>203.6		

[a] Exposed for 24 hr.

[b] CL denotes confidence.

[c] Relative toxicity = LD_{50} value of benzyl benzoate/ LD_{50} vaule of each chemical.

The acaricidal activity of 31 compounds and three acaricides: benzyl benzoate, deet, and dibutyl phthalate against adult *D. pteronyssinus* was examined by using direct contact toxicity bioassay (Table 15). As judged by 24 h LD_{50} values, *a*-cedrol (LD_{50}, 14.84 $\mu g/cm^2$) was the most toxic acaricide but was lower than that of benzy benzoate (LD_{50}, 4.31 $\mu g/cm^2$), against adult *D. farinae*. The toxicity of *α*-Terpineol and bornyl acetate (LD_{50}, 22.56-30.34 $\mu g/cm^2$) were more toxic than dibutyl phthalate (31.62 $\mu g/cm^2$). Also, linalool, terpinyl acetate, linalyl aceatate and terpinene-4-ol (LD_{50}, 38.42-54.32 $\mu g/cm^2$) were more toxic than DEET (LD_{50}, 75.64, $\mu g/cm^2$). Little or no acaricidal activity (>203.6 $\mu g/cm^2$) was observed with the other 22 compounds against adult *D. farinae*. There was no mortality in the untreated controls.

Table 15. Toxicity of *C. obtusa* oil compounds against *D. pteronyssinus* using the direct toxicity bioassay during 24hr exposure

Compound[a]	slope(±SE)	$LD_{50}(\mu g/cm^2)$	95% cl[b]	RT[c]
α-Pinene	6.5±6.8	140.67	136.59~143.98	0.03
Camphene		>203.6		
Sabinene		>203.6		
β-pinene	3.4±5.9	164.32	160.32~169.01	0.03
Myrcene		>203.6		
Phellandrene		>203.6		
α-Terpinene		>203.6		
4-Cymene		>203.6		
Limonene	5.5±0.63	163.72	159.84~166.79	0.03
γ-Terpinene	9.4±0.97	164.32	160.88~167.89	0.03
Terpinolene	3.37±0.20	137.64	130.22~143.25	0.03

Table 15. (continued)

Linalool	1.92±0.19	38.74	35.53~42.02	0.11
Terpinene-4-ol	7.72±3.87	54.32	50.21~57.85	0.08
α-Terpineol	3.91±0.55	22.56	21.02~24.68	0.19
α-Fenchyl acetate		>203.6		
Linalyl acetate	6.24±0.82	38.42	36.21~40.26	0.11
Bornyl acetate	3.59±4.58	30.34	27.58~32.95	0.14
α-Thujone		>203.6		
Terpinyl acetate	5.85±6.45	38.45	35.55~42.12	0.11
Longpinene		>203.6		
Isolongifolene		>203.6		
α-Cedrene		>203.6		
trans-Caryophyllene		>203.6		
β-Caryophyllene		>203.6		
α-Humulene	4.72±3.8	102.9	99.8~104.98	0.04
β-Chmigrene		>203.6		
Neryl isobutyrate		>203.6		
cis-Nerolidol		>203.6		
trans-Nerolidol		>203.6		
α-Cedrol	7.89±9.0	14.84	11.81~16.41	0.28
β-Eudesmol		>203.6		

[a] Exposed for 24 hr.

[b] CL denotes confidence.

[c] Relative toxicity = LD_{50} value of benzyl benzoate/ LD_{50} vaule of each chemical.

3. Repellency of *C. obtusa* against Hard tick

The repellency of *C. obtusa* oil was evaluated in comparison with the two commercial repellents (Icaridin and DEET) against *H. longicornis* (Table 16). In filter-paper diffusion method, *C. obtusa* oil (3.2 μg/cm^2) gave 100% repellency at 120 min after exposure. Whereas Icaridine (3.2 μg/cm^2) gave 96.1 % repellency, respectively, at 120 min.

Table 16. Repellency of *C. obtusa* oil and two repellent against *H. longicornis*

Material	Dose (μg/cm$^{2)}$	Repellency[a], (%)(mean±SE)					
		Min after application					
		30 min	60 min	90 min	120 min	150 min	180 min
C. obutsa oil	3.2	100±0.0a	100±0.0a	100±0.0a	100±0.0a	96.3±3.6ab	92.4±2.6ab
Icaridine	3.2	100±0.0a	100±0.0a	100±0.0a	96.1±1.8ab	87.5±2.5ab	82.7±2.3ab
DEET	3.2	100±0.0a	100±0.0a	100±0.0a	100±0.0a	100±0.0a	95.5±3.6ab

[a] Means with a column followed by the same letter are not significantly different (P = 0.05, Scheffe's test [SAS Institute, 2004]).

The repellency of the 31 compounds against *H. longicornis* was compared with that of DEET at 3.2 mg/cm^2 (Table 17). Responses varied according to the test compound and exposure time. *α*-Terpinene, terpinene-4-ol and *α*-terpineol gave 100% repellency 30 min post-treatment but 85.5, 56.4 and 68.9% repellency at 120 min, respectively. *α*-Pinene and *β*-Pinene gave 97.6 and 89.5 % repellency at 10 min, post-treatment but 67.4 and 0 % repellency at 120 min respectively. Limonene, γ-Terpinene, Terpinolene, Isobornyl

acetate, and Terpinyl acetate gave -50% repellency at 30 min but repellency of these compounds was significantly low at 30 min.

Table 17. Repellent activity of *C. obtusa* copomounds against *H. longicornis*

Compounds	Repellency[a], (%)(mean±SE)				
	Min after application				
	10 min	30 min	60 min	90 min	120 min
α-Pinene	97.6±4.2ab	92.5±3.6ab	86.5±4.2ab	72.4±5.2bc	67.4±3.4cd
Camphene	34.2±3.4ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g
Sabinene	14.5±4.2fg	0±0.0g	0±0.0g	0±0.0g	0±0.0g
β-Pinene	89.5±4.6ab	74.8±5.2ab	63.4±4.2cd	43.5±2.7ef	0±0.0g
Myrcene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
Phellandrene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
α-Terpinene	100±0.0a	100±0.0a	97.5±3.2ab	93.6±2.8ab	85.5±2.7ab
4-Cymene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
Limonene	85.4±3.4ab	67.5±3.8cd	47.4±3.6ef	0±0.0g	0±0.0g
γ-Terpinene	88.2±4.3ab	74.5±2.6bc	66.4±3.6cd	47.8±2.5ef	0±0.0g
Terpinolene	83.5±3.5ab	74.6±2.9bc	56.7±4.3cdf	0±0.0d	0±0.0g
Linalool	48.4±2.3ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g
Terpinene-4-ol	100±0.0a	100±0.0a	87.4±3.2ab	72.4±4.2bc	56.4±3.8cd
α-Terpineol	100±0.0a	100±0.0a	92.5±3.7ab	83.5±4.6bc	68.9±3.4cd
α-Fenchyl acetate	43.2±3.5ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g
Linalyl acetate	36.5±3.6ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g

Table 17. (continued)

Bornyl acetate	38.9±1.8ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g
α-Thujone	45.6±4.2ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g
Terpinyl acetate	78.4±2.7bc	64.5±4.2cd	48.6±3.3ef	0±0.0g	0±0.0g
Longpinene	57.8±3.4cd	0±0.0g	0±0.0g	0±0.0g	0±0.0g
Isolongifolene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
α-Cedrene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
trans-Caryophyllene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
β-Caryophyllene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
α-Humulene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
β-Chmigrene	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
Neryl isobutyrate	16.5±4.2ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g
cis-Nerolidol	24.6±6.3ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g
trans-Nerolidol	42.5±4.5cd	0±0.0g	0±0.0g	0±0.0g	0±0.0g
α-Cedrol	0±0.0g	0±0.0g	0±0.0g	0±0.0g	0±0.0g
β-Eudesmol	35.7±4.7ef	0±0.0g	0±0.0g	0±0.0g	0±0.0g

[a] Means with a column followed by the same letter are not significantly different (P = 0.05, Scheffe's test [SAS Institute, 2004]).

4. Contact and fumigant toxicity of *C. obtusa* oil against German cockroach

The toxicity of *C. obutsa* oil and four insecticides, deltamethrin, dichlorvos, permethrin, and propoxur, to adult female *B. germanica* was evaluated by comparing the LC_{50} values estimated from direct contact toxicity bioassay (Table 18). As judged by the 24 h LC_{50} values, *C. obutsa* oil (2.77 mg/cm^2) was less effective than propoxur (LC_{50}, 0.18 mg/cm^2), dichlorvos (0.007 mg/cm^2), deltamethrin (0.013 mg/cm^2), or permethrin (0.05 mg/cm^2). There was no mortality in the solvent-treated controls.

Table 18. Toxicity of *C. obutsa* oil and four inscticides against adult female *B. germanica* using the filter-paper contact toxicity bioassay during a 24hr exposure

Compound	n[a]	slope(±SE)	LC_{50}(μg/cm^2)	95% cl[b]	RT[c]
C. obtusa oil	240	9.53 ± 2.77	2.77	2.50-3.05	0.06
β-Thujaplicin	80		>10		
Deltamethrin	240	1.87 ± 0.66	0.013	0.006-0.018	13.85
Dichlorvos	240	2.18 ± 0.53	0.007	0.005-0.010	25.71
Permethtin	240	4.60 ± 0.82	0.05	0.04 -0.06	3.60
Propoxur	240	2.99 ± 0.64	0.18	0.14-0.23	1.00

[a] The number of *B. germanica* females tested.

[b] CL denotes confidence limit.

[c] Relative toxicity, LD_{50} value of propoxur/LD_{50} value of the other compound.

The insecticidal activity of 31 compounds used against adult female *B. germanica* was compared with those of deltamethrin, dichlorvos, permethrin, and propoxur as above (Table 19). Potencies varied according to compound tested. As judged by the 24 h LC_{50} values, the adulticidal activity of *a*-thujone (LC_{50}, 0.09 mg/cm^2) were comparable to that of permethrin. The toxicity of linalool and *a*-terpineol ranging from 0.10 to 0.12 mg/cm^2, was higher than that of propoxur. Moderate contact toxicity (LC_{50}, 0.30 - 2.81 mg/cm^2) was observed with *α*-pinene, *β*-pinene, myrcene, phellandrene, *α*-terpinene, limonene, *γ*-terpiene, terpinene-4-ol, *α*-cedrene, *α*-humulene and *α*-cedrol. Weak or no contact toxicity was produced from the other 20 compounds. No mortality was observed in the solvent-treated controls.

Table 19. Toxicity of *C. obutsa* oil compounds against adult female *B. germanica* using the filter-paper contact toxicity bioassay during a 24hr exposure

Compound	n[a]	slope(±SE)	LC_{50} (μg/cm^2)	95% cl[b]	RT[c]
α-Pinene	240	9.53 ± 2.77	2.77	2.50 – 3.05	0.06
Camphene	80		>10		
Sabinene	80		>10		
β-pinene	240	3.03 ± 0.79	1.23	1.00 – 1.79	0.15
Myrcene	240	7.77 ± 2.03	2.81	2.47 – 3.15	0.06
Phellandrene	150	6.72 ± 1.29	0.28	0.25 – 0.31	0.64
α-Terpinene	240	6.49 ± 1.99	2.61	2.08 – 2.95	0.07
4-Cymene	80		>10		
Limonene	240	7.56 ± 2.72	2.58	2.05 – 2.86	0.07
γ-Terpinene	240	2.80 ± 0.61	0.50	0.38 – 0.64	0.36

Table 19. (continued)

Terpinolene	80		>10		
Linalool	240	3.49 ± 0.60	0.12	0.10 – 0.15	1.50
Terpinene-4-ol	150	3.80 ± 0.81	0.42	0.35 – 0.51	0.43
α-Terpineol	240	2.68 ± 0.67	0.10	0.07 – 0.14	1.80
α-Fenchyl acetate	80		>10		
Linalyl acetate	80		>10		
Bornyl acetate	80		>10		
α-Thujone	120	3.47 ± 0.68	0.09	0.07 – 0.11	2.00
Terpinyl acetate	80		>10		
Longpinene	80		>10		
Isolongifolene	80		>10		
α-Cedrene	150	6.30 ± 2.09	0.30	0.26 – 0.43	0.60
trans-Caryophyllene	80		>10		
β-Caryophyllene	80		>10		
α-Humulene	240	4.04 ± 1.15	2.67	2.15 – 3.15	0.07
β-Chmigrene	80		>10		
Neryl isobutyrate	80		>10		
cis-Nerolidol	80		>10		
trans-Nerolidol	80		>10		
α-Cedrol	150	5.25 ± 1.23	1.26	1.11 – 1.48	0.14
β-Eudesmol	80		>10		

[a] The number of *B. germanica* females tested.

[b] CL denotes confidence limit.

[c] Relative toxicity, LD_{50} value of propoxur in Table 2/LD_{50} value of the other compound.

The fumigant toxicity of 31 compounds tested to adult female *B.germanica* was compared with that of dichlorvos (Table 20). On the basis of 24 h LD_{50} values, *a*-terpineol (21.89 mg/L of air) was the most toxic fumigant followed by Linalool (26.20 mg/L of air) and *a*-thujone (36.22 mg/L of air). Moderate toxicity (LD_{50}, 56.75 - 341.08 mg/L air) was observed with *α*-pinene, *β*-pinene, myrcene, phellandrene, *α*-terpinene, *ɣ*-terpiene, terpinene-4-ol, *α*-cedrene, *α*-humulene *α*-cedrol and limonene. The other 20 compounds exhibited weak or no fumigant activity. All tested compounds were less effective than dichlorvos (LD_{50}, 0.007 mg/L of air).

Table 20. Fumigant activity of *C. obutsa* oil and its constituents compounds against adult female *B. germanica* using vapor phase toxicity bioassay during a 24hr exposure

Compound	n[a]	slope(±SE)	LD_{50}(µg/cm^2)	95% cl[b]
α-Pinene	150	3.85 ± 0.87	218.17	182.96 – 269.87
Camphene	80		>800	
Sabinene	80		>800	
β-pinene	150	3.05 ± 0.82	143.76	115.22 – 226.97
Myrcene	240	4.84 ± 1.19	157.95	137.08 – 185.51
Phellandrene	150	7.06 ± 1.36	80.61	72.88 – 89.86
α-Terpinene	150	6.57 ± 1.14	332.29	307.44 – 336.91
4-Cymene	80		>800	
Limonene	150	6.65 ± 1.63	341.08	309.72 – 418.21
γ-Terpinene	150	2.84 ± 0.78	206.86	162.98 – 278.63
Terpinolene	80		>800	
Linalool	150	4.42 ± 0.81	26.20	22.90 – 30.15

Table 20. (continued)

Terpinene-4-ol	150	2.96 ± 0.48	56.75	45.96 – 69.99
α-Terpineol	150	5.90 ± 1.24	21.89	19.43 – 24.80
α-Fenchyl acetate	80		>800	
Linalyl acetate	80		>800	
Bornyl acetate	80		>800	
α-Thujone	150	4.27 ± 0.35	36.22	34.04 – 38.29
Terpinyl acetate	80		>800	
Longpinene	80		>800	
Longifolene	80		>800	
α-Cedrene	150	3.59 ± 0.68	63.61	52.35 – 80.00
trans-Caryophyllene	80		>800	
β-Caryophyllene	80		>800	
α-Humulene	150	3.21 ± 0.75	196.25	157.10 – 246.04
β-Chmigrene	80		>800	
Neryl isobutyrate	80		>800	
cis-Nerolidol	80		>800	
trans-Nerolidol	80		>800	
α-Cedrol	150	2.42 ± 0.79	291.99	218.31 – 431.80
β-Eudesmol	80		>800	

[a] The number of *B. germanica* females tested.

[b] CL denotes confidence limit.

5. AChE inhibition of test compounds

The German cockroach AChE inhibitory activity of 31 compounds were tested. Base on IC_{50} values, α-pinene (IC_{50}, 0.38 mM) exhibited the strongest inhibition. Moderate activity was observed with terpinene-4-ol (IC_{50}, 0.62 mM), β-pinene (IC_{50}, 0.68 mM), limonene (IC_{50}, 0.74 mM), linalool (IC_{50}, 0.77 mM) and α-terpinene (IC_{50}, 0.98 mM). The other 25 compounds exhibition no inhibitory activity.

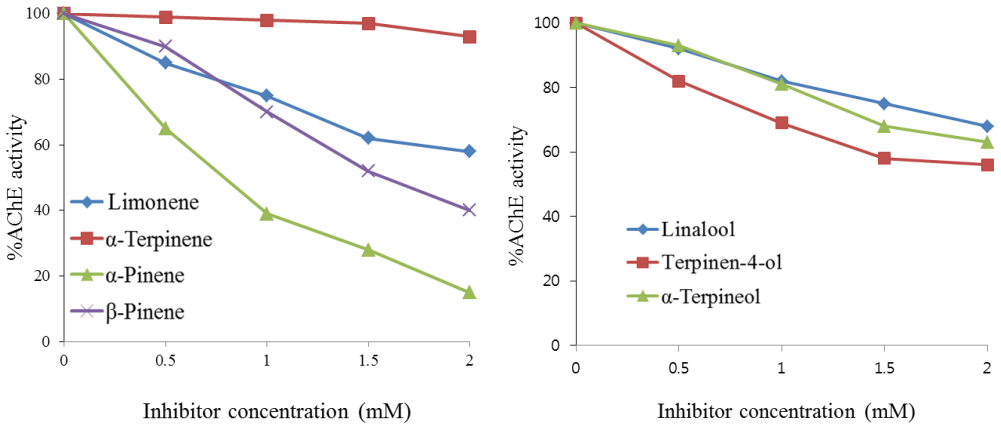


Fig. 16.The percentage of enzyme activity values for the inhibitors was calculated as compared to the control activity

Table 21. *In vitro* inhintion of AChE extracted from *B. germanica* head by 31 compounds

Compound[a]	IC_{50} (mM)	Compound[a]	IC_{50} (mM)	Compound[a]	IC_{50} (mM)
α-Pinene	0.38	Linalool	0.77	trans-Caryophyllene	NA
Camphene	ND[b]	Terpinene-4-ol	0.62	*β*-Caryophyllene	NA
Sabinene	NA[c]	α-Terpineol	0.75	*α*-Humulene	ND
β-pinene	0.68	*α*-Fenchyl acetate	NA	*β*-Chmigrene	NA
Myrcene	ND	Linalyl acetate	NA	Neryl isobutyrate	NA
Phellandrene	ND	Bornyl acetate	NA	cis-Nerolidol	ND
α-Terpinene	0.98	*α*-Thujone	ND	trans-Nerolidol	NA
4-Cymene	ND	Terpinyl acetate	NA	*α*-Cedrol	ND
Limonene	0.74	Longpinene	NA	*β*-Eudesmol	NA
γ-Terpinene	ND	Isolongifolene	NA		
Terpinolene	ND	*α*-Cedrene	ND		

[a] Concentration of compound (treatment) required for 50% enzyme inhibition as calculated from the dose-response curve.

[b] ND, not determined, means the maximum level of inhibition below 50%.

[c] NA, not acitve (1mM) tested.

Discussion

Supercritical fluid extraction (SFE) has an enormous interest nowadays, with thousands of references dealing with SFE. It is now a real option for product development, mainly those that will be used for human consumption, such as new foods, food ingredients/additives, insecticide or pharmaceutical products. Moreover, SFE has also demonstrated some advantages in the environmental field; for example, to reduce solvent waste, to get new useful compounds from industrial by-products, and to allow quantification and/or removal of toxic compounds from the environment. The supercritical fluid extraction involving of volatile components from plant materials. Emphasis is placed on optimization of extraction parameters (temperature, pressure, extraction time, modifier, etc.) for complete recovery of analytes from their matrices (Lili, 2011). In this study, Supercritical carbon dioxide (SC-CO_2) extraction was used to determine the essential oil composition of the leaves of *C. obtusa*. The optimal extraction conditions for the oil yield within the experimental range of variables examined were paticle size (0.5 - 2.0 mm), temperature (40 - 60℃) and pressure (100 - 500) bar. The chemical composition of the essential oils was analyzed by GC-MS. The major components were terpinyl acetate, bornyl acetate and sabienen.

Plants are potential products for dust mite control because many of them are selective to pests, have no or little harmful effects on nontarget organisms and the environment (Pollart *et al.,* 1977; Lee, 2004), and may be applied to dust mite nests such as beds, carpeted floors, furniture, and sofas in the same way as other conventional acaricides (Tovey, 1981). Many plant extracts and phytochemicals are known to possess

acaricidal activity against house dust mites (Lee, 2004). The reported naturally occurring acaricidal compounds against dust mites include fenchone, estragol, and thymol derived from *Foeniculum Vulgare* (Lee, 2004), anisaldehyde and 3-carene from the leaves from *Pimpinella anisum* seed (Lee, 2004), and cinamaldehyde, cinnamyl alcohol, and salicylaldehyde from *Cinnamomum cassia* (Kim, 2001). It has been reported that susceptibility to some plants such as bitter almond, caraway, and perilla was greater in *D. farinae* adults than in *D. pteronyssinus* adults (Kim, 2001). However, *D. farinae* adults are found to be more tolerant to the wood of *Thuga heteropylla* and *Cryptomeria japonica* than *D. pteronyssinus* adults (Miyazaki, 1989). However, no significant difference in acaricidal activity of cinnamaldehyde, cinnamyl alcohol, or salicylaldehyde between *D. farinae* and *D. pteronyssinus* has been noted (Kim, 2001). In this study, results are similar with cinnamaldehyde, cinnamyl alcohol, and salicylaldehyde derived from Cinnamomum cassia bark. In this study, the acaricidal constituent of *C. obtusa* oil was identified as *β*-thujaplicin against adult *D. farinae* and *D. pteronyssunus*. The active constituent of the *C. obtusa* branch was identified as *a*-thujaplicin ($C_{10}H_{12}O_2$) by spectroscopic analyses. Responses varied with dose. The toxicity of *C. obtusa* oil and *β*-thujaplicin and the acaricides to adult *D. farinae* were examined by the direct contact bioassay. As judged by 24 h LD_{50} values, acaricidal activity of *C. obtusa* oil (LD_{50}, 3.42 μg/cm^2) and *β*-thujaplicin (LD_{50}, 4.02 μg/cm^2) used was high than that of benzy benzoate (LD_{50}, 4.31 μg/cm^2), dibutyl phthalate (LD_{50}, 31.62 μg/cm^2) and DEET (LD_{50}, 78.34 μg/cm^2). Also, The toxicity of *C. obtusa* oil and *β*-thujaplicin and the acaricides to adult *D. pteronyssinus* were examined by the direct contact bioassay. As judged by 24 h LD_{50} values, acaricidal activity of *C. obtusa* oil (LD_{50}, 4.12 μg/cm^2) and *β*-thujaplicin (LD_{50}, 3.84 μg/cm^2) used

was high than that of benzy benzoate (LD_{50}, 4.21 µg/cm^2), dibutyl phthalate (LD_{50}, 30.72 µg/cm^2) and DEET (LD_{50}, 75.64, µg/cm^2). Potent acaricidal activity against both mite species was also obeseved in *a*-pinene, *β*-pinene, myrnene, phellandrene, *a*-terpinene, limonene, *ɣ*-terpinene, linalool, terpinene-4-ol, *a*-terpineol, *a*-thujone, *a*-cedrene, *a*-humulene and *a*-cedrol. These results indicate that acaricidal activity of *C. obtusa* likely results from by *a*-thujaplicin. *a*-Thujaplicin merits further study as potential house dust mite control agents or lead compounds.

The hard tick *Haemaphysalis longicornis*, which is widely distributedin East Asia and Oceania can act as a vector for viruses, chlamydia, rickettsia, bacteria, and protozoa. In this study, the repellencies of *C. obtusa* oil was evaluated in comparison with the two commercial repellency (Icaridin and DEET) against *H. longicornis*. In filter-paper diffusion method, *C. obtusa* oil (3.2 µg/cm^2) gave 100% repellency at 120 min after exposure. Whereas Icaridine (3.2 µg/cm^2) gave 96.1 % repellency, respectively, at 120 min. The repellency of the 31 compounds against *H. longicornis* was compared with that of DEET at 3.2 mg/cm^2. Responses varied according to the test compound and exposure time. *α*-Terpinene, terpinene-4-ol and α-terpineol gave 100% repellency 30 min post-treatment but 85.5, 56.4 and 68.9% repellency at 120 min, respectively. *α*-Pinene and *β*-Pinene gave 97.6 and 89.5 % repellency at 10 min, post-treatment but 67.4 and 0 % repellency at 120 min respectively. Limonene, γ-terpinene, terpinolene, terpinyl acetate, and longpinene gave 50% repellency at 30 min but repellency of these compounds was significantly low at 30 min. *C. obtusa* oil and *α*-terpinene, terpinene-4-ol and *α*-terpineol described merit further study as potential fumigants or leads for the control of *H. longicornis*.

Various compounds, including phenolics, terpenoids, and alkaloids, exist in plant essential oils and jointly or independently they contribute to bioefficacy such as insecticidal, ovicidal, repellent, and antifeeding activities against various insect species (Isman, 2000, 2001; Shigeharu, 2003; Yang *et al.*, 2003; Ngoh *et al.*, 1998). Much effort has been focused on the determination of the distribution, nature, and practical use of plant essential oil-derived chemical substances that have insecticidal activity. Ngoh et al. (1998) reported the contact and fumigant toxicity of benzene derivatives eugenol, methyleugenol, isosafrole, and safrole but neither contact nor fumigant toxic effects of the terpenoids cineole, *p*-cymene, limonene, and *a*-pinene of essential oils against adult female *Periplaneta americana* L.

In this study, the toxicity of *C. obtusa* oil and 31 compounds against adult females of the German cockroach, *Blattella germanica* L., was examined using direct contact and vapor phase toxicity bioassays and compared with those of deltamethrin, dichlorvos, permethrin, and propoxur, four commonly used insecticides. As judged by the 24 h LC_{50} values, *C. obutsa* oil (2.77 mg/cm^2) was less effective than propoxur (0.18 mg/cm^2), dichlorvos (0.007 mg/cm^2), deltamethrin (0.013 mg/cm^2) or permethrin (0.05 mg/cm^2). The insecticidal activity of 31 compounds used against adult female *B. germanica* As judged by the 24 h LC_{50} values, the adulticidal activity of *a*-thujone (0.09 mg/cm^2) was comparable to that of permethrin (0.05 mg/cm^2). The toxicity of linalool and *a*-terpineol ranging from 0.10 to 0.12 mg/cm^2, was higher than that of propoxur (0.18 mg/cm^2). Moderate contact toxicity (0.30-2.81 mg/cm^2) was observed with α-pinene, β-pinene, myrcene, phellandrene, α-terpinene, limonene, γ-terpiene, terpinene-4-ol, α-cedrene, α-humulene and α-cedrol. These compounds were less effective than either deltamethrin

(0.013 mg/cm^2) or dichlorvos (0.007 mg/cm^2).

Elucidation of the mode of action of essential oils and their constituents is of practical importance for insect control because it may give useful information on the most appropriate formulation, delivery means, and resistance management. Volatile compounds of many plant extracts and essential oils consist of alkanes, alcohols, aldehydes, and terpenoids, particularly monoterpenoids (Coats *et al.*, 1991; Visser, 1983), and exhibit fumigant activity (Ahn, 1988; Chang, 2001; Yang *et al.*, 2003; Kim, 2001). In vapor phase toxicity tests, *a*-terpineol (21.89 mg/L of air) was the most toxic fumigant followed by linalool (26.20 mg/L of air) and *a*-thujone (36.22 mg/L of air) On the basis of 24 h LC_{50} values. Moderate toxicity (LC_{50}, 56.75-341.08 mg/L air) was observed with *α*-pinene, *β*-pinene, myrcene, phellandrene, *α*-terpinene, *γ*-terpiene, terpinene-4-ol, *α*-cedrene, *α*-humulene *α*-cedrol and limonene. The other 20 compounds exhibited weak or no fumigant activity. All tested compounds were less effective than dichlorvos (LC_{50}, 0.07 mg/L of air). Dichlorvos (0.07 mg/L of air) was the most potent fumigant. *C. obtusa* oil *a*-terpineol described merit further study as potential fumigants or leads for the control of *B. germanica*.

Acetylcholinesterase is the target site for carbamate and organophosphate insecticides. The inhibition of AChE by carbamate or organophosphate insecticides occurs via a reversible complex formation followed by carbamylation or phosphorylation (Shi *et al.*, 2002). AChE is a key enzyme in the transmission of nerve impulses, specifically in termination of cholinergic synaptic transmission in mammals and insects. The primary mechanism of acute toxicity of carbamate and organophosphate insecticides has been reported as its inhibition of AChE in the cholinergic synapse of the nervous

system of the cockroach (Qian1a, 2001). In this study, the German cockroach AChE inhibitory activity of 31 test compounds were tested. Base on IC_{50} values, α-Pinene (IC_{50}, 0.38 mM) exhibited the strongest inhibiton. Moderate activity was observed with terpinene-4-ol (IC_{50}, 0.62 mM), β-pinene (IC_{50}, 0.68 mM), limonene (IC_{50}, 0.74 mM), linalool (IC_{50}, 0.77 mM) and α-terpinene (IC_{50}, 0.98 mM).

References

Ahn Y.J., Kwon M., Park H.M., and Han C.G. 1997. Potent insecticidal activity of *Ginkgo biloba*-derived trilactone terpenes against *Nilaparvata lugens*. In: Phytochemical Pest Control Agents, Hedin P. A., Hollingworth R., Miyamoto J., Masler E. and Thompson D. G. Eds., ACS Symposium Series 658, American Chemical Society, Washington, DC., pp. 90–105.

Ahn Y.J., Lee S.B., Lee, H.S., Kim, G.H. 1998. Insecticidal and acarcidal activity of carvacrol and *a*-thujaplicin derived from *Thujopsis dolabrata* var. hondai sawdust. J. Chem. Ecol. 24: 81-90.

Akgerman A., Roop R.K., Hess R.K., Yeo S.D. 1991. Environmental application of supercritical extraction. In Supercritical Fluid Technology-Reviews on Modern Theory and Application; Bruno T.J., Ely J.F., Eds.; CRC Press: Boca Raton, FL, USA. pp. 479.

Amer A. Mehlhorn H. 2006a. Larvicidal effects of various essential oils against Aedes, Anopheles, and Culex larvae (Diptera, Culicidae). Parasitol. Res. 99:466-472

Amer A. Mehlhorn H. 2006b. Persistency of larvicidal effects of plant oil extracts under differnet storage conditions. Parasitol. Res. 99:43-477.

Arlian L.G. 1977. Humidity as a factor regulating feeding and water balance of the house dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. J. Med Entomol. 14: 484-488.

Arlian L.G. 1989. Biology and ecology of house dust mites, Dermatophagoides spp. and Euroglyphus spp. Immunol. Allergy Clin. N. Am. 9: 339-356.

Arlian L.G. 1992. Water balance and humidity requirments of house dust mites. Exp. Appl. Acarol. 16: 15—35.

Arlian L.G. 2002. Arthropod allergens and human health. Annu. ReV. Entomol. 47: 395-433.

Arnason J.T., Philogene B.J.R., Morand P. 1989. Insecticides of plant orgin; ACS Symposium Series 387; American Chemical Society: Wahington, DC.

Arruda L. K., Chapman M.D. 2001. The role of cockroach allergens in asthma. Curr. Opin. Pulm. Med. 7: 14–19.

Arruda L.K., Vailes L.D., Platts-Mills T.A.E, Hayden M.L., Chapman M.D. 1997. Induction of IgE antibody responses by glutathione-*S*-transferase from the German cockroach (*Blattella germanica*). J. Biol. Chem. 272: 7–12

Barton A.F.M. 1999. Industrial use of Eucalyptus oil. Proceedings, The Oil Mallee Profitable Landcare Seminar; Oil Mallee Association of Western Australia: Perth, Australia. pp 43-54.

Birnbaum J., Orlando J.P., Charpin D. Vervloet D. 1995. Cockroaches and mites share the same beds. J. Allergy Clin. Immunol. 56: 561–562.

Bocevska M., Sovova H. 2007. Supercritical CO_2 extraction of essential oil from yarrow. J. Supercrit. Fluids. 40: 360–367.

Bronswijk J.E. and Sinha R.N. 1971. Pyroglyphid mites (Acari) and house dust allergy. J. Allergy. 47: 31-52.

Chang K.S. 2001. Insecticidal activity of phenylpropene (*E*)-anethole identified in *Illicium verum* fruit against three medical insect pests, MS thesis, Seoul National University, Suwon, Republic of Korea.

Chang K.S. 2013. Insecticide resistance monitoring and resistance breaking control strategies using plant-dervied active constituents against major vectors. PhD dissertation, Seoul National University, Seoul, Republic of Korea.

Chang K.S., Ahn Y.J. 2001. Fumigant activity of (*E*)-anethole identified in *Illicium Verum* fruit against *Blattella germanica*. Pest Manag. Sci. 58: 161-166.

Chapman M.D., Vailes L.D., Hayden M.L., Platts-Mills T.A.E., Arruda L.K. 1996. Cockroach allergens and their role in asthma. pp. 942–951, in Allergy and Allergic Diseases, Ed. A. B. Kay.

Chuang M.H., Brunner G. 2006. Concentration of minor components in crude palm oil. J. Supercrit. Fluids. 37: 151–156.

Cloarec A., Rivault C., Fontaine F., Le Guyader A. 1992. Cockroaches as carriers of bacteria in multifamily dwelling. Epidemiol. Infect. 109: 483–490.

Coats J.R., Karr L.L., Drewes C.D. 1991. Toxicity and neurotoxic effects of

monoterpenoids in insects and earthworms. In Naturally Occurring Pest Bioregulators; Hedin, P. A., Ed.; ACS Symposium Series 449; American Chemical Society: Washington, DC. pp 305-316.

Cochran D.G. 1989. Monitiring for insecticide resistance in fieldcollected strains of the German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. 82: 336-341.

Coelho L.A.F., Oliveira J. V., d'Avila S.G. 1996. The effects of temperature and solvent density on the characteristics of the extracts from SCFE of rosemary oil, Braz. J. Chem. Eng. 13: 51.

Colloff M.J., Ayres J.F., Carswell P.H., Howarth T.G., Merrett E.B., Mitchell M.J., Walshaw J.O., Warner J.A., Woodcock A.A. 1992. The control of allergens of dust mites and domestic pets: a position paper. Clin. Exp. Allergy. 22：1-28.

Colwell D.D. et al. 2011. Vector-borne parasitic zoonoses: emerging scenarios and new perspectives. Vet. Parasitol. 182, 14–21

Dantas-Torres F. 2007. Rocky Mountain spotted fever. Lancet Infect. Dis. 7, 724–732

de Saint Georges-Gridelet. 1987. J. Med. Entomol. 24: 408-411.

Dean J.R. 1998. Extraction Methods for Environmental Analysis. John Wiley & Sons, Inc.UK. pp 107–109, 123–126.

Diaz-Maroto M.C., Perez-Coello S., Cabezudo M.D. 2002. Supercritical carbon dioxide extraction of volatiles from spices comparison with simultaneous distillation-extraction. J. Chromatogr. A. 947: 23-29.

Downing A.S., Wright C.G. and Farrier M.H. 1993. Population growth of *Dermatophagoides farinae* Hughes (Acari：Epidermoptidae) suppressed by methprene and hydroprene. J. Med. Entomol. 30: 531-536.

Eggleston P. 1996. For the national cooperative inner city asthma study. Allergens in inner city homes. J. Allergy Clin. Immunol. 97: 301 (Abstr.).

Enan E. 2001. Insecticidal activity of essential oils: octopaminergic sites of action. Comp. Biochem. Physiol. 130C: 325–37

Erika M.V. 2005. Supercritical fluid extraction of plants and the functional properties of the extracts. Budapest university of technology and economics.

Erkey C., Madras G., Orejuela M., Akgerman A. 1993. Supercritical carbon dioxide of organics from soil. Environ. Sci. Technol. 27: 1225-1231.

Fain A., Guerin B., Hart B. J. 1990. Mite and Allergic Disease; Allerbio: Varennes en Argonne, France, pp 190.

FAO-Food and Agriculture Organization. 1998. Regional coordination for the integrated tick and tick. borne disease control programme in Eastern, Central and Southern Africa. Report for the OAU/IBAR organization of Africa unit/interbureau for animal resources.

Fernandes F.F., Paula Souza Freitas E. 2007. Acaricidal activity of an oleoresinous extract from *Copaifera reticulata* (Leguminosae: Caesalpinioideae) against larvae of the southern cattle tick, *Rhipicephalus* (Boophilus) *microplus* (Acari: Ixodidae). Veterinary Parasitology 147: 150–154.

Fotedar R., Shriniwas U.B., Banerjee U. 1991. Nosocomial infections: cockroaches as possible vectors of drug resistant Klbsiella. J. Hosp. Infect. 18: 155–159.

Fujisaki K., Kawazu S., Kamio T. 1994. The taxonomy of the bovine Theileria spp. Parasitology Today. 10: 31–33.

Furuno T., Terdad Y., Yano S., Uehara T., Jodai S. 1994. Activities of leaf oils and their components from Lauraceae trees against house dust mite. Mokuzai Gakkaishi 40: 78-87.

Gazim Z.C., Demarchi I.G., Lonardoni M.V., Amorim A.C., Hovell A.M., Rezende C.M., Ferreira G.A., Lima E.L., Cosmo F.A., Cortez D.A., 2011. Acaricidal activityof the essential oil from *Tetradenia riparia* (Lamiaceae) on the cattle tick *Rhipicephalus* (Boophilus) *microplus* (Acari; Ixodidae). Experimental Parasitology 129: 175–178.

Gómez A.M., López C.P., Dela O. and E.M. 1996. Recovery of grape seed oil by liquid and supercritical carbon dioxide extraction: A comparison with conventional solvent extraction. Chem. Eng. J. 61: 227-231.

Gornemann T., Nayal R, Pertz H.H. and Melzig, M.F. 2008. Antispasmodic activity of essential oil from Lippia dulcis Trev. Ethnopharmacol. 117: 166.

Gracia I., Garcia M.T., Rodríguez J.F., Fernández M.P. and de Lucas A. 2009. Modelling of the phase behaviour for vegetable oils at supercritical conditions. J. Supercrit.

Fluids. 48: 189-194.

Guan G.Q., Yin H., Luo J.X. 2002. Transmission of Babesia sp. to sheep with fieldcollected *Haemaphysalis qinghaiensis*. Parasitology Research 88: S22–S24.

Hayes W. J., Jr. Laws E. R. Jr. 1991. Handbook of Pesticide Toxicology; Academic: San Diego, CA, 1.

Helm R. M., Squillace D.L., Jones R.T., Brenner R.J. 1990. Shared allergenic activity in Asian (*Blattella asahinai*), German (*Blattella germanica*), American (*Periplaneta americana*), and Oriental (*Blatta orientalis*) cockroach species. Int. Arch. Allergy Appl. Immunol. 92: 154–161.

Helson G.A.H. 1971. House-dust mites and possible connection with sudden infant death syndrome. N. Z. Med. J. 74: 209.

Hong C.H., Kim C.S., Kim N.K., Kim Y.H. 2001. Compositionof essential oils from the leaves and the fruits of *Chamaecyparis obtusa* and *Chamaecyparis pisifera*. J. Korean Soc. Agric. Chem. Biotechnol. 44: 116-121.

Hostettmann K., Potterat O. 1997. Strategy for the isolation and analysis of antifungal, molluscicidal, and Jarvicidal agents from tropical plants. In Phytochemicals for Pest Control; Hedin, P.A., Hollingworth, R.M., Masler, E.P., Miyamoto J., Thompson D. G., Eds.; ACS Symposium Series 658; American Chemical Society: Washington, DC. pp 14-26.

http://parasitipedia.net/index.php?option=com_content&view=article&id=2549&Itemid=2826

http://www.cdc.gov/lyme/stats/chartstables/reportedcases_statelocality.html

Ibáñez E., Palacios J., Señoráns F.J., Santa-María G., Tabera J., Reglero G. 2000. Isolation and separation of tocopherols from olive by-products with supercritical fluids. J. Am. Oil Chem. Soc. 77: 2.

Ignatowicz S. 1981. Effect of inorganic salts upon biology and development of acaricidal mites. VII. Rapid desiccation of the copra mite *Tyrophagus putrescentiae* (Schrank), and other mites with tricalcium phosphate and ferric phosphate. Pol. Pismo Entomol. 1981, 51, 471-482.

Iori A., Grazioli D., Gentile E., Marano G. 2005. Acaricidal properties of the essential oil of Melateuca alternifolia Cheel (tea tree oil) against nymphs of *ixodes ricinus*. Veterinary Parasitology 129: 173–176.

Isman M.B. 1997. Neem and other botanical insecticides: barriers to commercialization. Phytoparasitica 25: 339-344.

Isman M.B. 1999. Pesticides based on plant essential oils. Pestic. Outlook. 10:68–72

Isman M.B. 2000. Plant essential oils for pest and disease management. Crop Prot. 19, 603–608.

Isman M.B. 2001. Pesticides based on plant essential oils for management of plant pests and diseases. In International Symposium on Development of Natural Pesticides from Forest Resources; Korea Forest Research Institute: Seoul, Korea, pp 1-9.

Isman M.B. 2005. Problems and opportunities for the commercialization of botanical insecticides, pp. 283-291. *Biopesticides of Plant Origin*, C. Regnault-Roger, B.J.R. Philogene and C.Vincent (eds.), Lavoisier Tech & Doc.

Isman M.B. 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and increasingly regulated world. Annu. Rev. Entomol. 51, 45–66.

Iwuala M.O.E., Osisiogu I.U.W., Agbakwuru E.O.P. 1981. Dennettia oil, a potential new insecticide: test with adults and nymphs of *Periplaneta americana* and *Zonocerus Variegatus*. J. Econ. Entomol. 74: 249-252.

Ixtaina V.Y., Vega A., Nolasco S.M., Tomas M.C., Gimeno M., Barzana E., Tecante A. 2010. Supercritical carbon dioxide extraction of oil from Mexican chia seed (*Salvia hispanica* L.): Characterization and process optimization. J. Supercrit. Fluids 55: 192–199.

Jang Y.S., Jeon J.H., Lee H.S. 2005. Mosquito larvicidal activity of active constituent derived from *Chamaecyparis obtusa* leaves against 3 mosquito species. J. Am. Mosq. Control Assoc. 21: 400–403.

Jirovetz L., Buchbauer G., Stoilova I., Stoyanova A., Krastanov A., Schmidt E. 2006. Chemical composition and antioxidant properties of clove leaf essential oil. J Agric Food Chem. 54: 6303-6307.

Jongejan F., Uilenberg, G. 2004. The global importance of ticks. Parasitology 129: S3–

S14

Jung W.C. 2006. Insecticidal acitivity of *Myristica fragrans* seed-dervied compounds againt *Blattella germanica* (Dictyoptera; Blattellidae). MS thesis, Seoul National Univ.: Republic of Korea.

Kang B.C., Zhou K., Lai Y.L., Hong C.B. 1996. Experimental asthma developed by room air contamination with cockroach allergen. Int. Arch. Allergy Immunol. 111: 299–306.

Kim D. H., Ahn Y. J. 2001. Contact and fumigant activities of constituents of *Foeniculum Vulgare* fruit against three coleopteran stores-product insects. Pest Manag. Sci. 57: 301-306.

Kim D.H. 2000. Insecticidal and repellent activities of *Foeniculum vulgare* fruit-derived materials. M.S. thesis, Seoul National University, Suwon, Republic of Korea.

Kim D.H., Ahn Y.J. 2001. Contact and fumigant activities of constituents of *Foeniculum vulgare* fruit against three coleopteran stored-product insects. Pest Manag. Sci. 57:301-306.

Kim E.H. 2002. Acaricidal activity of phenylpropenes identified in essential oil from Eugenia caruophyllata against *Dermatophagoides farinae*, *Dermatophagoides pteronyssius* (Acari： Pyroglyphidae), and Tyrophagus putrescentiae (Acari： Acaridae) MS thesis, Seoul National University. Suwon. Republic of Korea.

Kim H.K. 2001. Acaricidal activities of phenylpropenes identified in *Cinnamomum cassia* bark against Dermatophagoides spp. (Acari: Pyroglyphidae). M.S. Thesis, Seoul National University, Suwon, Republic of Korea.

Kim H.K. 2001. Acaricidal activities of phenylpropenes identified in *Cinnamomum cassia* bark against Dermatophagoides spp. (Acari：pyroglyphidae). MS thesis, Seoul National Univ.：Suwon, Republic of Korea.

Kim H.K. 2007. Acaricidal and allergen denaturing activities of plant-derived compounds toward three medical mite species. PhD dissertation, Seoul National University, Seoul, Republic of Korea.

Kim N.J. 2011. Resistance monitoring in common mosquitoes and toxicity evaluation of

constitutents from three plants against mosquito species. PhD dissertation, Seoul National University, Seoul, Republic of Korea.

Kim S.I. Mosquito repellency of cassia, fennel, and xanthoxylum essential oil compounds and larvicidal activity of *Zanthoxylum piperitum* bark compounds. PhD dissertation, Seoul National University, Seoul, Republic of Korea.

Kim S.I., Chang K.S., Yang Y.C., Kim B.S., Ahn Y.J. 2004. Repellency of fennel oil-containing aerosol and cream products to mosquitoes under laboratory and field conditions. Pest Manag. Sci. 60: 1125-1130.

Kostyukovsky M, Rafaeli A, Gileadi C, Demchenko N, Shaaya E. 2002. Activation of octopaminergic receptors by essential oil constituents isolated from aromatic plants: possible mode of action against insect pests. Pest Manag. Sci. 58:1101–6

Koyama S., Yamaguchi Y., Tanaka S., Motoyoshiya J. A. 1997. new substance (Yoshixol) with an interesting antibiotic mechanism from wood oil of Japanese traditional tree (kino-hinoki), *Chamaecypris obtusa.* Gen. Pharmacol. 28: 797-804.

Kwon J.H., Ahn Y. J. 2002. Acaricidal activity of butylidenphthalide Identified in *Cnidium ojficinale* against rhizome against *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* and *Tyrophagus putrescentiae* (Acari：Pyroglyphidae). J. Agric. Food Chem. 50: 4479-4482.

L. K. Chao, K. F. Hua, H.Y. Hsu, S.S. Cheng, J.Y. Liu, S.T. and Chang, 2005. Study on the antiinflammatory activity of essential oil from leaves of Cinnamomum osmophloeum. J. Agric. Food Chem. 53: 7274-7278.

Lawless J. 2002. The Encyclopedia of Essential Oils; Thorsons: London, U.K.

Lee H.S. 2004. Acaricidal activity of constituents identified in *Foeniculum Vulgare* fruit oil against Dermatophagoides spp. (Acari: Pyroglyphidae). J. Agric. Food Chem. 52: 2887-2889.

Lee H.S. 2004. *p*-Anisaldehyde: Acaricidal Component of *Pimpinella anisum* L. Seed Oil against the House Dust Mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus.* Planta Med. 70: 279-281.

Lee S. E., Kim J. E., Lee H. S. 2001. Insecticide resistance in increasing interest. Agric.

Chem. Biotechnol. 44: 105-112.

Lehrer S., Horner W.E., Menon P., Stankus R.P. 1991. Comparison of cockroach allergenic activity in whole body and fecal extracts. J. Allergy Clin. Immunol. 7: 574–580.

Létisse M., Rozières, M., Hiol, A., Sergent M., Comeau L. 2006. Enrichment of EPA and DHA from sardine by supercritical fluid extraction without organic modifier I. Optimization of extraction conditions. J. Supercrit. Fluids. 38: 27-36.

Li Y., Luo J., Guan G. 2009. Experimental transmission of Theileria uilenbergi infective for small ruminants by *Haemaphysalis longicornis* and *Haemaphysalis qinghaiensis*. Parasitology Research 104: 1227–1231.

Lili X., Xiaori Z., Zhaowu Z., Rong C., Haifeng L., Tian X. and Shuling W. 2011. Recent advances on supercritical fluid extraction of essential oils. African Journal of Pharmacy and Pharmacology. Vol. 5(9), pp. 1196-1211

Lind P. 1985. Purification and partial characterization of two major allergens from the house dust mite *Dermatophagoides pteronyssinus*. J. Allergy Clin. Immunol. 76: 753-761.

Lindquist R.K., Adams A.J., Hall F.R., Adams I.H.H. 1990. Laboratory and greenhouse valuations of Margosan-O against bifenthrin-resistant and -susceptible greenhouse whiteflies, Trialeurodes Vaporarium (Homoptera: Aleyrodidae). Proceedings of the USDA Neem Workshop; USDA-ARS 86; USDA: Beltsville, MD. pp 91-99.

Liu W., Fu Y.J., Zu Y.G., Tong M.H., Wu N., Liu X.L., Zhang S. 2009. Supercritical carbon dioxide extraction of seed oil from *Opuntia dillenii* Haw and its antioxidant activity. Food Chem. 114: 334–339.

McHugh A.M., Krukonis J.V. Supercritical fluid extraction. Principles and practice. Butterworth Heinemann. Newton, MA, USA 1994; pp 1-26, 99-134, 293-310.

Mendoza J., Snyder F.D. 1970. Cockroach sensitivity in children with bronchial asthma. Ann. Allergy 28: 159–163

Menon P., Menon V., Hillman B., Sanjkus R., Lehrer S.B. 1991. Skin test reactivity to whole body and fecal extracts of American (*Periplaneta americana*) and German (*Blattella germanica*) cockroaches in atopic asthmatics. Ann. Allergy 67: 573–577

Mimica-Dukić N., Bozin B., Soković M., Mihajlović B. and Matavulj M. 2003. Antimicrobial and antioxidant activities of three Mentha species essential oils. J. Agric. Food Chem. 69: 413-419.

Mitchell E.B., Willkins S.R., Deighton J.M. and platts—Mills, T.A.E. 1985. Reduction of house dust allergen levels in the home- Use of the acaricide pirimiphos-methyl. Clin. Allergy. 15: 235-240.

Miyazaki Y., Yatagai M., Takaoka M. 1989. Effect of essential oils on the activity of house dust mite. Jpn. J. Biometeorol. 26: 105-108.

Mohamed R.S., Mansoori G.A. 2002. The Use of Supercritical fluid extraction technology in food processing; featured article—food technology magazine, The World Markets Research Centre: London, UK.

Mohammed J.H., Mohammed Z.S., Sahena F., Mohd Y.A.M., Nik N.N.A.R, Mohd O. A.K. 2012. Applications of supercritical fluid extraction (SFE) of palm oil and oil from natural sources. Molecules. 17: 1764-1794.

Mukhopadhyay M. 2000. Natural extracts using supercritical carbon dioxide. CRC Press. Florida, USA. pp 3-5, 13-20, 83-93, 97-100, 109.

Mumcuoglu K. Y., Miller J., Zamir C., Zentner G., Helbin V., Ingber A. 2002. The in vivo pediculicidal efficacy of a natural remedy. Isr. Med. Assoc. J. 4: 790-793.

Mumcuoglu K.Y., Gat Z., Horowitz T., Miller J., Tana R. B., Zvi A. B., Naparstek Y. 1999. Abundance of house dust mites in relation to climate in contrasting agricultural settlements in Israel. Med. Vet. Entomol. 13: 252-258.

Mumcuoglu K.Y., Gat Z., Horowitz T., Miller, J., Tana R. Bar., Zvi A. Ben., Naparstek, Y. 1999. Abundance of house dust mites in relation to climate in contrasting agricultural settlements in Israel. Med Vet. Entomol. 13: 252-258.

Ngoh S.P., Choo L.E.W., Pang F.Y., Huang, Y., Kini M.R., Ho S.H. 1998. Insecticidal and repellent properties of nine volatile constituents of essential oils against the American cockroach, *Periplaneta americana* (L.). Pestic. Sci. 54: 261-268.

Nicholson, W.L. et al. (2010) The increasing recognition of rickettsial pathogens in dogs and people. Trends Parasitol. 26, 205–212

Nik-Norulaini N.A., Setianto W.B., Zaidul I.S.M., Nawi N.A., Azizi C.Y.M., Mohd Omar A.K. 2009. Effects of supercritical carbon dioxide extraction parameters on virgin coconut oil yield and medium-chain triglyceride content. Food Chem. 116: 193-197.

Nong, X., Ren, Y.J., Wang, J.H., Fang, C.L., Xie, Y., Yang, D.Y., Liu, T.F., Chen, L., Zhou, X.,Gu X.B., Zheng W.P., Peng X.R., Wang S.X., Lai S.J., Yang G.Y. 2013. Clinical efficacy of extract from *Eupatorium adenophorum* against the scab mite, *Psoroptes cuniculi*. Veterinary Parasitology 192, 247–252.

Patarroyo J.H., Vargas M.I., Gonzáles C.Z., Gusmán F., Martins-Filho O.A., Afonso L.C.C., Valente F.L., Peconick A.P., Marciano A.P., Patarroyo V.A.M., Sossai S. 2009. Immune response of bovines stimulated by synthetic vaccine SBm7462 against *Rhipicephalus* (Boophilus) *microplus*. Veterinary Parasitology 166: 333–339.

Pellerin P. 1991. Supercritical fluid extraction of natural raw materials for the flavor and perfume industry. Perfumer and Flavorist. 16: 37 – 39.

Penaud A., Nourrit J., Timon-David P., Charpid J. 1977. Results of a controlled trial of the acaricide paragerm on Dermatophagoides spp. in dwelling houses. Clin. Allergy. 749-753.

Peterson C.J., Nemetz L.T., Jones L.M., Coat J.R. 2002. Behavioral activity of catnip (Lamiaceae) essential oil components to the German cockroach (Blattodea: Blattellidae). J. Econ. Entomol. 95: 377-380.

Piesman J., Eisen L. 2008. Prevention of tick-borne diseases. Annu. Rev. Entomol. 53: 323–343

Platts-Mills T.A.E., de Weck A. 1989. Dust mite allergens and asthma a worldwide problem. J. Allergy Clin. Immunol. 83: 416-427.

Poliakoff M., King P. 2001. Phenomenal fluids. Nature . 412: 125.

Pollart S.M., Chapman, M.D. and Platts-Mills T.A.W. 1987. House dust sensitivity and environmental control. Prim. Care. 14: 591-603.

Pollart S.M., Mullins D. E., Vailes L.D., Hayden M.L., Platts-Mills T.A.E. 1991. Identification, quantitation and purification of cockroach allergens using monoclonal antibodies. J. Allergy Clin. Immunol. 87: 511–521.

Pollart S.M., Smith T.F., Morris E.C., Gelber L.E., Platts-Mills T.A.E., Chapman M.D. 1991. Environmental exposure to cockroach allergens: analysis with monoclonal antibody-based enzyme immunoassays. J. Allergy Clin. Immunol. 87: 505–510.

Pollart S.M., Ward G.W. Jr., Platts-Mills T.A.E. 1987. House dust sensitivity and environmental control. Immun. Allergy Clin. North America 7: 447-461.

Pourmortazavi S.M., Hajimirsadeghi S.S. 2007. Supercritical fluid extraction in plant essential and volatile oil analysis. J. Chromatogr. A. 1163: 2-24.

Priestley C.M, Williamson E.M, Wafford K.A, Sattelle D.B. 2003. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human GABA receptors and a homo-oligomeric GABA receptorfrom Drosophila melanogaster. Br. J. Pharmacol. 140:1363–1372

Qian1a K., Wei1b X.Q., Zeng X.P., Liu T., Gao1e X.W. 2001. Stage-dependent tolerance of the German cockroach, *Blattella germanica* for dichlorvos and propoxur. Journal of Insect Science: Vol. 10

Rasheed M., Afshan F., Tariq R.M., Siddiqui B.S. Gulzar T., Mahmood A., Begum S., Khan B. 2005. Phytochemical studies on the seed extract of piper nigrum Linn. Nat. Prod. Res. 19:703-712.

Regassa A., 2000. The use of herbal preparations for tick control in western Ethiopia. Journal of the South African Veterinary Association 71: 240–243.

Reverchon E., Marrone C. 2001. Modeling and simulation of the supercritical CO_2 extraction of vegetable oils. J. Supercrit. Fluids. 19: 161-175.

Reverchon E., Taddeo R. 1995. Extraction of sage oil by supercritical CO_2: Influence of some process parameters. J. Supercrit. Fluids. 8: 302–309.

Rial-Otero R., Gaspara E.M., Moura I. and Capeloa J.L. 2007. Gas chromatography mass spectrometry determination of acaricides from honey after a new fast ultrasonic-based solid phase micro-extraction sample treatment. Talanta .71: 1906-1914.

Ribeiro V.L.S., Santos J.C.D., Bordignon S.A.L., Apel M.A., Henriques A.T., von Poser G.L. 2010. Acaricidal properties of the essential oil from *Hesperozygis ringens* (Lamiaceae) on the cattle tick *Riphicephalus* (Boophilus) microplus. Bioresource Technology 101, 2506–2509.

Rice P.J., Coats J.R. 1994. Insecticidal properties of several monoterpenoids to the house fly (Diptera: Muscidae), red flour beetle (Coleoptera: Tenebrionidae) and the southern corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 87: 1172-1179.

Rizvi S.S.H., Yu Z.R., Bhaskar, A.R., Raj C.C.B. 1994. Fundamentals of processing with supercritical fluid. In Supercritical Fluid Processing of Food and Biomaterials; Rizvi, S.S.H., Ed.; Chapman and Hall: Glasgow, UK. pp. 1–26.

Rosenstreich D.L., Eggleston P., Kattan M., Baker D., Slavin R.G. 1997. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. N. Engl. J. Med. 336: 1356–1363.

Roth, L. M., Willis E.R. 1952. A study of cockroach behavior. Amer. Midi. Nat. 47:66-129.

Rothe M. J., Grant-Kels J. M. 1996. Diagnostic criteria for atopic dermatitis commentary. Lancet 348: 769-770.

Rozendaal J.A. 1997. Vector Control. Methods for Use by IndiViduals and Communities; World Health Organization: Geneva, Switzerland, 7-177.

Sahena F., Zaidul I.S.M., Jinap S., Yazid A.M., Khatib A., Norulaini N.A.N. 2010 Fatty acid compositions of fish oil extracted from different parts of Indian mackerel (Rastrelliger kanagurta) using various techniques of supercritical CO_2 extraction. Food Chem. 120: 879-885.

Salgin U., Yildiz N., Köroglu F. and Calimli, A. 2007. A parametric study of phenolic compounds desorption performance from organobentonite by supercritical fluids. J. Supercrit. Fluids. 39: 296-303.

Sanchez Ramos I., Catanera P. 2001. Acaricidal activity of natural monoterpenes on Tyrophagus putrescentiae (Schrank), a mite of stored food. J. Stored Prod. Res. 37: 93-101.

Schal C., Hamilton R. L. 1990. Integrated suppression of synanthropic cockroaches. Annu. ReV. Entomol. 35: 521-551.

Schober G., Wetter G., Bischoff E., van Bronswijk, Kniest F. M. 1987. Control of house dust mites (Pyroglyphidae) with home disinfectants. Exp. Appl. Acarol 3: 179-189.

Schou C., Lind P., Fernandez-Caldas E., Lockey R.F., Lowenstein H. 1990. Identification and purification of an important cross-reactive allergen from American and German cockroach. J. Allergy Clin. Immunol. 86: 935–46

Schulaner F.A. 1970. Sensitivity to cockroachin three groups of allergic children. Pediatrics 45: 465–466

Sharma. P., Mohan L., Srivastava C.N. 2006. Phytoextract-induced developmental deformities inmalaria vector. Bioresour, Technol. 97:1599-1604.

Shi M.A, Yuan J.Z., Wu J., Zhuang P.J., Wu X. F., Tang Z. H. 2002. Kinetic analysis of acetylcholinesterase in a propoxur-resistant strain of housefly (Musca domestica) from Shanghai, China. Pesticide Biochemistry and Physiology 72: 72-82.

Shigeharu I. 2003. Laboratory evaluation of gaseous essential oils (Part 2). Int. J. Aromather. 13: 173-184.

Siddiqui B.S., Gulzar T., Mahmood A., Begum S., Khan B., Afshan F. 2004. New insecticidal amides from prtroleum ether extract of dired Piper nigrum L. whole fruits. Chem Pharm Bull (Tokyo). 52:1349-1352.

Silva Vaz Jr I.D., Imamura S., Nakajima C., Cardoso F.C., Ferreira C.A.S., Renard G., Masuda A., Ohashi K., Onuma M. 2005. Molecular cloning and sequence analysis of cDNAs encoding for Boophilus microplus, *Haemaphysalis longicornis* and Rhipicephalus appendiculatus actins. Veterinary Parasitology. 127: 147–155.

Singh G., Upadhyay R.K. 1993. Essential oils: a potent source of natural pesticides. J. Sci. Ind. Res. 52: 676-683.

Stroh J, Wan M.T, Isman M.B, Moul D.J. 1998. Evaluation of the acute toxicity to juvenile Pacific coho salmon and rainbow trout of some plant essential oils, a formulated product, and the carrier. Bull. Environ. Contam. Toxicol. 60:923–30

Sukumar K., Perich M.J., Boobar L. R. 1991. Botanical derivatives in mosquito control: a review. J. Am. Mosq. Control Assoc. 7: 210-237.

Susan M. P., G. W. Ward, Jr. 1987. House dust sensitivity and environmental control. Immunol. Allergy North Amer. 7: 447-461.

Tanaka T., Maeda H., Matsuo T., Boldbattar D., Umemiya-Shirafuji R., Kume A., Suzuki H., Xuan X., Tsuji N., Fujisaki K. 2012. Parasiticidal activity of *Haemaphysalis*

longicornis longicin P4 peptide against Toxoplasma gondii. Invertebrate Neuropeptides XII 34: 242–250.

Temelli F. 2009. Perspectives on supercritical fluid processing of fats and oils. J. Supercrit. Fluids. 47: 583–590.

Tiziana F., Gonzalo V., Erika V., Monica R. Garcia R., Guillermo R. 2012. Isolation of essential oil from different plants and herbs by supercritical fluid extraction. Journal of Chromatography A, 1250:34-48

Tomasko D.L., Macnaughton S.J., Foster N.R. 1999. General Introduction to Supercritical Fluids. Chem. Eng. 733: 1-10.

Tovey E. R., Chapman M. D., Wells C. W., Platts-Mills T.A.E. 1981. The distribution of dust mite allergen in the houses of patients with asthma. Am. ReV. Respir. Dis. 124: 630- 635.

Tsao R., Lee S., Rice P.J., Jensen C., Coats J.R. 1995. Monoterpenoids and their synthetic derivatives as leads for new insectcontrol agents. In Synthesis and Chemistry of Agrochemicals IV.

Tuberoso C.I.G., Kowalczyk A., Coroneo V., Russo M. T., Dessì S. P., Cabras J. 2005. Composition and Antioxidant, Antimicrobial, and Antifungal Activities of the Essential Oil of Achillea ligustica All. Agric. Food Chem. 53: 10148-10153.

U.S. EPA. 1996. Exemption of certain pesticide substances from federal insecticide, fungicide, and rodenticide act requirements. U.S. Environmental Protection Agency, Final Rule, 40 CFR 152.25(g), May 6.

Van Bronswijk J.E.M.H., Sinha R.N. 1971. Pyroglyphid mites (Acari) and house dust allergy. J. Allergy 47: 31-52.

van Wijnen J.H., Verhoeff A.P., Mulder-Folkerts D.K.F., Brachel H.J.H., Schou C. 1997. Cockroach allergen in house dust. Allergy 52: 460–464.

Veal L. 1996. The potential effectiveness of essential oils as a treatment for headlice, Pediculus humanus capitis. Complement Ther. Nurs. Midwifery. 2: 97-101.

Visser J.H. 1983. Differential sensory perceptions of plant compounds by insects. In Plant Resistance to Insects; Hedin P.A. Ed., ACS Symposium Series 208; American Chemical Society: Washington, DC. pp 215-230.

Wharton G. W. 1976. J. Med. Entomol. 12: 577-621.

Wharton G.W., Duke K.M., Epstein H.M. 1979. Water and the physiology of house mites. In: Rodriguez, J. G. (ed.), Recent Advances in Acarology, vol. 1. Academic Press, New York.

Wirtz R.A. 1984. Allergic and toxic reactions to nonstinging arthropods. Annu. ReV. Entomol. 29: 47-69.

Wooster M.T., Ross M.H. 1989. Sublethal responses of the German cockroaches to vapors of commercial pesticide formulations. Entomol. Exp. Appl. 52: 49-55.

Yang Y.C., Lee S.H., Lee W.J., Choi D.H., Ahn Y.J. 2003. Ovicidal and adulticidal effects of Eugenia caryophyllata bud and leaf oil compounds on Pediculus capitis. J. Agric. Food Chem. 51: 4884-4888.

Zaidul I.S.M., Norulaini N.A.N., Mohd Omar A.K. 2006. Separation/fractionation of triglycerides in terms of fatty acid constituents in palm kernel oil using supercritical CO_2. J. Sci. Food Agric. 86: 1138-1145.

Zaidul I.S.M., Norulaini N.A.N., Mohd Omar A.K., Sato Y., Smith R.L. 2007. Separation of palm kernel oil from palm kernel with supercritical carbon dioxide using pressure swing technique. J. Food Eng. 81: 419-428.

Zermanea A., Meniai A.H., Larkeche O., Cramponc C., Barth D. 2012. Extraction and Modeling of Algerian Rosemary essential oil using supercritical CO2: Effect of pressure and temperature. Energy Procedia 18: 1038 – 1046

Zorloni A., Penzhorn B.L., Eloff J.N., 2010. Extracts of Calpurnia aurea leaves from southern Ethiopia attract and immobilise or kill ticks. Veterinary Parasitology. 168: 160–164.

Chapter II. Anti-inflammatory activities and safety properties of supercritical fluid extract from *Chamaecyparis obtusa*

Introduction

The Inflammation is physiological response of a body to a variety of stimuli, such as infections and tissue injury. Inflammatory response protects a body from inflammatory stimuli by restricting the tissue damage at the site of infection or tissue injury (Kang *et al.,* 2008). As the primary interface between the body and the external environment, the skin provides the first line of defense against traumatic injury and invasion by microbial pathogens. In addition to its properties as a physical barrier, the skin has many active defense mechanisms (Kupper and Fuhlbrigge, 2004) and regulation of these mechanisms is crucial, as inappropriate or misdirected immune activity is implicated in the pathogenesis of a large variety of inflammatory skin disorders (Chi *et al.,* 2003). While some of these conditions are easily remedied, treatments for chronic inflammatory diseases such as psoriasis and atopic dermatitis are not 100% successful (Chi *et al.,* 2003; Kang *et al.,* 2008).

High levels of inflammatory cytokines and reactive oxygen species are proposed to contribute to the pathophysiological mechanisms associated with various inflammatory skin disorders (Trouba *et al.,* 2002). It is widely recognized that cutaneous inflammation is produced and maintained by the interaction of various inflammatory cell populations that migrate to the inflammation site in response to the release of soluble pro-inflammatory mediators such as cytokines, prostaglandins, and leukotriene (Briganti and

Picardo, 2003; Lee *et al.*, 2003). Current therapies focus on treating symptoms of skin disorders with a combination of moisturizers, antihistamines, antibiotics, and corticosteroids, with the aim of repairing barrier function, and reducing itch, secondary infections, and inflammation (Kang *et al.*, 2008). However, steroids can disrupt a number of cytokine networks involved in lymphocyte function, resulting in immunosuppression, and long-term topical use can decrease collagen synthesis, leading to skin atrophy (Oikarinen *et al.,* 1998). Because of these risks, new therapeutic approaches are being intensively investigated (Kang *et al.,* 2008). A number of inflammatory mediators, including interleukin-4 (IL-4), interleukin-6 (IL-6) and nitric oxide (NO), are produced by inflammatory stimuli (Kang *et al.,* 2008). Although an appropriate production of inflammatory mediator is beneficial, excessive or persistent production of inflammatory mediator is involved in a variety of pathological conditions, such bacterial sepsis and rheumatoid arthritis (Dinarello, 1997; Palladino *et al.,* 2003; Kang *et al.,* 2008).

Essential oils are a mixture of volatile and natural substances, characterized by a strong odor and produced by aromatic plants as secondary metabolites (Rita *et al.*, 2013). They have a wide range of applications and have been commercially important for the pharmaceutical, food, cosmetic and perfume industries (Rita *et al.,* 2013). The variety of pharmacological activities found in essential oils is remarkable (Rita *et al.,* 2013). This class of natural products is attracting the interest of many researchers to investigate its potential as drugs for the treatment of various diseases (Rita *et al.*, 2013). The number of clinical and pre-clinical studies about essential oils and their chemical constituents is increasing every year (Rita *et al.,* 2013). Furthermore, there are many bioactive substances that are synthesized from constituents of essential oils. Some pharmacological

activities of these oils, such as antitumoral and antinociceptive actions are related to their anti-inflammatory effects (De Sousa, 2012; Santos and Rao, 2000; Valério *et al.,* 2007; Juergens and Stober, 1998; Wu *et al.*, 2004; Rita *et al.,* 2013). The monoterpenes are natural products belonging to the chemical group of terpenes and the main constituents of essential oils (Rita *et al.,* 2013). They are found in many bioactive essential oils and medicinal plants (De Sousa, 2012). Considering that the monoterpenes are common in many plant species and are used in cosmetic and pharmaceutical preparations, as well as in the food industry, it is important to review the pharmacological potential of monoterpenes with the anti-inflammatory activity (Rita *et al.*, 2013).

Anti-inflammatory potential of constituent compounds from *C. obutsa* oil utilizing supercritical fluid extraction has not been studied until now. Therefore, the constituent compounds of *C. obutsa*, examined their anti-inflammatory effect *in vitro* and in *vivo*. In *vitro*, Anti-inflammatory effects were assessed by measuring the levels of secretory proteins and mRNA of TNF-α, IL-4 and IL-6 production. Furthermore, an in *vivo* anti-inflammatory study was performed using a TPA-induced skin inflammation mouse model. In addition, *C. obutsa* oil designed to determine the safety profile, based on acute oral toxicity, primary dermal irritation and primary eye irritation studies.

Literature review

2.1. Inflammation

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (Ferrero, 2007). Inflammation is part of the non-specific immune response that occurs in reaction to any type of bodily injury and in which the cardinal signs of inflammation can be explained by increased blood flow, elevated cellular metabolism, vasodilation, release of soluble mediators, extravasation of fluids and cellular influx (Ferrero *et al.*, 2006; Sunita, 2010). Inflammation can be classified based on duration of inflammation as acute and chronic inflammation (Sunita, 2010).

2.2. Acute Inflammation

Acute inflammation is the early (almost immediate) response of a tissue to injury. It is nonspecific and may be evoked by any injury short of one that is immediately lethal (Chandrasoma and Taylor, 1998; Sunita, 2010). All of these processes bring the defence systems of the body to the affected area. In acute inflammation polymorphonuclear neutrophils usually predominate, whereas macrophages and lymphocytes predominate in chronic inflammation. Eosinophils are often prevalent in sites of helminth infections. Hence the characteristics of inflammation are determined both by the tissue in which it occurs and by the initiating agent and its persistence (http://www.open.edu/openlearn/science-maths-technology/science/biology/introduction-histopathology/content-section-1.2).

Acute inflammation is a process typical of vascularized tissues whereby interstitial

fluid and white blood cells accumulate at the site of injury (Sunita, 2010). Upon injury or damage, an increase in microvascular permeability is an early event that leads to edema formation during inflammation. After this change, many other mechanisms are activated, contributing to the amplification of the inflammatory response and tissue damage (Bucci *et al.*, 2005; Sunita, 2010). The cardinal signs of acute inflammation are redness (rubor), heat (calor), swelling (tumor), pain (dolor), and loss of function (function laesa) (Gallin and Snyderman, 1999; Sunita, 2010). Redness and heat are due to increased blood flow to the inflamed area; swelling is due to accumulation of fluid; pain is due to release of chemicals that stimulate nerve endings; and loss of function is due to a combination of factors (Chandrasoma and Taylor, 1998; Sunita, 2010).

2.3. Chronic Inflammation

Chronic inflammation is a prolonged process (weeks to months) in which active inflammation with mononuclear cells, tissue destruction and attempts at healing may all occur simultaneously (Kumar *et al.*, 2005; Sunita, 2010). Chronic inflammation is characterised by the dominating presence of macrophages in the injured tissue. These cells are powerful defensive agents of the body, but the toxins they release (including reactive oxygen species) are injurious to the organism's own tissues as well as invading agents. Consequently, chronic inflammation is almost always accompanied by tissue destruction (Sunita, 2010).

Inflammation has very specific characteristics, whether acute or chronic, and the innate immune system plays a pivotal role, as it mediates the first response. Infiltration of innate immune system cells, specifically neutrophils and macrophages, characterizes the acute inflammation, while infiltration of T lymphocytes and plasma cells are features of

chronic inflammation. Monocyted/macrophages play a central role in both, contributing to the final consequence of chronic inflammation which is respresented by the loss of tissue function due to fibrosis (Ferrero *et al*., 2006; Sunita, 2010).

Inflammation is a complex pathophysiological process mediated by a variety of signaling molecules produced by leukocytes, macrophages, mast cells, platelets, etc. Macrophages play a crucial role in the generation of pro-inflammatory molecules like nitric oxide (NO) (Saha *et al.*, 2004; Sunita, 2010).

2.4. Biological significance of nitric oxide

Nitric oxide (NO, formula N=O) is one of the inflammatory mediators causing inflammation in many organs. Nitric oxide, an inorganic free radical, is involved in various physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation, in organ systems (Aktan, 2004; Tewtrakul and Itharat, 2007; Sunita, 2010). At low concentration NO is shown to play a role as a neurotransmitter and NO production at high concentration is implicated in having a role in the pathogenesis of stroke, septic stroke, and other imflammatory diseases (Kim *et al.*, 2000; Sunita, 2010). NO is enzymatically synthesized via the oxidation of L-arginine by a family of nitric oxide synthase (NOS), which are either constitutive (cNOS) or inducible (iNOS). The three isoforms of NOS are encoded by distinct genes. NOS-I, also known as neuronal or brain NOS (nNOS), is found in high concentrations in neuronal and some nonneuronal tissue. NOS-II, also known as macrophage NOS or inducible NOS (iNOS), is originally found in macrophages. Furthermore, it exists in a variety of cell types including hepatocytes, vascular smooth muscle cells, fibroblasts, and epithelial cells. NOS-III, also known as endothelial NOS (eNOS), is identified as the enzyme that

produces endotheliumderived relaxing factor. Both NOS-I and NOS-III, often grouped together as constitutive NOS (cNOS), are usually constitutively expressed, and their activities are regulated by intracellular calcium concentration via calmodulin (Davis *et al.*, 2001; Hobbs *et al.*, 1999; MacMicking *et al.*, 1997; Sunita, 2010). NOS-II, inducible NOS (iNOS), is not present in resting cells but can be induced by immunostimulatory cytokines, bacterial products or infection in number of cells, including endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells. It generates NO independently of intracellular calcium concentrations (Aktan, 2004; Hobbs *et al.*, 1999; MacMicking *et al.*, 1997; Tezuka *et al.*, 2001; Sunita, 2010). The isoforms of nitric oxide synthase and their major physiological functions and implications in various diseases are summarized The nitric oxide is produced by the oxidation of L-arginin by inducible nitric oxide synthase (iNOS) in cells (Tezuka *et al.*, 2001; Sunita, 2010). The overall reaction consists of a two step oxidative conversion of L-arginine to NO and Lcitrulline via Nw-hydroxy-L-arginine (NOHarginine) as an intermediate, with monooxygenase I and monooxygenase II, each step representing a mixed-function oxidation (Aktan, 2004; Sunita, 2010).

2.5. NO production

One of the most common methods for detecting NO from a wide variety of samples and matrices is the diazotization assay, also known as the Griess assay. The reaction involves reacting NO_2^- with sulfanilamide (SA) and N-(1-Naphthyl) ethylenediamine Dihydrochloride (NED) under acidic (phosphoric acid) conditions to yield an azo dye, whose concentration could then be used as an indirect indicator of NO_2^- (and NO) concentration in the sample. This method is the procedure widely used today (Hetrick and

Schoenfisch, 2009; Sunita, 2010).

After stimulation with lipopolysaccharide (LPS), a major component of the outer membranes of Gram-negative bacteria (Kim *et al*., 2004; Sunita, 2010), many cells including macrophages express the iNOS which is responsible for the production of large amount of NO. This inducible enzyme is one of the essential components of the inflammatory response and is implicated in the pathogenesis of several inflammatory diseases (Saha *et al.,* 2004). The Griess assay is used for the determination of NO metabolites in RAW 264.7 cells induced by LPS. The Griess reaction involves first reacting NO_2^- with sulfanilamide (SA) under acidic conditions to form a diazonium salt intermediate. The diazonium salt intermediate is then coupled to *N*-(1-Naphthyl)ethylenediamine Dihydrochloride (NED) to form the stable water-soluble azo dye. The NO_2^- concentration is determined by comparing the absorbance of the azo dye solution to a calibration curve prepared with known concentrations of NO_2^- (Hetrick and Schoenfisch, 2009).

2.6. Tumor necrosis factor-alpha (TNF-*a*)

Tumor necrosis factor (TNF-*a*), cachexin or cachectin and formally known as tumor necrosis factor-alpha) is a polypeptide cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction (Sunita, 2010). It is produced chiefly by activated macrophages (M1), although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells and neurons. The primary role of TNF is in the regulation of immune cells (Sunita, 2010). TNF is able to induce apoptotic cell death, to induce inflammation, and to inhibit tumorigenesis and viral replication (Sunita, 2010). Dysregulation of TNF production has been implicated in

variety of human diseases, as well as Alzheimer's disease, cancer and major depression (Locksley *et al.*, 2001; Swardfager, 2010).

The major source of TNF-*α* is the cells of the monocyte/macrophage lineage, with T lymphocytes, neutrophils, mast cells, and endothelium also contributing under different circumstances (Sunita, 2010). All potentially noxious stimuli, ranging from the physical (ultraviolet light, X-radiation, heat) to the chemical and immunological, can rapidly induce TNF-*α* production and release (Sunita, 2010). *In vivo* TNF-*α* is the most rapidly produced pro-inflammatory cytokine, with serum levels detectable in mice in 30 min. TNF-*α* comes from preformed stores by cleavage of membrane TNF-α on macrophages, neutrophils, and activated T cells by TNF-*α* converting enzyme (TACE/ADAM17), and release of cytoplasmic granules from mast cells and eosinophils. Subsequent release of TNF-α is due to new synthesis, chiefly in macrophages and T lymphocytes (Feldmann & Maini, 2001; Sunita, 2010). Large amounts of TNF-*a* are released in response to lipopolysaccharide, other bacterial products, and Interleukin-1 (IL-1). A local increase in concentration of TNF-*a* will cause the cardinal signs of inflammation to occur: heat, swelling, redness, and pain (Sunita, 2010). If the rapid release of TNF-*α* at times of stress is blocked, the expression of other pro-inflammatory cytokines, such as IL-1 and IL-6, is reduced (Sunita, 2010). This and analogous *in vitro* data suggest that TNF-*α* *in vivo* coordinates the cytokine response to injury and acts as a fire alarm (Sunita, 2010). The induction by TNF-*α* of multiple chemokines and adhesion molecules is of major importance in rapidly attracting immune and inflammatory leukocytes to the site of injury and TNF-*α* release (Sunita, 2010). TNF-*α* also acutely upregulates the function of the immune system, but following prolonged exposure to an excess of TNF-*α*, it is immunosuppressive (Feldmann & Maini, 2001; Sunita, 2010).

2.7. Macrophages

The inflammatory process is usually tightly regulated, involving both signals that initiate and maintain inflammation and signals that shut the process down (Sunita, 2010). An imbalance between the two signals leaves inflammation unchecked, resulting in cellular and tissue damage (Sunita, 2010). Macrophages are a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes, and tissue macrophages (Sunita, 2010). From the blood, monocytes migrate into various tissues and transform macrophages (Sunita, 2010). In inflammation, macrophages have three major function; antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors (Sunita, 2010). Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation (Sunita, 2010). They are activated and deactivated in the inflammatory process (Sunita, 2010). Activation signals include cytokines (interferon gamma, granulocyte-monocyte colony stimulating factor, and tumor necrosis factor alpha), bacterial lipopolysaccharide, extracellular matrix proteins, and other chemical mediators (Sunita, 2010). Inhibition of inflammation by removal or deactivation of mediators and inflammatory effector cells permits the host to repair damages tissues (Sunita, 2010). Activated macrophages are deactivated by anti-inflammatory cytokines (interleukin 10 and transforming growth factor beta) and cytokine antagonists that are mainly produced by macrophages (Sunita, 2010). Macrophages participate in the autoregulatory loop in the inflammatory process (Sunita, 2010). Because macrophages produce a wide range of biologically active molecules participated in both beneficial and detrimental outcomes in inflammation, therapeutic interventions targeted macrophages and their products may open new avenues for controlling inflammatory diseases (Fujiwara and Kobayashi, 2005; Sunita, 2010).

Materials and Methods

2.1. Chemicals

Fourteen compounds used in this study were as follows: *α*-pinene, sabinene, *β*-pinene, 4-cymene, limonene, *γ*-terpienen, bornyl acetate, linalyl acetate, terpinyl acetate, longipinene, *α*-cedrol and terpinolene from ChromaDex (California, United States); *β*-eudesmol was supplied by Sigma (St. Louis, MO); *β*-thujaplicin was supplied by Tokyo Kasei (Tokyo, Japan).

2.2. T lymphocyte proliferation

Total splenocytes were prepared and cells were plated at 1×10^6 cells/ml in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT, USA), glutamine and 2-mercaptoethanol. Concanavalin A (ConA) was used as an inducer of T lymphocyte. K6PC-9 (1, 10 or 20 μM) was pretreated for 1 h before being incubated with ConA (1 μg/ml) for 72 h. Cells were pulsed with 1 μCi/well of [^{3}H]-thymidine (113 Ci/nmol, NEN, Boston, MA, USA) for the last 18 h and harvested with an automated cell harvester (Inotech, Dottikon, Switzerland). The amount of incorporated [^{3}H]-thymidine was measured using a Wallac Microbeta scintillation counter (Wallac, Turku, Finland).

2.3. Nitric oxide production

NO_2^- accumulation was used as an indicator of nitric oxide production in the medium. Mouse peritoneal macrophages macrophages were harvested by sterile peritoneal lavage using PBS, washed, resuspended in culture medium and plated. Non-adherent cells were removed by repeated washing after 2 h incubation at 37 ℃. Macrophages were stimulated with lipopolysaccharide (LPS, 200 ng/ml) in the presence or absence of K6PC-9p (10, 20 or 50 lm) for 24 h and culture supernatants were collected for subsequent assay. The supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2% phosphoric acid) and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 540 nm.

2.4. *β*-Hexosaminidase release assay

Degranulation of RBL-2H3 cells was evaluated by measuring the activity of the granule-stored enzyme-bhexosaminidase secreted in the extracellular medium. Cells were cultured in 24 well plates (2×10^5 cells/well) overnight. The cells were sensitized with anti-DNP-IgE (100 ng/mL) for 16 h at 37℃. After washing the cells with TGCM buffer (136 mM NaCl, 2.68 mM KCl, 0.36 mM $NaH_2PO_2H_2O$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$,

11.9 mM $NaHCO_3$, 5 mM dextrose, 1 g/L gelatin, pH 7.4), they were pretreated with ALBE (1, 10, 100 μg/mL) for 30 min and then treated with DNP-BSA (1 μg/mL) for 30 min at 37℃. Aliquots of the cellular supernatant (15 μL) were transferred to 96-well plates and incubated with 60 μL of substrate (1 mM p-nitrophenyl-N-acetyl-b-D-glucosaminide in citrate 0.05 M, pH 4.5) for 60 min at 37℃. The cells were lysed with 0.1% Triton X-100 before removing the supernatant to measure the total b-hexosaminidase activity. The reaction was stopped by adding 150 μL of Na_2CO_3-$NaHCO_3$ buffer 0.1 M, pH 10. The absorbance at 405 nm was measured with a microplate reader (Themo Labsystems). The results were presented as the percentage of total β-hexosaminidase content of the cells determined by cell lysis with 0.1% Triton X-100.

2.5. XTT assay

XTT assay was performed using Cell proliferation Kit II (XTT) purchased from Roche Applied Science (Mannheim, Germany) according to manufacturer's instruction. Briefly, XTT labelling mixture was prepared by mixing 50 volumes of 1 mg/ml sodium 3´-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulphonic acid hydrate (in RPMI 1640) and one volume of 0.383 mg/ml of *N*-methyldibenzopyrazine methyl sulphate (in PBS). XTT labeling mixture was added to culture and incubated for 1 h at 37 ℃. Absorbance was measured at 490 nm wave length.

2.6. Inhibitory effects on LPS-induced TNF-*a* release

Inhibitory effects on the release of TNF-*a* from RAW 264.7 cells were evaluated using Quantikine mouse TNF- *a* ELISA test kit. Briefly, the cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37 ℃ in a humidified atmosphere containing 5% CO_2. After that, the medium was replaced with fresh medium containing 5 μg/ml of LPS together with test samples at various concentration levels and incubated for 48 h. The supernatant (50 $\mu\ell$) was then transferred into 96-well ELISA plate and TNF- *a* concentrations were determined.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The expression of mRNA transcripts of TNF-*a* (forward: 5'-aggttctgtccctttcactcactg-3', reverse: 5'-agagaacctgggagtagacaaggta-3'), IL-6 (for ward: 5′-atggcaatgttcctgaactca act-3′, reverse: 5′-caggacaggtatagattctttccttt-3′) and *β*-actin (forward: 5'-atggcaatgttcctg aactcaact-3', reverse: 5′-caggacaggtatagattcttt ccttt-3') was determined by real-time RT-PCR. Total RNA was isolated using TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Equal amounts of RNAwere reverse transcribed into cDNA using oligo (dT) 15 primers. iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and iCycler iQ™ Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) were used for real-time PCR analysis. For amplification, samples were

heated to 94 ℃ for 8min and cycled 45 times at, 94 ℃ for 30 s, and 56 ℃ for 30 s, and 72 ℃ for 45 s. Using standards, the amount of TNF-α, IL-6 and E-selectin cDNAwas determined and normalized by the amount of *β*-actin cDNA.

2.8. TPA-induced skin inflammation

Skin inflammation was induced by topical application of 20 ㎕ of 12-O-tetradecanoylphorbol-13-acetate (TPA, 15 mg/mL in AOO) to each ear of BALB/c mice. The indicated concentrations of RS (0.05, 0.5 or 5 μg/ear, dissolved in AOO) were treated 30 min before TPA treatment. Ear thickness was measured using a digital thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo, Japan) right before and 4 h after TPA treatment, and increase n ear thickness was determined by substracting ear thickness at 0 h from that of 4h.

2.9. Statistical analysis of anti-inflammatory evaluation

The results are expressed as mean ± SD. Paired *t*-test was used to compare two groups and one-way ANOVA and Dunnett's t-test was used for multiple comparisons using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The criterion for statistical significance was set at $P < 0.05$.

2.10. Acute oral toxicity

The acute oral toxicity evaluation was conducted in rats to determine the potential of *C. obtusa* oil to produce acute oral toxicity from a single dose through the oral route. Seven healthy young adult female, nulliparous and non-pregnant sprajue dawley. Dawley rats were obtained from Samtako Bio Korea Co., Ltd. The rats were singly housed in suspended stainless steel cages with mesh floors conforming to the size recommendations in the toxicological test guideline (KFDA Notification No. 2012-86, 2012.8.24). Litter paper was placed beneath the cage and was changed at least three times per week. The rats were maintained at controlled temperature (22 - 23 ℃), relative humidity (50 ± 1 %), and light/dark cycles (12 h light/12 h dark).

All animals were observed for mortality, signs of gross toxicity and behavioral changes during the first several hours post-dosing and at least once daily for 14 d after dosing. Individual body weights were recorded prior to administration and again on days 7 and 14 (termination) following dosing. Necropsies were performed on all animals at terminal sacrifice. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavioral pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea and coma. All rats were euthanized by CO_2 inhalation at the end of the 14-d observation period and gross necropsies were performed on all animals. Tissues and organs of the thoracic and abdominal cavities were examined.

2.11. Acute dermal toxicity

The acute dermal toxicity evaluation was conducted in rats to determine the potential for *C. obtusa* oil to produce toxicity from a single topical application. Seven healthy young adult albino Sprague. Dawley rats were obtained from Samtako Bio Korea Co., Ltd. The rats were singly housed in suspended stainless steel cages with mesh floors, and exposed to the conditions described above. On the day prior to *C. obtusa* oil application, the animals were prepared by clipping the dorsal area. After clipping and prior to application, the animals were examined for health, weighed (initial) and the skin was checked for any abnormalities. Individual doses were calculated based on the initial body weights, taking into account the concentration of the test oil. Prior to the application, *C. obtusa* oil was then applied to a 2 × 3-inch 4-ply gauze pad and placed on a dorsal area of ca. 2 × 3 inches (ca. 10% of the body surface). The gauze pad and entire trunk of each animal were then wrapped with a 3-inch Durapore tape to avoid dislocation and to minimize the loss of test substance. The rats were then returned to their designated cages. The day of application was considered as day 0 of the study. After 24 h of exposure to *C. obtusa* oil, the pads were removed, and the test sites were gently cleansed of any residual test substance. Individual body weights of the animals were recorded prior to NEXT-II application (initial) and again on days 7 and 14 (termination). The animals were observed for mortality, signs of gross toxicity and behavioral changes during the first several hours after application and at least once daily thereafter for 14 d. Observations included gross

evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity, and behavioral pattern. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhea and coma. All rats were euthanized via CO_2 inhalation on day 14. Gross necropsies were performed on all animals at terminal sacrifice. Tissues and organs of the thoracic and abdominal cavities were examined.

Table 22. Classification systems of World Health Organization (WHO) for hazardous substances for acute toxicity testing

Exposure Routes	WHO class (LD_{50}, mg/kg bw.)					
	Active Ingredient		Formulations			
	Toxic	Hazardous	I	Ⅱ	Ⅲ	IV
Acute oral	<200	<2,000	<20	<200	<2,000	> 2,000
Acute dennal	<400	<2,000	<40	<400	<4,000	>4,000

I , Extremely; Ⅱ, Highly; Ⅲ, Moderately, IV, Slightly.

2.12. Primary skin irritation

The primary skin irritation test was conducted in three young nulliparous non-pregnant adult New Zealand albino rabbits to determine the potential for *C. obtusa* to cause irritation after a single topical application. The rabbits were obtained from Samtako Bio Korea Co., Ltd., and singly housed in suspended stainless steel cages with mesh

floors, which conform to the size recommendations in the toxicological test guideline (KFDA Notification No. 2012-86, 2012.8.24). Litter paper was placed beneath the cage and was changed at least three times per week. The rabbits were allowed free access to lab chow and filtered tap water ad libitum, and maintained at controlled temperature (20 ℃ - 22 ℃), relative humidity (50 ± 1 %) and light/dark cycles (12 h light/12 h dark). Animals were acclimated to laboratory conditions for a period of 3 d prior to the initiation of dosing. The day before the application, rabbits were prepared by clipping the dorsal area and the trunk. On the day of dosing but prior to application, the rabbits were critically examined for health and the skin checked for any abnormalities. Six healthy rabbits without pre-existing skin irritation were selected for the testing. Individual dose sites were scored according to the Draize Scoring System (Draize *et al.*, 1944) at 30-60 min, 24, 48 and 72-scoring intervals after patch removal. Test oil was then applied to 2.5 cm × 2.5 cm, 4-ply gauze pad and placed on one 6 cm^2 intact dose site on each rabbit. The pad and entire trunk of each rabbit were then wrapped with a semi-occlusive 3 inch Micropore tape to avoid dislocation of the pad. After 24 h of exposure to *C.obtusa* oil, the pads and collars were removed and the test sites were gently cleansed with water of any residual test substance. The classification of irritancy was obtained by adding the average erythema and edema scores for the 30-60 min, 24, 48 and 72-scoring intervals. The animals were also observed for signs of gross toxicity and behavioral changes at least once daily during the test period (Tables 23 and 24).

Table 23. Grading scale of skin irritation

Description	Score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edge of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

Table 24. Irritation index skin

Rating	Primary irritation index (P.I.I)
None irritant	< 1.0
Mild irritant	1.1 - 2.0
Moderate irritant	2.1 - 5.0
Severe irritant	> 5.1

Draize *et al.*, 1944.

2.13. Primary eye irritation

The primary eye irritation test was conducted in rabbits to etermine the potential for *C. obtusa* oil to produce irritation from a single instillation through the ocular route. Nulliparous and non-pregnant New Zealand albino rabbits were obtained from Samtako Bio Korea Co., Ltd. Litter paper was placed beneath the cage and was changed at least three times per week. The rabbits were allowed free access to lab chow and filtered tap water ad libitum, and maintained at controlled temperature (20 ℃ - 22 ℃), relative humidity (50 ± 1 %) and light/dark cycles (12 h light/12 h dark). Animals were acclimated to laboratory conditions for a period of 5 d prior to the initiation of dosing. Prior to test initiation, both eyes of rabbits were examined using a white light source and a fluorescein dye procedure. One drop of 2 % ophthalmic fluorescein sodium was instilled into both eyes of each rabbit. The eyes were rinsed with physiological saline (0.9 % NaCl) ca. 30 s after instillation of the fluorescein and then evaluated for corneal damage using an ultraviolet light source. Prior to test substance instillation, the eyes were re-examined and scored for gross abnormalities according to the "Scale for Scoring Ocular Lesions" (Draize *et al.,* 1944). Twelve healthy animals without pre-existing ocular irritation were selected for the testing. Prior to instillation 2 - 3 drops of ocular anesthetic (tetracaine hydrochloride ophthalmic solution, 0 % - 5 %) were placed into both the treated and controlled eye of each rabbit. The upper and lower lids were then gently held together for about 1 s before releasing to minimize loss of the test substance. The left (control) eye of each animal remained untreated and served as a control. The rabbits were then returned to their designated cages. Ocular irritation was evaluated macroscopically

using a high-intensity white light (Mag Lite) in accordance with Draize et al. (1944) at 1, 24, 48 and 72 h post-instillation. The fluorescein eye evaluation was used at 24 h to verify the absence of corneal damage. Individual irritation scores were recorded for each animal. In addition to observations of the cornea, iris and conjunctivae, any other lesions were noted. The average score for all rabbits at each scoring period was calculated to aid in data interpretation. Time intervals with the highest mean score (Maximum Mean Total Score; MMTS) for all rabbits were used to classify the test substance (*C. obtusa* oil) by the system of Kay & Calandra (1962). The animals were also observed for signs of gross toxicity and behavioral changes at least once daily during the test period. Observations included gross evaluation of skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea and coma. Data are shown in Tables 25, 26.

Table 25. Irritation index of eye

Rating	Index of acute ocular irritation (I.A.O.I)
None irritant	< 10
Mild irritant	10.1 ~ 30
Moderate irritant	30.1 ~ 60.0
Severe irritant	> 60.1

Table 26. Grading scle of eye irritation

(1) Co meal	
(A) Corneal opacity : The level of eye's on the rich colors.	
- There was no suppuation or corneal opacity.	0
- Close formation or dispersion of comeal opacity (not to be on the same normal transparency has a little slowed) clearly observed terminal of the Iris.	1
- Translucent can easily find but, unclear a bit of a terminal of the Iris.	2
- That is painted in opal color, not founded terminal of the Iris and founded the size of pupil barely.	3
- Corneal is opaque, not founded terminal of the Iris because the corneal opacity.	4
(B) Range of comeal haze.	
- Under a quarter (but, not zero).	1
- More than a quarter under one half.	2
- More than a quarter under three quarters.	3
- More than a three quarters under one.	4
A x B x 5 The maximum value = 80	
(2) Iris	
(A) Reaction numericalness.	
- Normality.	0
- A marked of wrinkle, bloodshot, lump, around the comeal bloodshot of single or severe combined, Iris is respond to a light (underreaction is benign).	1
- Not a response to light, hemorrhage, and almost deniaged.	2
A x 5 The maximum value = 10	
(3) Conjunctiva	
(A) Rubefaction (A case of the Eyelid and conjunctiva).	
- The blood vessels in regular.	0
- Some blood vessels clearly bleeding.	1
- A wide crimson, each blood vessels can not easily find.	2
- A light crimson.	3
(B) Chemosis	
- Not swelling.	

- A little swelling more than normal (Add to nictitating niembrance).	0
- This is noticable lump accompanied with eversion that part of an eyelid.	1
- Lump of an eyelid with half-closed eyes.	2
- Lump of an eyelid with more than half-closed eyes.	3
(B) Discharge	
- Not founding.	0
- A little discharge (except in the case that inner eyes of normal animal).	1
- Steep Eyelash and Eyelid with discharge.	2
- The part of fairly large size around eyes, steep Eyelash and Eyelid with discharge.	3
Grade (A + B +C) x 2 The maximum value = 20	

2.14. Statistical analysis of safety evaluation

Significance was judged at a probability value of $p < 0.05$. Rats were evaluated separately. Group means and standard deviations were calculated for body weight, daily body weight gain, daily food consumption, organ weight, and organ-to-body/brain weight ratio. Data within groups were compared using a one-way Analysis of Variance (ANOVA), followed by comparison of the treated groups to control by Dunnett's Multiple Comparisons test. Data were evaluated for homogeneity of variances and normality by the Bartlett test.

Results

2.1. Inhibitory effects on ConA-induced T cell proliferation

Fourteen compounds examined the effects on ConA-induced T cell proliferation for 72 h to examine the immunomodulatory effect of *C. obtusa* oil. The result revealed that terpinyl acetate demonstrated the most potent inhibitory activity 36 % at 30 μg/mL concentration. Followed, the inhibitory activity of sabinene and *β*-pinene demonstrated 30 and 21 % at 30 μg/mL concentration. Additionally, the inhibitory activity of Limonene demonstrated 41 and 88 % at 30 and 100 μg/mL (Table 27). Other compounds did not influence much on the suppression on ConA-induced T cell proliferation.

2.2. Inhibitory effects on Nitric oxide production

Fourteen compounds of *C. obtusa* oil examined the effects on nitric oxide (NO) production, LPS stimulated RAW 264.7 cells were used. The result revealed that inhibitory activity of *β*-thujaplicin demonstrated 46 and 75 % at 3 and 10 μg/mL, followed, *β*-pinene demonstrated 27, 58 and 88 % at 3, 10 and 30 μg/mL (Table 28). Other compounds did not influence much on the suppression on nitric oxide (NO) production.

Table 27. Inhibitory effects of fourteen constituent compounds of *C. obtusa* oil on T cell proliferation

Compounds	μg/ml	Mean ± SD [a], (%)	Compounds	μg/ml	Mean ± SD [a], (%)
β-Thujaplicin	0.003	99±1	Teripinolene	3	114±5
	0.01	101±1		10	101±2
	0.03	103±2		30	111±8
	0.1	2±1		100	34±4
α-Pinene	3	100±4	Linalyl acetate	3	108±3
	10	102±4		10	118±3
	30	115±7		30	96±3
	100	0±1		100	17±6
Sabinene	3	110±1	Bonyl acetate	3	98±2
	10	109±3		10	96±13
	30	70±17		30	106±2
	100	0±1		100	9±6
β-Pinene	3	114±4	*α*-Thujone	3	113±7
	10	113±7		10	113±4
	30	79±11		30	110±3
	100	-1±3		100	106±5
4-Cymene	3	101±5	Terpinyl acetate	3	98±4
	10	104±4		10	103±4
	30	103±5		30	64±10
	100	18±5		100	-1±2
Limonene	3	107±9	*α*-Cedrol	3	110±8
	10	102±7		10	109±1
	30	59±7		30	117±1
	100	22±5		100	55±12
γ-Terpinene	3	105±5	*β*-Endesrol	3	107±3
	10	108±1		10	108±2
	30	109±6		30	106±12
	100	20±6		100	84±3

[a] Values are presented as mean ± SD of the percentage of T cell formation of cultures treated with vehicle only.

[a] Statistically significant difference : $p<0.05$

Table 28. Inhibition of NO formation in LPS-stimulated RAW264.7 cells by fourteen constituent compounds of *C. obtusa* oil

Compounds	μg/ml	Mean ± SD [a], (%)	Compounds	μg/ml	Mean ± SD [a], (%)
	0.003	54±3		3	95±5
β-Thujaplicin	0.01	25±4	Teripinolene	10	90±3
	0.03	2±2		30	101±3
	0.1	0±1		100	54±12
	3	91±0		3	91±1
α-Pinene	10	79±4	Linalyl acetate	10	95±4
	30	54±6		30	89±8
	100	-3±1		100	62±2
	3	89±4		3	95±4
Sabinene	10	70±7	Bonyl acetate	10	92±4
	30	22±8		30	89±4
	100	-3±0		100	31±3
	3	73±1		3	89±6
β-pinene	10	42±2	*α*-Thujone	10	87±2
	30	12±3		30	87±3
	100	-3±1		100	74±9
	3	91±8		3	97±4
4-Cymene	10	94±5	Terpinyl acetate	10	99±3
	30	83±8		30	89±9
	100	8±1		100	10±8
	3	104±5		3	102±7
Limonene	10	102±5	*α*-Cedrol	10	107±9
	30	90±6		30	105±5
	100	22±2		100	86±14
	3	92±2		3	95±4
γ-Terpinene	10	89±5	*β*-endesrol	10	102±5
	30	89±10		30	90±7
	100	57±8		100	-1±1

[a] Values are presented as mean ± SD of the percentage of NO formation of cultures treated with vehicle only.

[a] Statistically significant difference : $p<0.05$

2.3. *β*-Hexosaminidase releasein IgE-sensitized mast cells

Rat mast cell line RBL-2H3 cells were used to determine the effect of 14 constituent compounds of *C. obtusa* oil on the secretion of *β*-hexosaminidase. Initially, we measured the cytotoxicity of fourteen constituent compounds of *C. obtusa* oil on RBL-2H3 cells using the XTT assay. *β*-thujaplicin suppressed the DNP-BSA induced *β*-hexosaminidase secretion in IgE-sensitized RBL-2H3 cells at 3, 10, and 30 μg/mL and the effects are 29, 49 and 79% (Table. 29).

Table. 29. Effects of fourteen constituent compounds of *C. obtusa* oil on cell viability and antigen-induced *β*-hexosaminidase in RBL-2H3 cells

Compounds	μg/ml	Mean ± SD [a], (%)	Compounds	μg/ml	Mean ± SD [a], (%)
	3	81±6		10	97±1
β-Thujaplicin	10	51±5	Teripinolene	30	69±5
	30	21±3		100	87±1
	3	99±11		10	85±4
α-Pinene	10	82±7	Linalyl acetate	30	72±5
	30	88±6		100	84±5
	3	102±1		10	105±3
Sabinene	10	100±5	Bonyl acetate	30	96±3
	30	102±4		100	105±3
	3	88±10		10	89±7
β-pinene	10	83±11	*α*-Thujone	30	94±4
	30	71±5		100	67±7
	10	64±11		10	92±3
4-Cymene	30	93±1	Terpinyl acetate	30	96±6
	100	84±3		100	102±6
	10	100±4		10	71±5
Limonene	30	97±4	*α*-Cedrol	30	82±4
	100	89±6		100	147±5
	10	48±17		3	103±1
γ-Terpinene	30	71±6	*β*-Endesrol	10	106±7
	100	123±5		30	109±3

[a] Values are presented as mean ± SD of the percentage of *β*-hexosaminidase of cultures treated with vehicle only.

[a] Statistically significant difference : $p<0.05$

2.4. Inhibitory effects on Nitric oxide production

Inhibitory effects of *β*-thujaplicin and *β*-pinene examined the effects on nitric oxide (NO) production, LPS stimulated RAW 264.7 cells were used. As illustrated in Figure 18. *β*-thujaplicin was a more potnet inhibitor of proligeration as compared to *β*-pinene under the treated conditions. To evaluate the inhibitory effect of *β*-thujaplicin on No production in LPS-induced RAW 264.7 cells, dmonstrated 33, 53 and 77 % at 1, 3 and 10 μg/mL. *β*-pinene demonstrated 33, 53 and 77 % at 3, 10 and 30 μg/mL (Table 30).

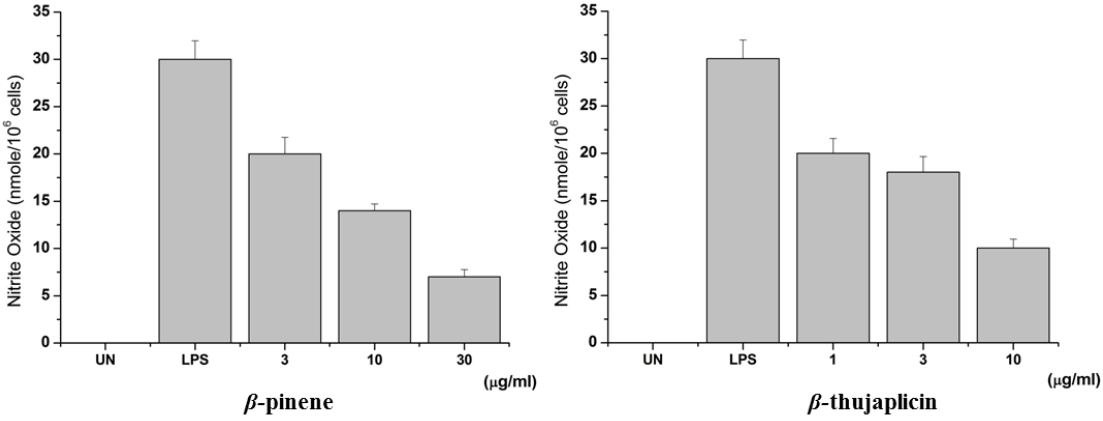


Fig 17. Inhibitory activity of *β*-pinene and *β*-thujaplicin on NO formation in LPS-induced RAW 264.7 cells.

Table. 30. Inhibitory activity of *β*-pinene and *β*-thujaplicin on NO formation in LPS-induced RAW264.7 cells.

μg/ml		Nitric Oxide (nmole/10^6 cells)[a]
UN		ND
DNP-BSA		30±1.96
β-pinene	3	20±1.761
	10	14±0.704
	30	7±0.755
β-thujaplicin	1	20±1.574
	3	18±1.661
	10	10±0.935

[a] Determined by measuring optical density at 540 nm

2.5. Effect of pro-inflammatory cytokines IL-6 and TNF-α (RT-PCR, ELISA)

To evaluate the effect of *β*-pinene and *β*-thujaplicin on the LPS-induced expression of inflammatory enzymes and proinflammatory cytokines, the mRNA levels of iNOS, IL-6, and TNF-α were analyzed by quantitative RT-PCR following treatment of RAW 264.7 cells. The results showed that *β*-pinene down-regulates mRNA expression of inflammatory genes and pro-inflammatory cytokines, such as iNOS and IL-6. *β*-thujaplicin down-regulates mRNA expression of inflammatory genes and pro-inflammatory cytokines, such as TNF-α (Figure 19).

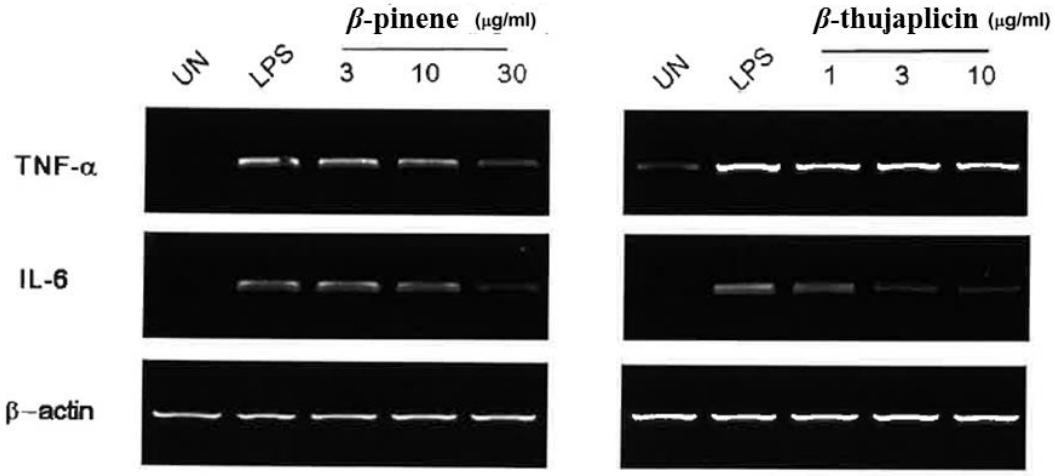


Fig 18. iNOS, IL-6, TNF-α gene expression

To evaluate the inhibitory effect of *β*-thujaplicin in LPS-induced RAW 264.7 cells, dmonstrated 43 and 63 % at 3 and 10 μg/mL on IL-6. Whereas, *β*-pinene demonstrated 50 and 79 % at 10 and 30 μg/mL on IL-6, respectively, demonstrated 16 and 74 % at 10 and 30 μg/mL on TNF-*α* (Table 31).

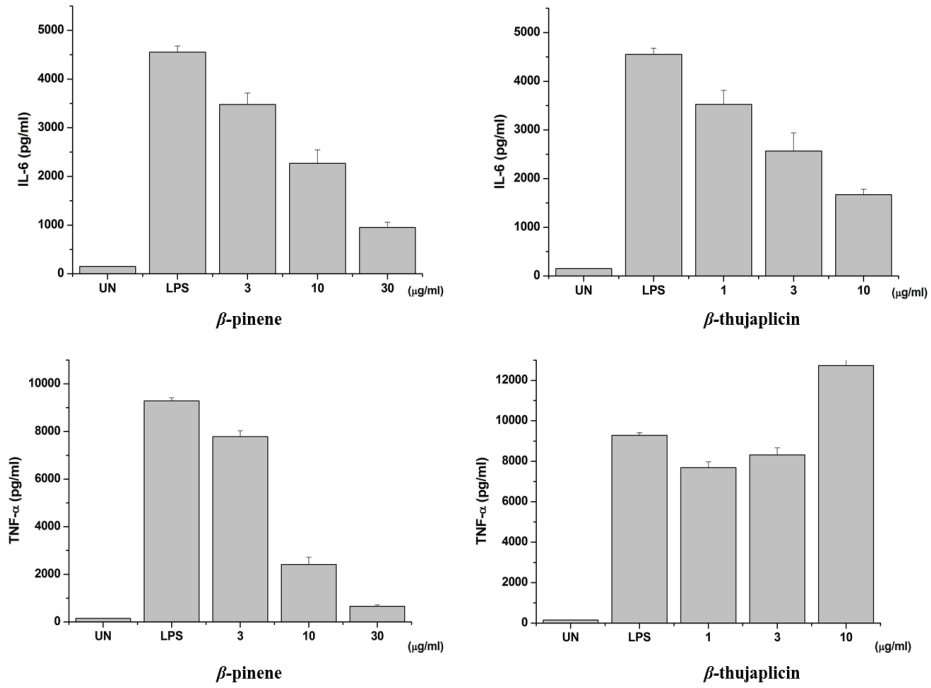


Fig 19. Inhibitory activity of *β*-pinene and *β*-thujaplicin on IL-6 and TNF-α in LPS-induced RAW264.7 cells.

Table 31. Inhibitory activity of *β*-pinene and *β*-thujaplicin on IL-6 and TNF-α in LPS-induced RAW 264.7 cells

μg/ml		IL-6 (pg/ml)	TNF-α (pg/ml)
UN		151±1	149±2
LPS		4551±130	9282±133
β-pinene	3	3478±236	7781±249
	10	2268±277	2412±310
	30	952±109	660±55
β-thujaplicin	1	3524±292	7685±293
	3	2568±370	8316±350
	10	1672±111	12738±285

2.6. *β*-Hexosaminidase secretion from antigen stimulated RBL Cells

RBL (rat basophilic leukemia) cells were employed to determine the effect of *β*-thujaplicin on the secretion of *β*-hexosaminidase, since *β*-hexosaminidase secretion is the hallmark of an allergic reaction resulting from allergen exposure. Antigen stimulation led to an increased secretion of *β*-hexosaminidase from RBL-2H3 cells in a dose-dependent manner (Fig. 21). *β*-thujaplicin suppressed the DNP-BSA induced *β*-hexosaminidase secretion in IgE-sensitized RBL-2H3 cells at 3, 10, and 30 μg/mL and the effects are 14, 45 and 81% Cell (Table. 32). The results was measured at non-toxic density.

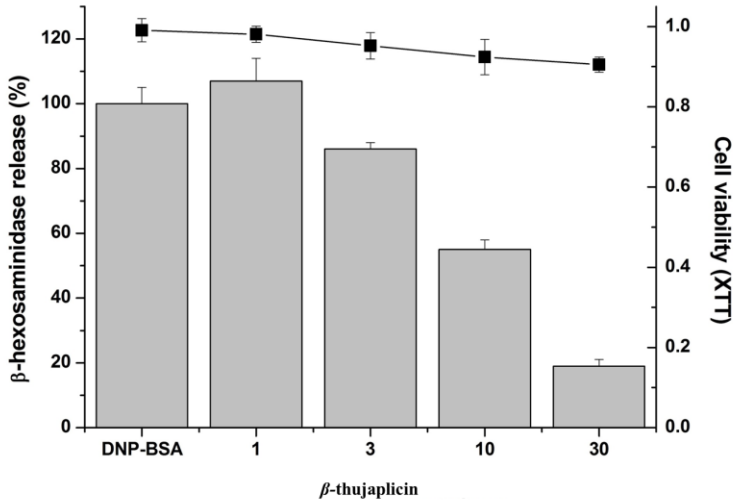


Fig 20. Effect of *β*-thujaplicin on *β*-hexosmamindase release and Cell viability (XTT)

Table 32. Effect of *β*-thujaplicin on *β*-hexosmamindase release and Cell viability (XTT)

μg/ml	*β*-hexosaminidase release (%)[a]	XTT
DNP-BSA	100±5	0.991±0.029
1	107±7	0.981±0.020
3	86±2	0.952±0.033
10	55±3	0.924±0.044
30	19±2	0.905±0.019

[a] Band area, % of control

2.7. IL-4, TNF-α gene and protein expression by *β*-thujaplicin (RT-PCR, Real-time PCR, ELISA)

Th2 cytokines, such as IL-4, stimulate the proliferation and differentiation of B lymphocytes to produce IgE. To evaluate the inhibitory effect of *β*-thujaplicin dmonstrated 25 and 78 % at 10 and 30 μg/mL on IL-4 using RT-PCR. Whereas, demonstrated 32, 66 and 80 % at 3, 10 and 30 μg/mL on TNF-α (Table 33).

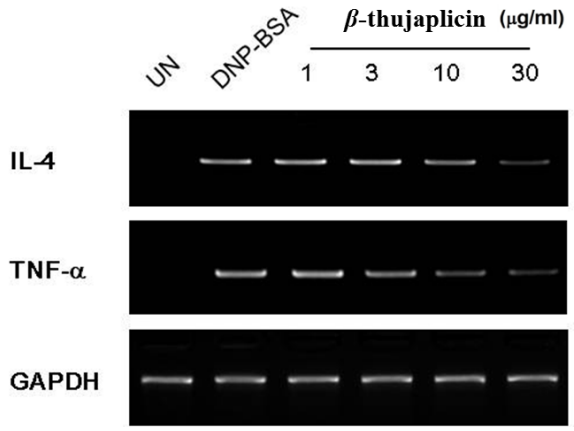


Fig 21. IL-4, TNF-α gene expression of *β*-thujaplicin (RT-PCR, band area)

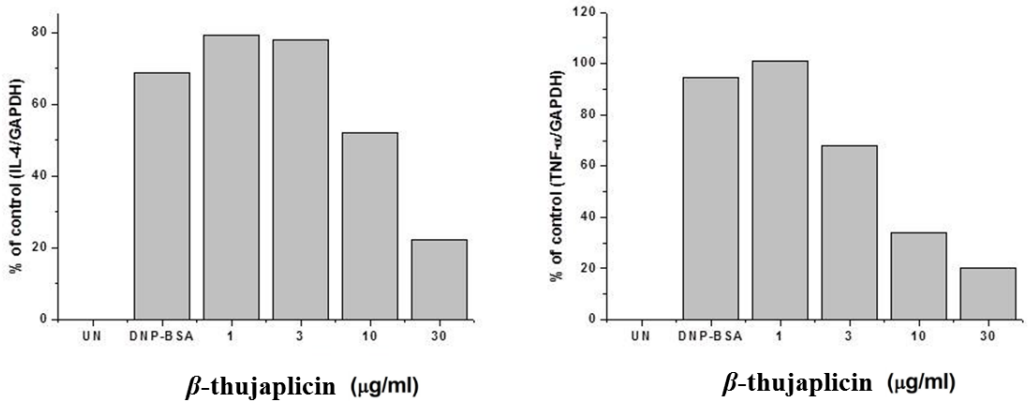


Fig 22. Inhibitory effects of by *β*-thujaplicin on the production of IL-4, TNF-α gene expression

Tabel. 33. Inhibitory effects of by *β*-thujaplicin on the production of IL-4, TNF-α gene expression

μg/ml	IL-4/GAPDH		TNF-α/GAPDH	
	ND	%[a]	ND	%[a]
DNP-BSA	69.27	100	94.58	100
1	79.26	114	101	101
3	77.91	112	68	68
10	52.01	75	33.85	34
30	22.19	32	20.06	20

[a] Band area, % of control

To evaluate the inhibitory effect of *β*-thujaplicin dmonstrated 22 and 71 % at 10 and 30 μg/mL on IL-4 using Real-time PCR. Whereas, demonstrated 32, 66 and 80 % at 20, 55 and 69 μg/mL on TNF-α (Table 34). Data-analysis utilizes the comparative CT (ΔΔCT) method.

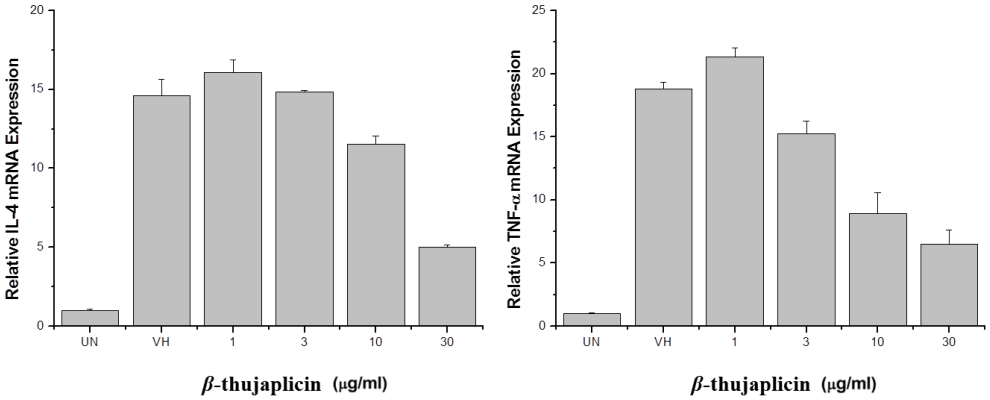


Fig. 23. Inhibitory activity by *β*-thujaplicin of IL-4, TNF-a gene expression

Table 34. Inhibitory activity by *β*-thujaplicin of IL-4, TNF-a gene expression

μg/ml	IL-4		TNF-α	
UN	1±0.11	%[a]	1±0.04	% [a]
DNP-BSA	14.61±1.03	100	18.78±0.51	100
1	16.05±0.82	111	21.33±0.73	114
3	14.84±0.11	102	15.26±0.96	80
10	11.52±0.53	77	8.92±1.63	45
30	4.99±0.17	29	6.5±1.09	31

[a] Data-analysis utilizes the comparative CT (ΔΔCT) method.

ΔCT (sample) = CT (target gene) - CT(GADPH)

ΔΔCT=ΔCT (treat sample)-ΔCT (NA sample) relative expression = $2^{-\Delta\Delta CT}$

To evaluate the inhibitory effect of *β*-thujaplicin dmonstrated 19 and 57 % at 10 and 30 μg/mL on IL-4 protein formation. Whereas, demonstrated 12 and 80 % at 10 and 30 μg/mL on TNF-α protein formation (Table. 35).

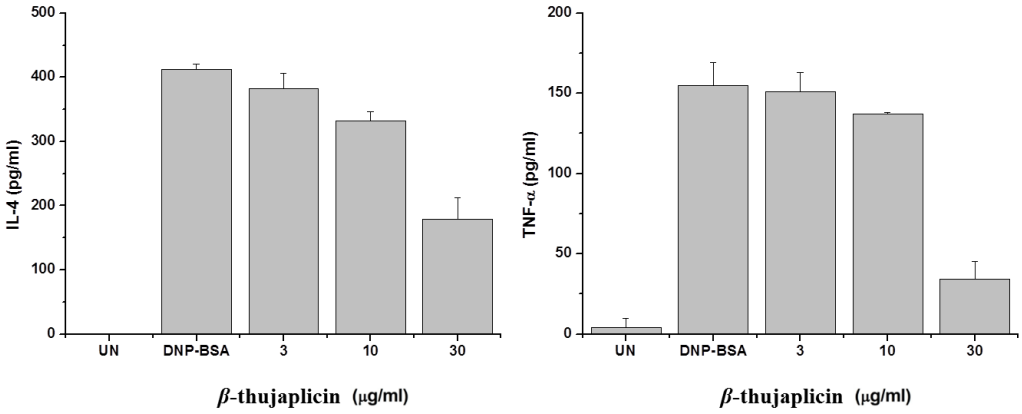


Fig 24. Inhibitory activity by *β*-thujaplicin of IL-4, TNF-*α* protein expression

Table 35. Inhibitory activity by *β*-thujaplicin of IL-4, TNF-*α* protein expression

μg/ml	IL-4 (pg/ml)		TNF-α (pg/ml)	
UN	ND	%	4±6	%
DNP-BSA	412±9	100	155±14	100
3	382±24	93	151±12	97
10	332±15	81	137±1	88
30	179±33	43	34±11	20

[a] Band area, % of control

2.8. Effect of *β*-pinene and *β*-thujaplicin on TPA-induced increase inflammation

Induction of acute skin inflammation by skin irritants causes vasodilation, leukocyte infiltration and edema, resulting in the increase of skin thickness. Therefore, change in ear thickness was used as a parameter of skin inflammation in experiment.

To investigate the anti-inflammatory effects of *β*-pinene and *β*-thujaplicin *in vivo*, we examined the effect of topically applied *β*-pinene and *β*-thujaplicin on TPA-induced skin inflammation. Fig. 26 shows that TPA (300 ng/ear) substantially increased ear thickness in BALB/c mice and this increase in ear thickness was dose-dependently suppressed by *β*-pinene and *β*-thujaplicin treatment. Treatment of mice with 3μg/ear of *β*-thujaplicin caused 43% inhibition of TPA-induced increase in ear thickness. Whereas, treatment of mice with 3*μ*g/ear of *β*-pinene caused 43% inhibition of TPA-induced increase in ear thickness (Table. 36)

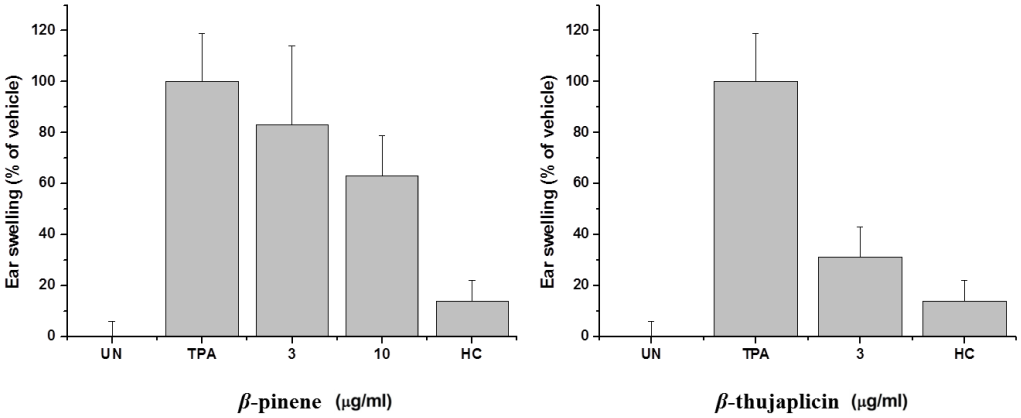


Fig 25. Effect of *β*-pinene and *β*-thujaplicin on TPA-induced increase inflammation

Table 36. Effect of *β*-pinene and *β*-thujaplicin on TPA-induced increase inflammation

Compound (μg)		Ear swelling	
		Increase in ear thichkness(mm)	% of vehicle
UN		0.000±0.006	0±6
TPA		0.103±0.020	100±19
β-pinene	1	0.085±0.032	83±31
	3	0.065±0.017	63±16
β-thujaplicin	10	0.032±0.013	31±12
1% Hydrocortisone		0.014±0.008	14±8

2.9. Acute oral toxicity

A single oral administration of *C. obtusa* oil was provided to male and female Sprague–Dawley rats to assess its acute toxicity. *C. obtusa* oil, at the limit dose of *C. obtusa* oil level of 5,000 mg/kg body weight, did not cause any mortality and did not demonstrate any signs of gross toxicity, adverse pharmacological effects or abnormal behavior in the treated female rats following dosing and during the observation period of 14 d thereafter. All animals survived, gained normal body weight and appeared active and healthy during the study. No gross abnormalities or pathological alterations were noted for any of the rats when necropsied at the conclusion of the 14 d observation period (Table 37). Based on these results and under the conditions of this study, the acute oral LD_{50} of *C. obtusa* oil is greater than 5,000 mg/kg of body weight in rats.

Table 37. Mortality in ICR mice treated orally with *C. obtusa* oil

Sex	Dose (mg/kg)	Days after treatment															Mortality (%)
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Male	0	0[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2,500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2,500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5,000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Number of dead animals

Table 38. Clinical signs in ICR mice treated orally with *C. obtusa* oil

Sex	Findings	Days after treatment														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Male	normal	7[a]	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	normal	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	normal	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Female	normal	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	normal	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	normal	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7

[a] No, of rats with the clinical signs

Table 39. Body weight change in male and female mice after single oral administration of *C. obtusa* oil

Sex	Dose (mg/kg)	Days after treatment				
		0	1	3	7	14
Male	0	237.8 ± 11.6[a]	254.4 ± 16.7	272.8 ± 12.3	303.2 ± 18.1	336.1 ± 17.5
	2,500	231.2 ± 3.7	233.6 ± 11.8	259.8 ± 10.1	298.8 ± 19.1	329.0 ± 10.2
	5,000	233.1 ± 7.7	230.3 ± 8.8	261.2 ± 9.1	295.4 ± 11.8	331.5 ± 13.6
Female	0	181.8 ± 11.6	184.3 ± 8.6	197.6 ± 9.1	209.5 ± 12.6	249.0 ± 12.3
	2,500	171.2 ± 9.2	174.3 ± 4.1	191.4 ± 7.3	210.4 ± 5.6	244.5 ± 4.7
	5,000	170.2 ± 6.7	169.5 ± 6.8	183.6 ± 7.4	207.4 ± 11.4	243.4 ± 3.9

[a] Values are presented as means ± SD (g)

2.10. Acute dermal toxicity

Acute dermal toxicity of *C. obtusa* oil was conducted in male and female Sprague–Dawley rats to determine the potential for *C. obtusa* oil to cause toxicity from a single topical application. All animals survived, gained normal body weight, and appeared active and healthy during the study. There were no signs of dermal irritation, gross toxicity, adverse pharmacological effects or abnormal behavior. No gross abnormalities were noted in any of the animals when necropsied at the conclusion of the 14-d observation period. The findings are summarized in Table 40. Under the conditions of this study, the single-dose acute dermal LD_{50} of *C. obtusa* oil is greater than 2,500 mg/kg of body weight in both male and female rats.

Table 40. Motality in SD rat treated dermally with *C. obtusa* oil

Sex	Dose (mg/kg)	Days after treatment															Mortality (%)
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Male	0	0[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1,250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2,500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1,250	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2,500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Number of dead animals

Table 41. Clinical sign in ICR mice treated dermally with *C. obtusa* oil

Sex	Dose (mg/kg)	Findings	Days after treatment														
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Male	0	normal	6[a)]	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	1,250	normal	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	2,500	normal	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Female	0	normal	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	1,250	normal	6	6	2	2	4	6	6	6	6	6	6	6	6	6	6
		crust formation	0	0	4	4	2	0	0	0	0	0	0	0	0	0	0
	2,500	normal	6	6	2	2	2	6	6	6	6	6	6	6	6	6	6
		crust formation	0	0	4	4	4	0	0	0	0	0	0	0	0	0	0

Table 42. Body weight change in male and female mice treated dermally with *C. obtusa* oil

Sex	Dose (mg/kg)	Days after treatment				
		0	1	3	7	14
Male	0	238.5 ± 13.3[a]	257.2 ± 17.8	278.1 ± 16.3	303.3 ± 19.1	337.4 ± 12.2
	1,250	249.7 ± 14.9	262.1 ± 34.3	282.7 ± 28.6	289.7 ± 20.0	338.3 ± 11.6
	2,500	255.2 ± 5.7	260.1 ± 7.2	282.7 ± 4.3	297.2 ± 7.4	342.2 ± 9.1
Female	0	179.7 ± 12.3	182.8 ± 9.3	200.2 ± 7.8	209.9 ± 7.9	245.8 ± 9.2
	1,250	179.5 ± 13.7	178.4 ± 14.2	189.7 ± 12.1	197.6 ± 12.2	239.2 ± 8.4
	2,500	185.5 ± 5.9	182.2 ± 1.4	193.1 ± 1.8	205.2 ± 8.7	246.2 ± 8.9

[a] Values are presented as means ± SD (g).

2.11. Primary skin irritation

Primary skin irritation was investigated in female New Zealand albino rabbits to evaluate the potential of *C. obtusa* oil to produce irritation after a single topical application. Following the application of *C. obtusa* oil, all animals appeared active and healthy. Apart from the skin irritation noted below, there were no signs of gross toxicity, adverse pharmacological effects or abnormal behavior. One hour after patch removal, very slight erythema was observed at all treatment sites. The overall incidence and severity of irritation decreased with time. All animals were free from dermal irritation within 24 h. A summary of the Draize primary dermal irritation scoring criteria for dermal reactions and descriptive rating for mean primary dermal irritation index (PDII) is presented in Table 24. Under the conditions of this study, the PDII for *C. obtusa* oil was calculated to be 0.17 at 5,000 mg/ 0.5ml, thus classifying *C. obtusa* oil to be slightly irritating to the skin (Tables 43 and 44).

Table 43. Skin irritation score in rabbits treated with *C. obtusa* oil

Site		Control								Test[a]							
Change		Erythema & Eschar				Edema				Erythema & Eschar				Edema			
Phases[b]		Intact		Abraded		Intact		Abraded		Intact		Abraded		Intact		Abraded	
		24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h
Animal No.	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sum		0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0
Mean		0	0	0	0	0	0	0	0	0	0.17	0	0.33	0	0	0	0
Total		0								0.5							
P.I.I[c]		0								0.13							

[a] Treated concentrarion (2,500 mg/ 0.5ml)

[b] Time after topical application

[c] P.I.I (Primary irritation index) = Total (Sum of mean of scores made at 24 and 72h)/4.

Table 44. Skin irritation score in rabbits treated with *C. obtusa* oil

Site		Control								Test[a]							
Change		Erythema & Eschar				Edema				Erythema & Eschar				Edema			
Phases[b]		Intact		Abraded		Intact		Abraded		Intact		Abraded		Intact		Abraded	
		24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h
Animal No.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sum		0	0	0	0	0	0	0	0	0	2	0	2	0	0	0	0
Mean		0	0	0	0	0	0	0	0	0	0.33	0	0.33	0	0	0	0
Total		0								0.67							
P.I.I[c]		0								0.17							

[a] Treated concentrarion (5,000 mg/ 0.5ml)

[b] Time after topical application

[c] P.I.I (Primary irritation index) = Total (Sum of mean of scores made at 24 and 72h)/4.

2.12. Primary eye irritation

Primary eye irritation test was conducted in New Zealand albino rabbits to determine the potential for *C. obtusa* oil to cause irritation from a single instillation. All animals appeared active and healthy. There were no signs of gross toxicity, adverse pharmacological effects or abnormal behavior. No corneal opacity or iritis was observed in any treated eye during the study. One hour following *C. obtusa* oil instillation, all treated eyes exhibited conjunctivitis (Table 26). Individual eye irritation scores are presented in Table 26 in accordance with the Draize Scale for scoring Eye Lesions and the Kay and Calandra Scheme for classifying eye irritants (Draize *et al.*, 1944; Kay & Calandra, 1962). The overall severity of irritation decreased with time (Tables 45 and 46). All animals were free of ocular irritation within 48 h. Under the conditions of this study, the MMTS of *C. obtusa* oil was determined to be 0.33 (Tables 8), classifying *C. obtusa* oil to be minimally irritating to the eye.

Table 45. Eye irritation score of rabbits treated *C. obtusa* oil during a 24 hr expoure

Animal No.	No eye washed			Score	Animal No.	Eye washed			Score
	Cornea	Iirs	Conjunctivae			Cornea	Iirs	Conjunctivae	
1	0	0	0	0	7	0	0	0	0
2	0	0	2	2	8	0	0	2	0
3	0	0	0	0	9	0	0	0	0
4	0	0	0	0	10	0	0	0	0
5	0	0	0	0	11	0	0	2	0
6	0	0	0	0	12	0	0	0	0
I.I.O.I[a]				2	I.I.O.I				0
M.I.O.I[b]				0.33	M.I.O.I				0
I.A.O.I[c]				0.33	I.A.O.I				0.33

[a] The Individual Index of Ocular Irritation = (A×B×5)+(C×5)+(D+E+F)

[b] Mean Index of Ocular Irritation.

[c] The Index of Acute Ocular Irritation = The maximum value of mean index ocular irritation.

Table 46. Eye irritation score of rabbits treated *C. obtusa* oil during 48 hr expoure

Animal No.	No eye washed			Score	Animal No.	Eye washed			Score
	Cornea	Iirs	Conjunctivae			Cornea	Iirs	Conjunctivae	
1	0	0	0	0	7	0	0	0	0
2	0	0	0	0	8	0	0	0	0
3	0	0	0	0	9	0	0	0	0
4	0	0	0	0	10	0	0	0	0
5	0	0	0	0	11	0	0	0	0
6	0	0	0	0	12	0	0	0	0
I.I.O.I[a]				0	I.I.O.I				0
M.I.O.I[b]				0	M.I.O.I				0
I.A.O.I[c]				0.33	I.A.O.I				0.33

[a] The Individual Index of Ocular Irritation = (A×B×5)+(C×5)+(D+E+F)

[b] Mean Index of Ocular Irritation.

[c] The Index of Acute Ocular Irritation = The maximum value of mean index ocular irritation.

Discussion

Inflammation is a physiological response of a body to stimuli, including infections and tissue injury. However, excessive or persistent inflammation causes a variety of pathological conditions, such as bacterial sepsis, rheumatoid arthritis and skin inflammation (Dinarello, 1997; Palladino *et al*., 2003). As the primary interface between the body and the external environment, the skin provides the first line of defense against traumatic injury and invasion by microbial pathogens. In addition to its properties as a physical barrier, the skin has many active defense mechanisms (Kupper and Fuhlbrigge, 2004) and regulation of these mechanisms is crucial, as inappropriate or misdirected immune activity is implicated in the pathogenesis of a large variety of inflammatory skin disorders. While some of these conditions are easily remedied, treatments for chronic inflammatory diseases such as psoriasis and atopic dermatitis are not 100% successful (Chi *et al.*, 2003). High levels of inflammatory cytokines and reactive oxygen species are proposed to contribute to the pathophysiological mechanisms associated with various inflammatory skin disorders (Trouba *et al*., 2002). It is widely recognized that cutaneous inflammation is produced and maintained by the interaction of various inflammatory cell populations that migrate to the inflammation site in response to the release of soluble pro-inflammatory mediators such as cytokines, prostaglandins, and leukotriene (Briganti and Picardo, 2003; Lee *et al.*, 2003; Sharif *et al*., 2010). Current therapies focus on treating symptoms of skin disorders with a combination of moisturizers, antihistamines, antibiotics, and corticosteroids, with the aim of repairing barrier function, and reducing

itch, secondary infections, and inflammation. However, steroids can disrupt a number of cytokine networks involved in lymphocyte function, resulting in immunosuppression, and long-term topical use can decrease collagen synthesis, leading to skin atrophy (Oikarinen *et al.,* 1998; Sharif *et al*., 2010). Because of these risks, new therapeutic approaches are being intensively investigated. A large number of plant species contain various bioactive compounds exhibiting health beneficial properties, anti-oxidative, anti-inflammatory and mainly antimicrobial effects, and their preventive and therapeutic use increases (Chien *et al.,* 2008; Lee *et al.,* 2009; Sharif *et al*., 2010). Numerous natural products have been already tested in various animal models for the development of new anti-inflammatory therapeutics (Chien *et al*., 2008; Lee *et al.,* 2009; Sharif *et al*., 2010).

In the present study, the constituent compounds of *C. obutsa*, examined their anti-inflammatory effect *in vitro* and *in vivo*. *In vitro*, Anti-inflammatory effects were assessed by measuring the levels of secretory proteins and mRNA of TNF-α, IL-4 and IL-6 production. The results showed that *β*-pinene induced nitric oxide production (LPS stimulated RAW 264.7 cells), down-regulates mRNA expression of inflammatory genes and pro-inflammatory cytokines, such as iNOS and IL-6. Also, *β*-thujaplicin suppressed the DNP-BSA induced *β*-hexosaminidase secretion in IgE-sensitized RBL-2H3 cells, down-regulates mRNA expression of inflammatory genes and pro-inflammatory cytokines, such as TNF-*α* and IL-4. Furthermore, an in *vivo* anti-inflammatory study was performed using a TPA-induced skin inflammation mouse model, and the results showed that *β*-pienen and *β*-thujaplicin reduced TPA-induced inflammation and attenuated the expression of iNOS in TPA-induced mouse skin tissue. Thus, *β*-pienen and *β*-thujaplicin demonstrated anti-inflammatory activity both in LPS-induced RAW 264.7 cells and TPA-

stimulated mouse skin and may therefore serve as a potential anti-inflammatory agent.

The present study was designed to determine the safety profile of *C. obutsa* oil utilizing supercritical flulid extraction, based on acute oral toxicity, primary dermal irritation and primary eye irritation studies. The acute oral LD_{50} of *C. obtusa* oil is greater than 5,000 mg/kg of body weight in rats. Also, the single-dose acute dermal LD_{50} of *C. obtusa* oil is greater than 5,000 mg/kg of body weight in both male and female rats. The primary dermal irritation index (PDII) of *C. obtusa* oil was calculated to be 0.17, thus classifying *C. obtusa* oil to be slightly irritating to the skin. In primary eye irritation studies, the maximum mean total score (MMTS) of *C. obtusa* oil was observed to be 0.33 and classifying *C. obtusa* oil to be minimally irritating to the eye.

Conclusion

The environmental problems caused by overuse of pesticides have been the matter of concern for both scientists and public in recent years (Isman and Machial, 2006). It has been estimated that about 2.5 million tons of pesticides are used on crops each year and the worldwide damage caused by pesticides reaches $100 billion annually (Isman and Machial, 2006). The reasons for this are two fold: (1) the high toxicity and nonbiodegradable properties of pesticides and (2) the residues in soil, water resources and crops that affect public health (Isman and Machial, 2006). Thus, on the one hand, one needs to search the new highly selective and biodegradable pesticides to solve the problem of long term toxicity to mammals and, on the other hand, one must study the environmental friendly pesticides and develop techniques that can be used to reduce pesticide use while maintaining crop yields (Isman and Machial, 2006). Natural products are an excellent alternative to synthetic pesticides as a means to reduce negative impacts to human health and the environment (Isman and Machial, 2006). The move toward green chemistry processes and the continuing need for developing new crop protection tools with novel modes of action makes discovery and commercialization of natural products as green pesticides an attractive and profitable pursuit that is commanding attention (Isman and Machial, 2006). The concept of “Green Pesticides” refers to all types of nature-oriented and beneficial pest control materials that can contribute to reduce the pest population and increase food production (Isman and Machial, 2006). They are safe and ecofriendly. They are more compatible with the environmental components than synthetic pesticides (Isman and Machial, 2006).

Thus in the present concept of green pesticides, some rational attempts have been made to include substances such as plant extracts, hormones, pheromones and toxins

from organic origin and also encompass many aspects of pest control such as microbial, entomophagous nematodes, plantderived pesticides, secondary metabolites from microorganisms, pheromones and genes used to transform crops to express resistance to pests. More recently, the encouragement of use of products from natural resources and even the extremely biodegradable synthetic and semisynthetic products in pest management, has been considered to constitute the umbrella of green pesticides (Koul *et al.*, 2003; Koul 2005; Dhaliwal and Koul, 2007; Koul, 2008).

Many monoterpenoids have insecticidal properties, the degree of toxicity of different compounds to one species differs considerably (Cornelius *et al.*, 1997). Pesticides based on plant essential oils or their constituents have demonstrated efficacy against a range of stored product pests, domestic pests, blood feeding pests and certain soft-bodied agricultural pests, as well as against some plant pathogenic fungi responsible for pre- and post-harvest diseases (Opender *et al.*, 2008). They may be applied as fumigants, granular formulations or direct sprays with a range of effects from lethal toxicity to repellence and/or oviposition deterrence in insects (Opender *et al.*, 2008). These features indicate that pesticides based on plant essential oils could be used in a variety of ways to control a large number of pests (Opender *et al.*, 2008).

In conclusion, *C. obtusa*-derived preparations containing active constituents described herein merit further study as potential insecticides for the control of insecticide – acaricide against house dust mites, repellency aganist hard tick and cockroach for protecting humans from bites and nuisance caused by vectors. Also, presented that possible applications of essential oil from *C. obtusa* as a useful anti-inflammatory agent. Moreover, the anti-inflammatory effects of the major pharmacological components present in the essential oil of *C. obtusa* might accelerate the development of new drugs for various inflammatory diseases.

References

Abbas A.K., Murphy K.M., Sher A. 1996. Functional diversity of helper T lymphocytes. Nature. 383: 787–793.

Abramovits W. 2005. Atopic dermatitis. J Am Acad Dermatol. 53: 86–93.

Ahn S.K., Bak H.N., Park B.D. 2006. Effects of a multilamellar emulsion on glucocorticoid-induced epidermal atrophy and barrier impairment. J. Dermatol. 33: 80–90.

Aktan F. 2004. iNOS-mediated nitric oxide production and its regulation. Life Sciences. 75: 639-653.

Barbato J.E., Tzeng E. 2004. Nitric oxide and arterial disease. J. Vasc. Surg. 40: 187-193.

Bohme M., Wickman M., Lennart N. S., Svartengren M., Wahlgren C. F. 2003. Family history and risk of atopic dermatitis in children up to 4 years. Clin Exp Allergy 33: 1226–1231.

Briganti S., Picardo M. 2003. Antioxidant activity, lipid peroxidation and skin diseases. What's new. J. Eur. Acad. Dermatol. Venereol. 17: 663–669.

Bruch-Gerharz D., Ruzicka T., Kolb-Bachofen V. 1998. Nitric oxide and its implications in skin homeostasis and disease - a review. Arch. Dermatol. Res. 290: 643-651.

Bucci M., Roviezzo F., Posadas I., Yu J., Parente L., Sessa W. C. 2005. Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation in vivo. Proceedings of the National Academy of Sciences. 3: 904-908.

Chandrasoma P., Taylor C. R. 1998. Part A. General Pathology, Section II. The Host Response to Injury. Concise Pathology (3rd ed.). New York, N.Y.: McGraw-Hill.

Chi Y.S., Lim H., Park H., Kim H. P. 2003. Effects of wogonin, a plant flavone from Scutellaria radix, on skin inflammation: in vivo regulation of inflammation associated gene expression. Biochem. Pharmacol. 66: 1271–1278.

Chien T.L., Chi J.C., Ting Y.L., Tung J.C., Sheng Y.W. 2008. Anti-inflammation activity of fruit essential oil from Cinnamomum insularimontanum Hayata. Bioresour. Technol. 99: 8783–8787.

Cho Y.H., Lee C.H., Kim S.G. 2003. Potentiation of lipopolysaccharideinducible

cyclooxygenase 2 expression by C2-ceramide via c-Jun Nterminal kinase-mediated activation of CCAAT ⁄ enhancer binding protein b in macrophages. Mol Pharm. 63: 512–523.

Choi J., Yoon B.J., Han Y.N., Lee K.T., Ha J., Jung H.J., Park, H.J. 2003. Antirheumatoid arthritis effect of *Rhus verniciflua* and of the active component, sulfuretin. Planta Med. 69: 899-904.

Chomczynski P., Mackey K. 1995. Substitution of chloroform by bromochloropropane in the single-step method of RNA isolation. Anal Biochem. 225: 163–164.

Cornelius M.L., Grace, J.K. and Yates III, J.R. 1997. Toxicity of monoterpenoids and other natural products to the Formosan subterranean termite (Isoptera: Rhinotermitidae). J. Econ. Entomol., 90: 320–325.

Davis K. L., Martin E., Turko I. V., Murad F. 2001. Novel effects of nitric oxide. Annual Review of Pharmacology and Toxicology. 41: 203-236.

De Sousa D.P. 2012. Medicinal Essential Oils: Chemical, Pharmacological and Therapeutic Aspects, 1st ed.; Nova Science Publishers: New York, NY, USA. p. 236.

De Vry.C.G., Valdez M., Lazarov M. 2005. Topical application of a novel immunomodulatory peptide, RDP58, reduces skin inflammation in the phorbol ester-induced dermatitis model. J Invest Dermatol. 125: 473–481.

Dhaliwal G. S., Koul O. 2007. Biopesticides and Pest Management: Conventional and Biotechnological Approaches. Kalyani Publishers, New Delhi.

Di-Nardo A., Wertz P., Giannetti A. and Seidenari S. 1998. Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. Acta Derm Venereol. 78: 27–30.

Dinarello C.A. 1991. Interleukin-1 and interleukin-1 antagonism. Blood. 77: 1627-1652.

Dinarello C.A. 1997. Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. Chest. 112: 321-329.

Draize J. H, Woodward G., Calvery H. O. 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membrane. J Pharm Exp Ther. 82: 377–390.

Feldmann M., Maini R. N. 2001. Anti-TNF-therapy of rheumatoid arthritis: What have we learned. Annual Review of Immunology. 19: 169-196.

Ferrero-Miliani L., Nielsen O.H., Andersen P.S., Girardin S.E. 2007. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1 generation. Clinical and Experimental Immunology. 147: 227-235.

Fisher C.J., Slotman G.J., Opal S.M., Pribble J.P., Bone R.C., Emmanuel G., Ng D., Bloedow D. C., Catalano M. A. 1994. Initial evaluation of human recombinant interleukin-1 receptor antagonist in the treatment of sepsis syndrome: a randomized, open-label, placebo-controlled multicenter trial. Crit. Care Med. 22: 12-21.

Fujiwara N., Kobayashi K. 2005. Macrophages in inflammation. Current Drug Targets-Inflammtion & Allergy. 2: 281-286.

Galli S.J., Nakae S., Tsai M. 2005. Mast cells in the development of adaptive immune responses. Nature Immunol. 6: 135–142.

Gallin J.I., Snyderman R. 1999. Overview. In D. T. Fearon, B. F. Haynes, & C. Nathan (Eds.), Inflammation Basic Principles and Clinical Correlates (3rd ed.). Philadelphia, USA: Lippincott Williams & Wilkins.

Gao X.K., Nakamura N., Fuseda K., Tanaka H., Inagaki N., Nagai H. 2004. Establishment of allergic dermatitis in NC／Nga mice as a model for severe atopic dermatitis. Biol Pharm Bull. 27: 1376–1381.

Glaser K.B., Sung M.L., Hartman D.A. 1995. Cellular and topical in vivo inflammatory murine models in the evaluation of inhibitors of phospholipase A2. Skin Pharmacol. 8: 300–308.

Han Y.H., Kwon J.H., Yu D.Y., Moon E.Y. 2006. Inhibitory effect of peroxiredoxin II (Prx II) on Ras-ERK-NFkB pathway in mouse embryonic fibroblast (MEF) senescence. Free Radic. Res. 40: 1182-1189.

Harris J.M., Cullinan P., Williams H. C. 2001. Environmental associations with eczema in early life. Br J Dermatol. 144: 795–802.

Hetrick E.M., Schoenfisch M.H. 2009. Analytical chemistry of nitric oxide. Annual Review of Analytical Chemistry. 2. 409-433.

Hiscott J., Marois J., Garoufalis J., D'Addario M., Roulston A., Kwan I., Pepin N., Lacoste J., Nguyen H., Bensi G., Fenton M. 1993. Characterization of a functional NF-κB site in the human interleukin 1b promoter: evidence for a positive autoregulatory loop. Mol. Cell. Biol. 13: 6231-6240.

Hobbs A.J., Higgs A., Moncada S. 1999. Inhibition of nitric oxide synthase as a potential therapeutic target. Annual Review of Pharmacology and Toxicology. 39: 191-220.

Homey B., Steinhoff M., Ruzicka T., Leung D.Y. 2006. Cytokines and chemokines orchestrate atopic skin inflammation. J Allergy Clin Immunol. 118: 178–189.

http://www.open.edu/openlearn/scie nce-maths-technology/science/biology/introduction-histopathology/content-section-1.2

Huwiler A., Kolter T., Pfeilschifter J., Sandhoff K. 2000. Physiology and pathophysiology of sphingolipid metabolism and signaling. Biochim Biophys Acta 1485: 63–99.

Imokawa G. 2001. Lipid abnormalities in atopic dermatitis. J Am Acad Dermatol. 45: 29–32.

Isman M.B., Machial C.M. 2006. Pesticides based on plant essential oils: from traditional practice to commercialization. In M. Rai and M.C. Carpinella (eds.), Naturally Occurring Bioactive Compounds, Elsevier, BV, pp 29–44.

Juergens U.R., Stober M.V. 1998. The anti-inflammatory activity of l-menthol compared to mint oil in human monocytes in vitro: A novel perspective for its therapeutic use in inflammatory diseases. Eur. J. Med. Res. 3: 539–545.

Kang J.S., Lee K., Han S.B. 2006. Induction of atopic eczema／dermatitis syndrome-like skin lesions by repeated topical application of a crude extract of *Dermatophagoides pteronyssinus* in NC／Nga mice. Int Immunopharmacol. 6: 1616–1622.

Kang J.S., Lee W.C., Lee K.H., Han M.H., Lee, H.J. Han, S.B. Kim H.C., Lee K.H., Park S.K., Kim H.M. 2008. Anti-inflammatory Activity of Methanolic Extract Isolated from Immature Fruit of Rhus succedanea. Lab. Anim. Res. 3: 297-302

Kang J.S., Yoon Y.D., Cho I.J., Han M.H., Lee C.W., Park S.K., Kim H.M. 2005. Glabridin, an isoflavan from licorice root, inhibits inducible nitric-oxide synthase expression and improves survival of mice in experimental model of septic shock. J. Pharmacol. Exp. Ther. 312: 1187-1194.

Kang J.S., Yoon Y.D., Lee K.H., Park S.K., Kim H.M. 2004. Costunolide inhibits interleukin-1β expression by down-regulation of AP-1 and MAPK activity in LPSstimulated RAW 264.7 cells. Biochem. Biophys. Res. Commun. 313: 171-177.

Kang J.S., Youm J.K., Jeong S.K. 2007. Topical application of a novel ceramide

derivative, K6PC-9, inhibits dust mite extract-induced atopic dermatitis-like skin lesions in NC／Nga mice. Int Immunopharmacol. 7: 1589–1597.

Kay J. H, Calandra J. C. 1962. Interpretation of eye irritation tests. J Soc Cos Chem. 13:281–9.

Kim B.H., Reddy A.M., Lee K.H., Chung E.Y., Cho S.M., Lee H. 2004. Inhibitory mechanism of chroman compound on LPS-induced nitric oxide production and nuclear factor B activation. Biochemical and Biophysical Research Communications. 325: 223-228.

Kim I.T., Park Y.M., Shin K.M., Ha J., Choi J., Jung H.J., Park H.J., Lee K.T. 2004. Anti-inflammatory and antinociceptive effects of the extract from Kalopanax pictus, Pueraria thunbergiana and Rhus verniciflua. J. Ethnopharmacol. 94: 165-173.

Kim N.Y., Kang T.H., Song E.K., Pae H.O., Chung, H.T., Kim Y.C. 2000. Inhibitory effects of butanol fraction of the aqueous extract of *Forsythia koreana* on the nitric oxide production by murine macrophage-like RAW 264.7 cells. Journal of Ethnopharmacology. 73: 323-327.

Kitawaki T., Kadowaki N., Sugimoto N. 2006. IgE-activated mast cells in combination with pro-inflammatory factors induce Th2-promoting dendritic cells. Int Immunol. 18: 1789–1799.

Kitts D.D., Lim K.T. 2001. Antitumorigenic and cytotoxic properties of an ethanol extract derived from Rhus verniciflua Stokes (RVS). J. Toxicol. Environ. 64: 357-371.

Koul O. 2008. Phytochemicals and insect control: An antifeedant approach. Crit. Rev. Plant Sci. 27: 1–24.

Koul O., Dhaliwal G.S., Marwaha S.S., Arora J.K. 2003. Future perspectives in biopesticides. In O. Koul, G.S. Dhaliwal, S.S. Marwaha and J.K Arora (eds.), Biopesticides and Pest Management., Vol.1, Campus Books International, New Delhi, pp. 386–388.

Kumar V., Abbas A.K., Fausto N. 2005. Robbin and Cotran pathologic basis of disease (7th ed.). Philadelphia: Elsevier Saunders.

Kupper T.S., Fuhlbrigge R. C. 2004. Immune surveillance in the skin: mechanisms and clinical consequences. Nat. Rev. Immunol. 4: 211–222.

Kwon Y.B., Kim C.D., Youm J.K. 2007. Novel synthetic ceramide derivatives increase

intracellular calcium levels and promote epidermal keratinocyte differentiation. J Lipid Res. 48: 1936–1943.

Lee D.Y., Choi G., Yoon T., Cheon M.S., Choo B.K., Kim H. K., 2009. Antiinflammatory activity of Chrysanthemum indicum extract in acute and chronic cutaneous inflammation. J. Ethnopharmacol. 123: 149–154.

Lee E.J., Suhr K.B., Lee J.H. 2003. The clinical efficacy of a multilamellar emulsion containing pseudoceramide in childhood atopic dermatitis: an open crossover study. Ann Dermatol. 15: 133–138.

Lee J.L., Mukhtar H., Bickers D.R., Kopelovich L., Athar M. 2003. Cyclooxygenases in the skin: pharmacological and toxicological implications. Toxicol. Appl. Pharmacol. 192: 294–306.

Lee K.Y., Jeon Y. J. 2005 Macrophage activation by polysaccharide isolated from Astragalus membranaceus. Int. Immunopharmacol. 5: 1225-1233.

Lee K.Y., Lee, M.H., Chang I.Y., Yoon S.P., Lim D.Y., Jeon Y.J. 2006. Macrophage activation by polysaccharide fraction isolated from *Salicornia herbacea*. J. Ethnopharmacol. 103: 372-378.

Lim K.T., Hu C., Kitts D.D. 2001. Antioxidant activity of a *Rhus verniciflua Stokes* ethanol extract. Food Chem. Toxicol. 39: 229-237.

Lin Y.M., Anderson H., Flavin M.T., Pai Y.H., Mata-Greenwood E., Pengsuparp T., Pezzuto J.M., Schinazi R.F., Hughes S.H., Chen F.C. 1997. In vitro anti-HIV activity of biflavonoids isolated from *Rhus succedanea* and *Garcinia multiflora*. J. Nat. Prod. 60: 884-888.

Lin Y.M., Flavin M.T., Schure R., Chen F.C., Sidwell R., Barnard D.L., Huffman J.H., Kern E.R. 1999. Antiviral activities of biflavonoids. Planta Med. 65: 120-125.

Locksley R.M., Killeen N., Lenardo M.J. 2001. The TNF and TNF receptor superfamilies: Integrating mammalian biology. Cell. 104: 487-501.

MacMicking J., Xie Q. W., Nathan, C. 1997. Nitric oxide and macrophage function. Annual Review of Immunology. 15: 325-350.

Matsuoka H., Maki N., Yoshida S. 2003. A mouse model of the atopic eczema ⁄ dermatitis syndrome by repeated application of a crude extract of house-dust mite *Dermatophagoides farinae*. Allergy. 58: 139–145.

Mihara K., Kuratani K., Matsui T., Nakamura M., Yokota K. 2004. Vital role of the itch-scratch response in development of spontaneous dermatitis in NC／Nga mice. Br J Dermatol. 151: 335–345.

Murphy J.E., Robert C., Kupper T.S. 2000. Interleukin-1 and cutaneous inflammation: a crucial link between innate and acquired immunity. J. Invest. Dermatol. 114: 602-608.

Natanson C., Eichenholz P.W., Danner R.L., Eichacker P.Q., Hoffman W.D., Kuo G.C., Banks S.M., MacVittie T.J., Parrillo, J. E. 1989. Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. J. Exp. Med. 169: 823-832.

Oikarinen A., Haapasaari K.M., Sutinen M., Tasanen K. 1998. The molecular basis of glucocorticoid-induced skin atrophy: topical glucocorticoid apparently decreases both collagen synthesis and the corresponding collagen mRNA level in human skin in vivo. Br. J. Dermatol. 139: 1106–1110.

Olesen A.B., Ellingsen A.R., Larsen F.S., Larsen P.O., Veien N.K., Thestrup-Pedersen K. 1996. Atopic dermatitis may be linked to whether a child is first- or second-born and／or the age of the mother. Acta Derm Venereol, 76: 457–460.

Opender K., Suresh W., Dhaliwal G.S. 2008. Essential Oils as Green Pesticides: Potential and Constraints. *Biopestic. Int.* 4(1): 63–84

Orie Y., Palma A.M., Hiroyoshi M., Manashi B., Yoshiaki S. 2013. Safety and toxicological evaluation of a novel, water-soluble undenatured type II collagen. Toxicol Mech Methods. 7: 491–499

Palladino M.A., Bahjat F.R., Theodorakis E.A., Moldawe L.L.. 2003. Anti-TNF-alpha therapies: the next generation. Natl. Rev. Drug Discov. 2: 736–746.

Park B.D., Youm J.K., Jeong S.K., Choi E.H., Ahn S.K., Lee S.H. 2003. The characterization of molecular organization of multilamellar emulsions containing pseudoceramide and type III synthetic ceramide. J Invest Dermatol. 121: 794–801.

Recio M.C., Giner R.M., Uriburu L. 2000. *In vivo* activity of pseudoguaianolide sesquiterpene lactones in acute and chronic inflammation. Life Sci. 66: 2509–2518.

Rim J.H., Jo S.J., Park J.Y., Park B.D., Youn J.I. 2005. Electrical measurement of moisturizing effect on skin hydration and barrier function in psoriasis patients. Clin Exp Dermatol. 30: 409–413.

Rita C.S.S., Luciana N.A., Damião P.S. 2013. A Review on Anti-Inflammatory Activity of Monoterpenes. Molecules. 18: 1227-1254.

Sae W.C. 2007. Studies on analgesic, antipyretic and anti-inflammatory activities of methanol extract of *Kaempferia galanga* L. in experimental animals. Unpublished master thesis, Prince of Songkla University, Faculty of Sciences.

Saha K., Lajis N.H., Israf D.A., Hamzah A.S., Khozirah S., Khamis, S. 2004. Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. Journal of Ethnopharmacology. 92: 263-267.

Santos F.A., Rao V.S.N. 2000. Antiinflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. Phytother. Res. 14, 240–244.

Sasakawa T., Higashi Y., Sakuma S. 2004. Topical application of FK506 (tacrolimus) ointment inhibits mite antigen-induced dermatitis by local action in NC／Nga mice. Int Arch Allergy Immunol. 133: 55–63.

Sharif M., Al R., Jung I.Y., Kim H.J., Kim J.S., Kang S.C. 2010. Anti-inflammatory activity of seed essential oil from *Zizyphus jujube*. Food and Chemical Toxicology. 48: 639−643

Shiohara T., Hayakawa J., Mizukawa Y. 2004. Animal models for atopic dermatitis: are they relevant to human disease. J Dermatol Sci. 36: 1–9.

Sohn E. H., Jang S. A., Joo H., Park S., Kang S. C., Lee C. H., Kim S. Y. 2011. Anti-allergic and anti-inflammatory effects of butanol extract from Arctium Lappa L. Clinical and Molecular Allergy. 9: 4

Sugimoto M., Arai I., Futaki N. 2006. Time course changes of scratching counts, dermatitis symptoms, and levels of cutaneous prostaglandins in NC／Nga mice. Exp Dermatol. 15: 875–882.

Sugimoto M., Arai I., Futaki N., Hashimoto Y., Honma Y., Nakaike S. 2006. COX-1 inhibition enhances scratching behaviour in NC／Nga mice with atopic dermatitis. Exp Dermatol. 15: 582–588.

Sunita M. 2010. Anti-inflammatory and anti-allergic activities of Thai traditional medicine preparation called prasaprohyai. MS thesis, Thammasat Univ.：Thailand.

Swardfager W, Lanctôt K, Rothenburg L, Wong A, Cappell J, Herrmann N. 2010. A meta-analysis of cytokines in Alzheimer's disease. Biol Psychiatry 68 : 930–941.

Takaoka A., Arai I., Sugimoto M. 2006. Involvement of IL-31 on scratching behavior in NC／Nga mice with atopic-like dermatitis. Exp Dermatol. 15: 161–167.

Tewtrakul S., Itharat A. 2007. Nitric oxide inhibitory substances from the rhizomes of *Dioscorea membranacea*. Journal of Ethanopharmacology. 109: 412-416.

Tezuka Y., Irikawa S., Kaneko T., Banskota A. H., Nagaoka T., Xiong Q. 2001. Screening of Chinese herbal drug extracts for inhibitory activity on nitric oxide production and identification of an active compound of *Zanthoxylum bungeanum*. Journal of Ethnopharmacology. 77: 209-217.

Titheradge M.A. 1999. Nitric oxide in septic shock. Biochim. Biophys. Acta 1411: 437-455.

Trouba K.J., Hamadeh H.K., Amin R.P., Germolec D. R. 2002. Oxidative stress and its role in skin disease. Antioxid. Redox Signal. 4: 665–673.

Valério D.A.R., Cunha T.M., Arakawa N.S., Lemos H.P., da Costa F.B., Parada C.A., Ferreira S.H., Cunha F.Q., Verri J.W.A. 2007. Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: Inhibition of cytokine production-dependent mechanism. Eur. J. Pharmacol. 562: 155–163.

von Mutius E. 2000. The environmental predictors of allergic disease. J Allergy Clin Immunol. 105: 9–19.

Wu X., Li X., Xiao F., Zhang Z., Xu Z., Wang H. 2004. Studies on the analgesic and anti-inflammatory effect of bornyl acetate in volatile oil from Amomum villosum. Zhong Yao Cai. 27: 438–439.

Xie Q.W., Kashiwabara Y., Nathan C. 1994. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J. Biol. Chem. 269: 4705-4708.

Zembower D.E., Lin Y.M., Flavin M.T., Chen F.C., Korba B. E. 1998. Robustaflavone, a potential non-nucleoside antihepatitis B agent. Antiviral Res. 39: 81-88.

초임계유체추출을 이용한 편백 성분의 살충, 기피, 항염효과 및 안전성 평가

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초 록

초임계유체추출(Supercritical fluid extraction)은 초임계 이산화탄소(Supercitical Carbon Dioxide)를 용매로 이용한 기술로 액체나 기체와 다른 초임계유체의 특성을 이용하여 추출 및 분리 공정에 적용 가능한 기술이다.

본 연구에서는 편백 (*Chamaecyparis obtusa)*을 초임계 유체 추출 (CO_2 Supercritical Fluid Extraction) 장비를 이용하여 추출 온도, 압력과 시간에 따른 시료의 추출 수율을 측정하였으며, 각 추출물을 GC/MS를 이용하여 분석을 진행하였다. 또한, 초임계유체추출물 및 분석을 통해 조사된 단일성분을 집먼지진드기 2종에 대해 살비활성, 작은소참진드기의 기피력 및 독일바퀴에 대한 살충 및 훈증 독성을 평가 하였으며, 아세틸콜린저해 활성, 항염 및 안정성 평가를 진행하였다.

편백잎을 초임계유체 추출장비를 이용하여 압력 및 분쇄 입자크기의 조건을 주어 진행한 결과 압력이 높을수록, 분쇄 입자가 작을수록 추출수율은 높아졌다. 편백

잎의 초임계유체추출물과 이들에 함유된 성분들 및 편백 가지의 용매 추출물의 순차 분획물을 HPLC를 이용하여 살비활성본체를 분리하여 분광학적 분석을 통해 얻은 *β*-thujaplicin을 큰다리집먼지진드기(*Dermatophagoides farinae*)와 세로무늬먼지진드기(*Dermatophagoides pteronyssinus*)에 대해 살비력을 3종의 상용살비제인 benzyl benzoate, *N, N*-dietyl m-touamide(DEET), dibutyl phtalate를 비교약재로 선정하여, 섬유확산법을 통해 검정하였다. 큰다리먼지진드기의 경우 편백초임계추출물과 *β*-thujaplicin이 LD_{50}값이 각각 3.42㎍/㎠, 4.02㎍/㎠ 였으며, 비교약재로 사용한 benzyl benzoate 4.31㎍/㎠, DEET 78.34㎍/㎠ 및 dibutyl phthalate 31.62㎍/㎠보다 높은 활성을 보였고, 세로무늬먼지진드기 역시 편백초임계추출물과 *β*-thujaplicin이 LD_{50}값이 각각 4.12㎍/㎠, 3.84㎍/㎠를 보여 비교약재로 사용한 benzyl benzoate (4.21㎍/㎠), DEET (78.64㎍/㎠) 및 dibutyl phthalate (30.72㎍/㎠)보다 높은 활성을 보였다. 작은소참진드기 기피력 평가에서는 상용기피제인 icaridin과 *N, N*-dietyl m-touamide(DEET)를 각각 10%를 처리하여 침입저지법 통해 비교 조사하였다. 결과, 편백 초임계유체추출물은 4㎍/㎠ 농도에서 100%, *α*-terpinene과 *α*-terpineol은 3.2㎍/㎠ 농도에서 노출 후 120분 후, 각각 85.5%와 68.9%의 기피력을 나타낸 반면, icaridin과 *N, N*-dietyl m-touamide (DEET)은 96.1% 및 100%의 기피력을 나타냈다. 독일바퀴 암컷에 대한 살충활성 평가는 4종의 상용살충제인 deltamethrin, dichlorvos, permethrin 및 propoxur를 비교 약재로 선정하여 직접접촉법 및 훈증독성을 평가하였으며, 편백 초임계유체추출물의 단일성분을 이용하여 독일바퀴 암컷 머리와 흉부의 아세틸콜린에스터라제(acetylcholinesterae, AChE) 저해 활성을 조사하였다. 접촉 시험에서는 편백 초임계추출물이 2.77 ㎍/㎠, phellandrene (0.28 ㎍/㎠), terpinene-4-ol (0.42 ㎍/㎠), *α*-thujone (0.09 ㎍/㎠)로 높은 활성을 보여, permethrin (0.05 ㎍/㎠), propoxur (0.18 ㎍/㎠)와 비슷한 농도 활성을 보였다. 하지만, 0.007~0.013 ㎍/㎠의 활성을 보인 deltamethrin과

dichlorvos에는 미치지 못했다. 훈증독성 평가 에서는 *α*-thujone (18.43 mg/L of air), linalool (26.20 mg/L of air)의 높은 활성을 보였지만 dichlorvos (0.07 mg/L of air)에는 미치지 못했다. 아세틸콜린에스터라제(acetylcholinesterae, AChE) 저해 활성을 조사에서는 *α*-Pinene, *β*-pinene, *α*-terpinene, limonene, linalool, terpinene-4-ol 및 *α*-terpineol이 AChE 저해활성(IC_{50}, 0.38 ~ 0.98 mM)을 보였다. 편백 초임계유체추출물의 단일성분에 대한 항염 효과를 *In vitro* assay를 통하여 효능을 검증하였고, T cell에서의 세포증식, Macrophages에서의 NO, Mast cell에서의 *β*-hexosaminidase release 에 대한 효능검증 수행하였다. 결과 *β*-Pinene는 NO 생성억제, IL-6, TNF-α의 유전자 발현 및 생성을 억제 하였고, *β*-thujaplicin은 NO 생성억제, IL-6 유전자 발현 및 생성을 억제 하였으며, DNP-BSA로 유도되어진 *β*-hexosaminidase release를 억제 및 염증성 cytokine인 IL-4, TNF-α의 유전자 발현 및 생성을 억제하였다. TPA(12-O-tetradecanoylphorbol-13-acetate)-induced inflammation 시험에서는 *β*-pinene, *β*-thujaplicin 모두 TPA에 유도 되어진 귀의 부종을 억제하였다. 편백 초임계유체추출물의 안전성 시험은 마우스와 랫드에 급성경구독성($LD_{50,}$ > 5,000mg/kg) 및 급성경피독성($LD_{50,}$ > 5,000 mg/kg)으로 나타나 저독성으로 판정되었다. 안점막자극성은 무자극성물질로 판정되어 매우 안전한 것으로 나타났다.

이상의 연구를 통해 편백을 소재로하여 초임계유체추출 기술을 적용한 최적 추출 조건을 확립 하였다. 3종(집먼지진드기, 작은소참진드기 및 독일바퀴)의 위생해충의 살충, 기피력 및 안정성 평가를 통해 기피제 및 훈증제로서 활용 할 수 있을 것으로 기대하며, 항염 평가를 통해 면역조절제로서의 가능성을 확일 할 수 있었다.

검색어: 초임계유체추출, 편백, 진드기, 바퀴, 항염, 안전성 평가

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