CHAPTER 4

DISCUSSION

Consumers and food processors demand for new self-preservatives that are biodegradable, toxic residue-free and environmental friendly to control spoilage in food. Essential oils compose of volatile aromatic compounds produced from plant secondary metabolism. They have been used as food flavouring and preservation (Rahman and Kang, 2009). Most of essential oils are classified as generally recognized as safe (GRAS) substances which have low risk on the human health and resistance development in microorganisms (Cardile et al., 2009). Thus, the essential oils from *A. graveolens* and *Z. piperitum* seeds are potentially applied as natural food preservatives.

In this study, Essential oils were extracted from *A. graveolens* and *Z. piperitum* seeds and chemically identified by GC-MS. *A. graveolens* seed oil constituents can be grouped into three-classes with monoterpene hydrocarbon 21.8%, oxygenated monoterpene 40.5% and phenylpropene 33.9%. The major compounds of the essential oil were *trans*-isodillapiole 33.1%, (+) -carvone 26%, (-) -limonene 19.2% and dihydrocarvone 12.3%. A previous study reported that main compound of *A. graveolens* seeds oil was carvone (75.21%), while the content of limonene was 21.56% and dihydrocarvone 3.02% (Radulescu et al., 2010) . Whereas the constituents of *Z. piperitum* seeds oil can be grouped into six sub-classes with monoterpene hydrocarbon 68.6%, oxygenated monoterpene 19.5%, sesquiterpene hydrocarbon 3.2%, oxygenated sesquiterpene 1.0%, phenylpropene 0.2%, and miscellaneous compounds 0.2%. The major components were β-phelladrene 23.2%, sabinene 14.6% and brevifolin 11.9%. Choochote et al., (2007) reported that the major compound of *Z. piperitum* seeds oil were (+) -limonene (37.9%), sabinene (13.3%), and β-myrcene (7.17%). Each element was different depending on species, sub-species and plant varieties, the portion of the plant used, different geographic locations where plants are grown, and weather

conditions during growth and the stage of growth at harvest (Burt, 2004; Nieblas et al., 2011; Silveira et al., 2012) . Essential oils containing terpenes (monoterpenes, sesquiterpenes and oxygenated derivatives) as major compoued were reported to exhibit antimicrobial activity (Cakir et al., 2004). The different compounds in one essential oil may exhibit synergism in antibacterial and antifungal activities (Reginer et al., 2008).

The essential oil of *A. graveolens* seeds showed pronounced antifungal efficacy against *A. flavus*. Mycelial growth curve of the fungus during the 9-day incubation period was shown in Figure 3.4. Mycelial growth was decreased when increasing concentrations of essential oils. While 2.0 µL/mL concentration of oil could completely inhibit mycelial growth of *A. flavus* in agar. Essential oil of *A. graveolens* seeds exhibited excellent performance for inhibition of *Aspergillus* spp growth at 6 µL/mL by poison food techniques (Singh et al., 2005). However, we found that the essential oil reduced mycelial growth with percentage reduction ranging from 71.1% to 92.1% at 0.25-1.5 µL/mL concentrations. Additionally, earlier report showed that *A. graveolens* seed oil inhibited growth of some food born microorganisms (Delaquis et al., 2002; Elgayyar et al., 2001; Fatope et al., 2006; Lopez et al., 2005; Sagdic and Ozcan, 2003). While *Z. piperitum* seed oil completely inhibited *A. flavus* at the MIC value of 4.5 µL/mL. The mycelial growth inhibition percentage was calculated on the 9th day shown in Figure 3.6. The oil significantly reduced fungal growth with inhibition percentage of 46.34, 62.50, 77.83, 82.78, 86.43, 89.03 and 91.04 at 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µL/mL concentrations, respectively. Prakash et al. (2012) reported that *Z. alatum* oil at 1.25 µL/mL was highly efficient for inhibition of *Aspergillus* spp. growth by poison food techniques. Furthermore, the essential oils from the seeds of *Zanthoxylum* species have been reported in suppressing food spoilage causing microorganisms and oral pathogens (Hurtado et al., 2003; Misra et al., 2013; Park et al., 2008; Tatsadjieu et al., 2003; Yeboa et al., 2005). In addition, effect restraining in the biomass of mycelia fungi was shown remarkably by the essential oil. (Dikbas et al., 2008; Kedia et al., 2014; Prakash et al., 2012; Tatsadjieu et al., 2009; Tian et al., 2012). As shown in Tables 3.5 and 3.6, the result indicated that 2.0 µL/mL of *A. graveolens* oil exhibited the highest inhibitory effect on the fungal biomass whereas *Z. piperitum* oil was shown the most efficiency at 4.5 µL/mL concentration. To sum up, the mycelial growth and the biomass mycelia of the *A. flavus* significantly restricted by the essential oils of *A. graveolens* and

Z. piperitum in a dosage-responsive manner. The effect of *A. graveolens* and *Z. piperitum* oils on *A. flavus* mycelial structure was examined under a light microscope. The microscopic observation results of fungus treated with 1.0 µL/mL by *A. graveolens* and *Z. piperitum* oil and control are shown in Figures 3.12 and 3.13. In vitro light microscope observations of the microstructure of *A. flavus* which is sensitive to essential oils revealed some mechanisms of the *A. graveolens* and *Z. piperitum* oils such as roundly twisted conidial heads and decreased hyphal diameters. The results obtained were similar to those from some previous work in which the microstructure of *Aspergillus* spp. treated with other essential oils was studied (Carmo et al., 2008; Sharma and Tripathi, 2008; Tian et al., 2011; Tolouee et al., 2010). In addition, the components of essentials oil may interfere with the enzymatic synthesis of cell walls (Tripathi et al., 2009). Also, some alteration induced by essential oil may cause a lack of cytoplasm, damage of integrity, the electron transport chain, H^+-ATPase inhibition, and finally cell death as in other essential oils (Burt, 2004).

The effect of *A.graveolens* and *Z. piperitum* oils on the ergosterol content in the plasma membrane of *A. flavus* was studied. Ergosterol, the end-product of the biosynthetic pathway and the main sterol in eukaryotic cells, is responsible for structural membrane character, such as fluidity and permeability same as cholesterol pathway of mammalian cells (Veen and Lang, 2005). Modification in the cell permeability of the plasma membrane led to damage of the normal shape of fungal cell. A previous study has shown the quantity reduction of ergosterol by other essential oils (Kedia et al., 2014; Pinto et al., 2009). In this study, the ergosterol content was determined by Tian et al., (2012) method based on the exclusive spectral absorption at 230 and 282 nm of the extracted sterols (ergosterol and 24(28) dehydroergosterol). The result indicated that the ergosterol content in the plasma membrane of *A. flavus* was reduced by 0.25 µL/mL of *A.graveolens* and 1.0 µL/mL of *Z. piperitum* oils. After incubation of *A. flavus* with 0.25 µL/mL of *A. graveolens* oil, a reduction of the ergosterol content in the plasma membrane was observed at 32% of the control. While 1 µL/mL of *Z. piperitum* oil decreased the ergosterol content by 28%. In previous works, the plasma membrane was the target of essential oils supported by the damage seen under SEM or TEM (Abyaneh et al., 2006; Khan et al., 2011; Nogueira et al., 2010; Tian et al., 2012; Tolouee et al.,

2010). Thus, the plasma membrane is probably an important antifungal target of *A. graveolens* and *Z. piperitum* oils.

The action mechanism of essential oils has not fully understood, but it is regarded as involving membrane destruction due to its hydrophilic or lipophilic characteristic (Cowan, 1999). However, *A. graveolens* L. oil exhibited higher antifungal activities than *Z. piperitum* oil, which may be attributed to the high contents of (-)-limonene, dihydro-carvone, (+)-carvone and *trans*-isodillapiole, high oxygenate monoterpenes and high phenylpropene. A recent report has shown that limonene and carvone were the most potent antifungal compounds against the variety of microorganisms that cause food spoilage such as *A. flavus* and *A. niger* (Aggarwal et al., 2001; Marei et al., 2012). Dillapiole, the main component of *Piper aduncum* oil showed antifungal activity against *Clinipellis perniciosa* (witches' broom) (Almeida et al., 2009). In other reports, oxygenated monoterpenes exhibited antimicrobial activity against plant pathogenic microorganisms (Mahmoud, 1994; Pauli, 2001 and Regnier et al., 2008). Furthermore, Zambonelli et al., (1996) reported that the oxygenated monoterpenes acted against pathogens because they prevented enzymatic reactions during synthesis of cell wall. A recent report has shown that some essential oil high phenylpropene such as *Foeniculum vulgare* Mill. ssp. vulgare var.azoricum (Mill.) Thell] leaves (rich in anethole) exhibited antimicrobial activity against Gram-positive bacteria (Senatore et al., 2013).

The main functional groups of *A. graveolens* L. oil were ketone and methoxy groups while hydrocarbons were the major functional group of *Z. piperitum* oils. The antifungal activity of essential oils is generally depending on lipophilicity of hydrocarbons structure and hydrophilicity of functional groups. The antimicrobial activity rank of essential oils is as follows: phenols> aldehydes> ketone> alcohol> ether> hydrocarbons (Kalemba and Kunicka, 2003). The mechanism action of ketone group (dihydro-carvone, (+)-carvone) and methoxy groups (*trans*-isodillapiole) may be disruption of the plasma membrane, increased permeability, extensive loss of the intracellular proteins and finally resulting in cell death similar to other phenylpropene (eugenol) (Devi et al., 2010) or ketone and methoxy groups may be able to form hydrogen bonds with the active sites of enzyme active center similar to phenol group (Bluma et al., 2008). Monoterpenes hydrocarbons which had low antimicrobial activity

may result from free functional group causing limitation of hydrogen bonding capacity or water solubility (Victório et al., 2008). However, it does not mean that monoterpene hydrocarbons has no antifungal activity, as it has been indicated that some of essential oils such as *Schinus molle* fruit (rich in α-phellandrene, β-pinene, β-phellandrene) exhibited antifungal activity against *Botrytis cinerea* (Ibrahim and Naser, 2014) and *S. terebinthifolius* fresh leaf (rich in sabinene and α-pinene) exhibited antibacterial and antifungal activities against pathogen (Gundidza et al., 2009). Moreover, Glisic et al., (2009) reported that fraction of α-pinene and mixture of α-pinene and sabinene from *Juniperus communis* L. showed the highest antimicrobial activity against bacteria, yeast and fungi. Furthermore, a previous study has shown that essential oils have a greater antimicrobial activity than the pure major components of essential oil (Gill et al., 2002; Morillon et al., 2002). Additionally, that minor components may also have important synergistic effect (Burt, 2004; Tripathi et al., 2009). However, the essential oil may be infiltrated into the lipid rich portion of the cell membrane to destroy or disrupt cell membrane by cross linkage reactions, damage of electrolytes, cytoplasm coagulation, damage the membrane protein, increased permeability leading to leakage of the plasma membrane, and reduction in level of amino acids, sugars and decreased the proton motive force, intracellular ATP synthesis and finally resulting in the death of the cell because essential oil, which has low molecular weight and highly lipophilic components passes simply through cell membranes and organization (Chao et al., 2005; Inouye et al., 2000; Nazzaro et al., 2013).

In the last experiment, we studied antifungal activity of essential oils applied on product surface. The potential application of essential oils to control spoilage fungi on agriculture products was previously reported (Kumar and Nambisan, 2013; Prakash et al., 2012; Tian et al., 2011; Tzortzakis, 2009). Dried bird chili, a model product, coated with *A. graveolens and Z. piperitum* oils was tested for potential to protect against *A. flavus* infection. Pour plate culture at 10^{-5} dilution of infected chili is shown in Figure 3.12. After incubation at 28 ^{o}C for 9 days, the concentrations of essential oil at 2.0 and 4.0 µL/mL of *A. graveolens* L. oil resulted in reduction of fungal development by 84.00% and 93.32% respectively. While *Z. piperitum* oil reduced 45.20% and 89.32 % at 4.5 and 9.5 µL/mL, respectively compared with the control. Thus, the essential oils of *A. graveolens* L. and *Z. piperitum* are likely to be developed into food preservatives.

The model product experiment showed the susceptibility to fungal infection because humidity conditions and nutrition in food favor spoilage fungi growth more than in laboratory media (Tzortzakis, 2009) . Thus, the model products are required higher concentrations of essential oils than in laboratory media in order to completely inhibit fungal growth. The use of essential oils can safely inhibit microorganisms in food without residues after storage. However, the essential oils have limitation of use because they have strong flavors and have applicability only in products with compatible flavor. In addition, the use of essential oil in food has to meet the standard safety limit values and sensory characteristics such as color, aroma, and firmness (Tian et al., 2011).