Antimicrobial Activity of Essential Oils and Their Application in Active Packaging to Inhibit the Growth of Molds on Bread

By

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Abstract

Bread spoilage caused by molds is a major concern for the bakery industry. Traditionally, bread is preserved by chemical preservatives, such as propionic and sorbic acids. Owing to consumer demand for "natural" foods with extended shelf life, there is an increasing interest in replacing chemical preservatives in bread with essential oils (EOs) as natural preservatives. In this study, a number of EOs were investigated for their antifungal activity against molds isolated from moldy bread. Cinnamon EO exhibited marked antimicrobial activity and its estimated minimum inhibitory concentration (MIC) was in the range of 31-125 ppm. The efficacy of sachets containing cinnamon oil in inhibiting filamentous fungi isolated from moldy bread was investigated *in vitro* and *in situ*, while *in situ* growth of molds was inhibited in the presence of sachets containing 25-1000 µL of cinnamon EO (CIN-03) in a sealed system for 14 days at room temperature. Sachets containing 50 µL or higher levels of CIN-03 showed fungicidal effect. Active packaging combined with 500 µL to 1000 µL of CIN-03 in sachets increased the shelf life of bread slices packaged in plastic bags by more than 14 days. The shelf life of whole wheat bread loaves was extended to 6 days using the sachets containing 250 µL to 1000 µL of CIN-03. The results of this work demonstrated that packaging of sliced bread with sachets containing cinnamon EO provides an innovative and effective means of extending the shelf life of bread products.

Résumé

La détérioration du pain causée par les moisissures est une préoccupation majeure pour l'industrie de la boulangerie. Traditionnellement, le pain est conservé par des conservateurs chimiques, tels que les acides propioniques et sorbiques. En raison de la demande des consommateurs pour des aliments "naturels" avec une durée de conservation prolongée, on s'intéresse de plus en plus au remplacement des conservateurs chimiques par des huiles essentielles (HE) en tant que conservateurs naturels dans le pain. Dans l'étude, plusieurs HE ont été étudiés pour leur activité antifongique contre les moisissures isolées du pain de moule. L'huile essentielle de cannelle a montré une activité antimicrobienne marquée et sa concentration minimale inhibitrice estimée (CMI) était dans la gamme de 31-125 ppm. L'efficacité des sachets contenant de l'huile essentielle de cannelle sur la suppression des champignons filamenteux isolés du pain moisi a été étudiée *in vitro* et *in situ*, tandis que la croissance *in situ* des moisissures a été inhibée en présence de sachets contenant 25-1000 µL d'HE de cannelle (CIN-03) dans un système scellé pendant 14 jours à température ambiante. Sachets contenant 50 µL ou plus de CIN-03 ont montré un effet fongicide. L'emballage actif combiné avec 500 µL à 1000 µL de CIN-03 en sachets augmente la durée de conservation des tranches de pain emballées dans des sacs en plastique par plus de 14 jours. La durée de conservation du pain de blé entier a été prolongée à 6 jours en utilisant les sachets contenant 250 µL à 1000 µL de CIN-03. Les résultats de ce travail ont démontré que l'emballage de pain tranché avec des sachets contenant de l'HE de cannelle constitue un moyen novateur et efficace de prolonger la durée de conservation des produits de boulangerie.

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Contents

List of Tables

List of Figures

List of Abbreviations

AP	Active packaging
ATR-FTIR	Attenuated total reflectance Fourier transform infrared
CAR	Carvacrol
CED	Cedar
CHI	Chili
CIN	Cinnamon
CIND	Cinnamaldehyde
CLO	Clove
CRA	Cranberry
CYP	Cypress
EC	European Commission
EO(s)	Essential oil(s)
EUG	Eugenol
FDA	U.S. Food and Drug Administration
GAR	Garlic
GRAS	Generally recognized as safe
HC	Health Canada
LAB	Lactic acid bacteria
LSPQ	Laboratoire de Santé Publique du Québec
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAP	Modified atmosphere packaging
MFC	Minimal fungicidal concentration
MIC	Minimum inhibitory concentration
MIZ	Mycelia inhibition zone
NaCl	Sodium chloride
ONI	Onion
ORE	Oregano
PDA	Potato dextrose agar
PIM	Piment
PIN	Pin
ROS	Rosemary
SAG	Sage
THY	Thyme

Chapter 1 Introduction

Bread is a highly perishable product which is very susceptible to microbial spoilage, especially mold spoilage. The common spoilage mold found in bread include *Penicillium*, *Aspergillus*, *Eurotium*, *Cladosporium,* and *Rhizopus* spp. (Legan, 1993; P. V. Nielsen & Rios, 2000). The bread products are more likely to be contaminated during post-baking periods, such as cooling, slicing, and wrapping operations. Due to the relatively high moisture content and water activity, the shelf life of preservative-free bread is typically of 3-7 days (Legan, 1993; Magan, Aldred, & Arroyo, 2012). Traditionally, industries use chemical preservatives to inhibit the microbial growth. The application of chemical preservatives including propionates, sorbic acids and sorbate can extend the shelf life of bread to over 14 days (Cauvain, 2015). However, over last few years, consumers have shown interest towards natural foods. Therefore, there is a demand for natural foods which motivates the industries to have "clean" labels by not using chemical preservatives in their products (Axel, Zannini, & Arendt, 2017). Essential oils (EOs), which are natural extracts from plants, have been shown to possess antimicrobial properties (Kalemba & Kunicka, 2003; Krisch, Tserennadmid, & Vagvolgyi, 2011; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Alvarez, 2007). Additionally, EOs are volatile and have been proven to be effective in vapor phase as well (Krisch, Rentskenhand, Horváth, & Vágvölgyi, 2013; López, Sánchez, Batlle, & Nerín, 2007), which provides the potential of applying EO vapor combined with the modified atmosphere packaging (MAP) or active packaging (AP). Many studies have demonstrated the application of EOs to preserve different food matrices including fruits, vegetables, meat, dairy and bakery products (Mendonca, Jackson-Davis, Moutiq, & Thomas-Popo, 2018; C. G. Otoni, Espitia, Avena-Bustillos, & McHugh, 2016; Suhr & Nielsen, 2003).

The main objective of the thesis is to develop an EO-based active packaging to extend the shelf life of preservative-free bread products. This will entail (1) the isolation and identification of molds from moldy bread; (2) the evaluation of antimicrobial effectiveness of EOs; (3) the elaboration of EO-containing sachets; (4) the evaluation of efficacy of EO-containing sachet against bread molds *in vitro* and *in situ*.

Chapter 2 Literature Review

2.1 Bread

Bread in its many forms is one of the most important staple foods. Bread is defined as "that food generally recognized as bread prepared from a dough of cereal flour and baked" (Legan, 1993). Traditionally, bread is made from wheat flour. Other kinds of cereals can also be milled to flour, but when mixing with water, only the proteins present in wheat flour and a few limited cereals can form gluten in the dough and makes bread become soft and light after baking (Cauvain, 2012a).

2.1.1 Main ingredients

There are many kinds of bread, which are derived from a variety of ingredients, formulations and processing techniques. The basic ingredients of bread include flour, salt, yeast, and water. The flour used for bread making is mainly wheat flour. It contains the gluten proteins which retain the carbon dioxide and enlarges the volume of the bread (Goesaert et al., 2005). Addition of salt in bread can not only contribute the flavor, but also control the action of the yeast. The yeast used for baking is normally *Saccharomyces cerevisiae*. The yeast can use the sugar from the flour and produce carbon dioxide during fermentation. Water is the solvent for flour to form bread dough. The amount of water can affect the properties of the bread dough. Too little or too much water will make the dough either be too firm or flowing. Other ingredients such as sugar, fat and improvers can also be added into bread to improve the characters of bread. Sugar can contribute the sweetness and crust color of bread. A high content of sugar can inhibit the fermentation by yeast. Fat can increase the gas retention of the dough which contributes to the softness and moistness of bread. The level of added fat depends on the type of the flour. Improvers are functional ingredients used

to improve the properties of bread, which including oxidising agents, reducing agents, emulsifiers, and enzyme-active agents (Cauvain, 2012b).

2.1.2 Bread processing

The bread making process varies for different types of bread. Overall, there are 6 steps as shown in the figure 2.1.

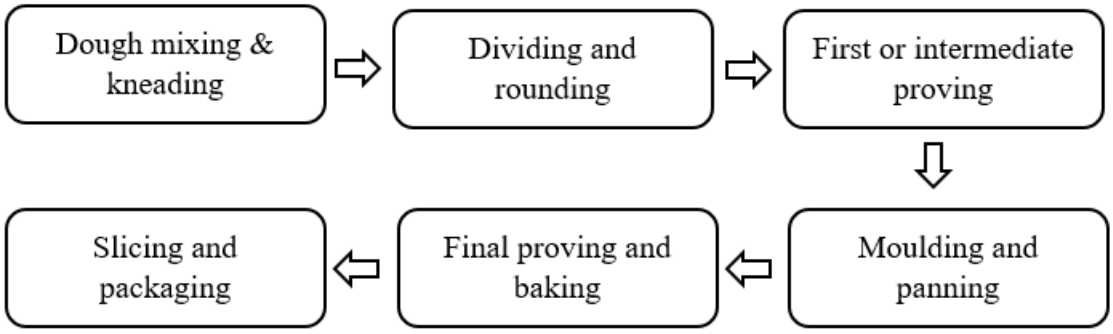


Figure 2.1 Flow chart of bread making process

Step1. Dough mixing and kneading. All the raw ingredients including flour, water, salt, yeast, and any other ingredients are homogenized to form dough and gluten. During kneading, air bubbles are incorporated within the dough for oxidation and yeast fermentation. The yeast uses oxygen and sugar and produces carbon dioxide.

Step 2. Dividing and rounding. The bulk bread dough is divided into the required shape and size for further processing (Gould, 2007). The dough must be divided into a predetermined weight with minimal damage of the dough. The weight accuracy depends on the homogeneity of the dough. The shape of the divided dough is then modified afterwards (Cauvain, 2012b).

Step 3. First or intermediate proving. This step allows the bread dough to relax before moulding. First proving provides time for yeast activity, therefore, more carbon dioxide is produced and the dough becomes sufficiently soft and extensible (Gould, 2007).

Step 4. Moulding and panning. This purpose of moulding is to shape the dough and modify the bread structure (Cauvain, 2012b). The dough pieces are then dropped into the pans which allows them to become softer.

Step 5. Final proving and baking. The final proving allows the dough to rest before baking. The dough pieces stay in the pans whereas the fermentation continues in a warm and humid condition. The size of gas bubbles within the dough increases and the dough expands. After proofing, the bread is baked. The heat causes the rapid expansion of gas in the dough, evaporation of water, gelatinization of starch and the coagulation of proteins, and finally, turns the dough to the loaf (Gould, 2007).

Step 6. Slicing and packaging. Bread is cooled before slicing and packing. The thickness of bread slices depends on the nature of the product and the preferences of costumers. Then, the slices and crusts are packed by machine or by hand.

2.2 Microbial Contamination of Bread

Bread has a high moisture content of about 40% and water activity of 0.94-0.97, which makes bread become perishable. The shelf life of preservative-free bread is about 3-7 days (Legan, 1993). Spoilage of bread is most commonly caused by mold. Bacterial spoilage of bread is less common, which is mainly due to the growth of *Bacillus* species. Yeast spoilage is also less common.

2.2.1 Mold Spoilage and Mycotoxins

During baking, heat treatment can kill most molds and spores. The contamination of bread occurs during the post-processing period, such as cooling, slicing and packaging. The environment of the processing area contains flour, dust, and mold spores which can contaminate the bread. Sliced and wrapped bread is more susceptible to mold spoilage. This is because bread is still hot when wrapped, then water droplets condense and at the same time, wrapping material prevents the moisture loss. Also, the slicing provides moist, cut surfaces on which mold can grow on (Cauvain, 2015; Legan, 1993; Magan et al., 2012)

Most common molds are *Penicillium* and *Aspergillus* spp. *Penicillium* spp. include *P. commune, P. roqueforti, P. solitum, P. crustosum, P. corylophilum, P. chrysogenum, , P. palitans*, and *P. polonicum* etc. (P. V. Nielsen & Rios, 2000; Suhr & Nielsen, 2003). Common *Aspergillus* spp. include *A. flavus, A. niger, A. candidus* and *A. glaucus*, etc. (Cauvain, 2015). The characteristics of some bread molds are summarized in Table 2.1.

The types of spoilage mold found in bread depend on the ingredients and processing used for breadmaking. Many molds found in wheat bread include *Penicillium*, *Aspergillus*, *Cladosporium, Rhizopus*, *Muco*r and *Neurospora* spp. (Legan, 1993). *P. roqueforti, P. paneum, P. carneum, P. corylpphilum, Eurotium repens* and *E. rubrum* are the most prevalent species found in rye bread (Filtenborg, Frisvad, & Thrane, 1996). *Aspergillus* spp. were found to be the most common spoilage mold in Indian breads (Legan, 1993). *Penicillium* spp. were reported to be the most common molds on spoiled Dutch rye breads (Hartog & Kuik, 1984). *Eurotium, Aspergillus* and *Penicillium* are the predominant molds found in Spanish bakery product (Guynot, Ramos, Sanchis, & Marin, 2005). For some sourdough breads, *P. roquefortii* is the predominant mold (Legan, 1993).

Table 2.1 Characteristics of common bread molds

Mold	Colony color	Colony appearance
Penicillium spp.	Blue/green	Fat, spreads rather slowly
A. niger	Black	Fluffy, spreads with spores
A. flavus	Olive green	
A. candidus	Cream	
A. glaucus	Pale green	
Cladosporium spp.	Dark olive green	Flat, spreads slowly
Neurospora sitophila	Salmon pink	Very fluffy and fast spreading
Rhizopus nigricans	Grey/ black	Very fluffy and fast spreading
Mucor spp.	Grey	

Adapted from Magan *et. al.*, 2012

The growth of mold on bread can be affected by the environmental factors, such as water activity, pH and temperature. Table 2.2 compares the growth rate of different molds affected by various pH (4.5 and 6.0) and water activity. From the table, it can be shown that *E. repens* has the highest growth rate when the pH and water activity are 6.0 and 0.97, respectively. All these molds grow faster at higher water activity, and the growth rate is also higher when pH is 6.0 than when pH is 4.5 (Magan et al., 2012). The storage temperature affects the growth of mold on bread. When the storage temperature increased from 4°C to 22-24°C, the incidence of *Penicillium* decreased from 82% to 42%, while *Aspergillus* spp. and *Rhizopus* spp. increased (Legan, 1993).

Table 2.2 Effects of pH and water activity on the growth rate (diametric growth rate, mm per day) of bread molds on bread when incubated at 25°C

	pH 4.5			pH 6.0		
	Water activity			Water activity		
	0.93	0.95	0.97	0.93	0.95	0.97
E. repens	2.8	2.6	4.6	4.1	7.5	9.3
P. verrucosum	0.8	1.3	1.4	1.1	1.8	1.9
P. verrucosum	0.8	1.1	1.6	1.1	2.2	2.3
A. ochraceus	1.5	2.3	2.7	1.8	2.8	3.3
P. coryolophilum	0.2	0.4	0.7	0.7	1.2	1.6
P. roquefortii	1.1	1.7	2.2	0.8	1.3	1.4

Adapted from Magan *et. al.*, 2012

Mold contamination can cause economic losses to the bakery industries, but also mold can produce mycotoxins which may induce health problems to consumers. Mycotoxins are secondary metabolites of filamentous fungi. Some mycotoxins may cause acute and chronic health effects on humans and animals (Filtenborg et al., 1996). Aflatoxin B1, aflatoxin G1, patulin, ochratoxin A, citrinin and sterigmatocystin can be produced by mycotoxigenic mold strains (Osborne, 1980). Toxigenic *Penicillium* including *P. cyclopium* and *P. citrinum* were isolated from wheat flour and bread in the USA (Bullerman & Hartung, 1973). In the UK, ochratoxin A was found to be produced by *P. cyclopium* (Osborne, 1980). In Italy, *A. ochraceus* was found to produce ochratoxin in moldy bread (Visconti & Bottalico, 1983).

The production of mycotoxins is susceptible to environmental factors including temperature, moisture, water activity, pH and oxygen concentration (Bosco & Mollea, 2012). The ability of mycotoxin to diffuse in the bread depends on its lipid or water solubility. In a study, *A. parasiticus, A. ochraceus* and *P. chrysogenum* were able to produce mycotoxins in presence of oxygen, but the diffusion of the mycotoxins aflatoxin B2 and M1 in whole wheat bread was limited; in the absence of oxygen, most of the toxins were undetectable, but only a 1 cm-diffusion of aflatoxins B1 ad G1 was observed (Reiss, 1981).

In developed countries, the health risk caused by mycotoxins is minimal. A large amount of mold is required to form mycotoxins in bread. In addition, consumers tend to reject the whole bread instead of cutting the visible moldy portion of the bread and eating the remainder of the bread (Cauvain, 2015).

2.2.2 Yeast Spoilage

Yeasts are usually added to bakery products as a leavening agent, but they can also spoil these products (Fleet, 2011). Like molds, yeasts are killed during baking, so the contamination of bread occurs during cooling, slicing and other following operations.

There are two types of yeast spoilage involved in bread spoilage: visible yeast spoilage and fermentative yeast spoilage (Cauvain, 2015). For visible yeast spoilage, yeasts have white, spreading growth, which looks like chalk dust on the surfaces of bread, so this type of yeast is also called “chalk molds”. Chalk molds are usually filamentous yeasts like *Pichia burtonii* and *Saccharomycopsis fibuligera* (Cauvain, 2015; Legan & Yoysey, 1991). The fermentative spoilage can cause alcoholic, fruity, or estery off-flavor of bread. The typical yeast is *S. cerevisiae* (Fleet, 2011).

2.2.3 Bacterial Spoilage

Ropiness is the major spoilage problem caused by bacteria, mainly the *Bacillus* genus including *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. cereus*, and *B. pumilus* (Bailey & Holy, 1993; Cauvain, 2015; Pepe, Blaiotta, Moschetti, Greco, & Villani, 2003) . The sources of contamination include raw ingredients, atmosphere, and equipment surfaces. During baking, if the core temperature of the loaf cannot achieve the baking temperature, the heat resistant *Bacillus* spores may survive. Bacteria spoilage of bread occurs within 12 hours after contamination, and it will cause an odour of pineapple at the beginning, then the bread crumb will become discolored and sticky (Rosenkvist & Hansen, 1995). Warm and moist conditions contribute to the ropey spoilage, for example, close stacking of bread loaves allows slower cooling and under-baking allows moister bread (Thompson, Christine, & Waites, 1993).

2.3 Preservation of Bread

Preservation of bread and extension of its shelf life can be achieved by preventing the access of microorganisms to the bread or inactivating the growth of microorganisms (College, 2012). If the bread is contaminated after baking, it is necessary to control the activity of microorganisms. The addition of preservatives is the most common way to preserve bread.

2.3.1 Conventional preservatives

Chemical preservatives are commonly used in bread preservation to inactivate the growth of microorganisms. These common chemical preservatives include propionate acids and their salts, sorbic acids and sorbate, acetic acid and its salts and ethanol (Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). The recommended levels of use of some chemical preservatives are summarized in Table 2.3.

Table 2.3 Antimicrobial properties of chemical preservatives used in bread preservation

Preservatives	Recommended level of use (%)
Propionic acid	0.1[1]
Calcium propionate	0.2[1]
Sodium propionate	0.2[1]
Sodium dipropionate (70% solution)	0.2[1]
Sorbic acid	0.1[2]
Acetic acid	0.1-0.2[2]
Sodium diacetate	0.3[2]
Ethanol	0.5-3.5[2]

Adapted from [1]Cauvain, 2015; [2]Legan, 1993

Propionic acid and its salts are mainly used to prevent bacterial spoilage caused by *Bacillus* spp. (specifically *B. subtilis* and *B. licheniformis*), and mold spoilage, but not for yeast spoilage (College, 2012; Legan, 1993). Though propionate has lower antimicrobial activity than propionic acid, propionate is more commonly used because it is more soluble and odor-free (Smith et al., 2004). Sorbic acids and their salts are used in bakery products to retard the growth of yeasts, molds and certain bacteria, such as *B. subtilis*. Acetic acid and its salts can control ropiness and mold spoilage problems in bakery products. Compared to propionic acid and its salts, the advantages of acetic acid and its salts are the fact that they are cheaper and less toxic. On a weight per weight basis, sorbic acid and its salts are the most effective followed by propionic acid and its salts, while acetic acid and its salts are least effective (Smith et al., 2004).

The mechanism of weak organic acids is associated with a disorder of the pH equilibrium in the cell of microorganisms. Weak acids, such as propionic acid, sorbic acid and acetic acid work better at lower pH due to their low pK_a value (4.19-4.87). At low pH conditions, they retain the uncharged and undissociated form of their structures, which is able to cross the cell membrane. After penetration, weak acids become dissociated because of the higher pH environment, which results in the release of protons. The accumulation of protons inside the membrane affects the metabolic functions, and finally may cause membrane disruption (Brul & Coote, 1999; Magan et al., 2012; Ray & Bullerman, 1982). The mechanism of the relative salts of organic acids are not fully understood. The preservation effect of sodium propionate may be related to its accumulation within the cell and interference with the carbohydrate metabolism (Heseltine, 1952).

There are disadvantages in using weak organic acids and their salts. For example, an addition of 0.2% of calcium propionate could lead to a 5-10% reduction in loaf volume (Magan et al., 2012), which in turn is due to the reduced yeast activity and altered dough rheology. High concentrations of preservatives can also affect the odour and flavor properties of bread (Legan, 1993). The preservatives mentioned above can be applied by spraying onto the surface of the bread after baking or cooling. However, it is difficult to apply the preservatives evenly to all surface areas (Cauvain, 2015).

Ethanol works as a mold inhibitor in bread. The addition of ethanol levels between 0.5 and 3.5% to bread can contribute to the extension of shelf life of bread (Legan, 1993). The shelf life of bread increased by 50% when 0.5% of ethanol was added. However, based on the sensory tests, the product may become unacceptable to consumers if more than 1% of ethanol was applied to the bread (Seiler, 1984). The application of ethanol can be done by spraying before packaging and sealing, so that it can act as a gaseous inhibitor. Ethanol, incorporated with active packaging can

also prevent microbial growth. Some studies used ethanol emitters and oxygen absorbers to preserve bread (Latou, Mexis, Badeka, & Kontominas, 2010; Salminen et al., 1996).

2.3.2 Alternative preservatives

The use of chemical preservatives in foods is controlled by different countries. Also, high quality and natural foods are desired by consumers these days. Therefore, there is an interest in using natural preservatives such as biopreservatives and essential oils (EOs).

2.3.2.1 Biopreservatives

Some microorganisms can produce antimicrobial compounds during fermentation to prevent bread spoilage and extend the shelf life of bread (College, 2012). Lactic acid bacteria (LAB) as biopreservatives have been commonly used in sourdough bread processing for a long time (Ryan, Dal Bello, & Arendt, 2008). Sourdough is a mixture of flour and water and fermented by LAB and yeasts (Dertli, Mercan, Arıcı, Yılmaz, & Sağdıç, 2016). The application of sourdough can decrease the usage of chemical preservatives or even replace them, which gives product a "clean" label. The LAB-fermented sourdough can achieve a pH drop due to the production of organic acids, which are mainly lactic and acetic acids. However, this kind of acidification has limited preservative effects against mold growth. LAB can also produce other antimicrobial metabolites such as phenyl and substituted phenyl derivates, cyclic dipeptides, hydroxy fatty acids (Axel et al., 2017). Many studies have been done to evaluate the antimicrobial activity of LAB *in vitro* and *in situ* (Dal Bello et al., 2007; Carla Luciana Gerez, Torino, Rollán, & Font de Valdez, 2009; Le Lay et al., 2016; Russo et al., 2017). *In vitro* tests, for instance, *Lactobacillus plantarum*, *Lb. reuteri*, and *Lb. brevis* were found to reduce bread molds including *Aspergillus*, *Fusarium*, and *Penicillium* spp. (Carla Luciana Gerez et al., 2009). In *in situ* tests, the antifungal LAB was added as a starter culture, and it was found that 40% *Lb. plantarum* was as effective as 0.2% calcium propionate. The antifungal

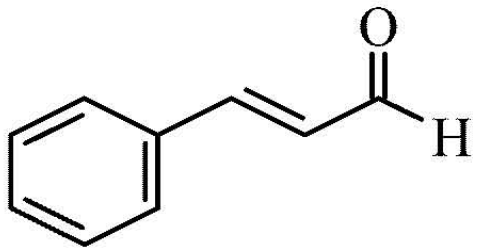
effect was due to the production of acetic, phenyllactic and lactic acids (C. L. Gerez, Torino, Obregozo, & Font de Valdez, 2010).

Yeasts excluding baker's yeast can also be applied as biopreservatives alone or combined with LAB in bakery products (Axel et al., 2017). *Pichia anomala*, *Meyerozyma guilliermondii*, and *Wickerhamomyces anomalus* have been studied as part of starter cultures added into wheat sourdough to extend the shelf life of bread (Coda et al., 2011; Coda et al., 2013; Mo & Sung, 2014). For example, slices of bread made from sourdough containing a combination of *W. anomalus* and *Lb. plantarum* had a shelf life of 28 days without contamination of fungi (Coda et al., 2011).

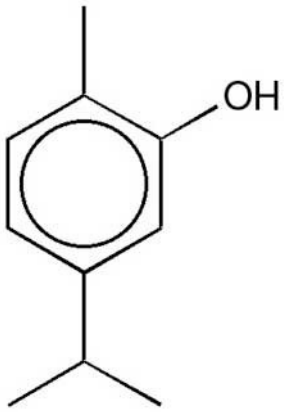
2.3.2.2 Essential oils (EOs)

In the last few decades, increased interest towards natural food additives has arisen the possible application of plants extracts and EOs in food preservation. EOs are aromatic and volatile liquid extracted from plants including cinnamon, thyme, oregano, clove, rosemary, garlic, sage, basil, marjoram, savory, pepper, and cardamon, etc. (Magan et al., 2012; Mendonca et al., 2018).

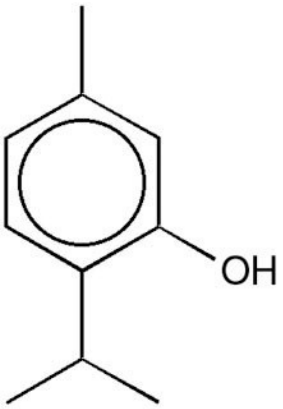
EOs are complex mixtures of low molecular weight organic compounds. The main effective compounds of cinnamon, thyme, oregano and clove essential oils are cinnamaldehyde, thymol, carvacrol, and eugenol, respectively (Ribes, Fuentes, Talens, & Barat, 2017). The chemical structures of the main active compounds are shown in Figure 2.2. EOs and their constituents have broad spectrum antimicrobial activity against microorganisms (Hyldgaard, Mygind, & Meyer, 2012; Mendonca et al., 2018).



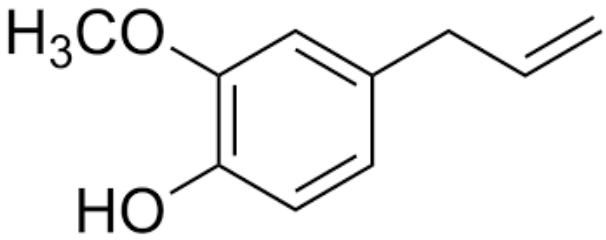
Cinnamaldehyde (cinnamon EO)



Carvacrol (oregano EO)



Thymol (thyme EO)



Eugenol (clove EO)

Figure 2.2 Chemical structures of the main active compounds of cinnamon, thyme, oregano, and clove EOs

Cinnamaldehyde is a major constituent in cinnamon EO. It acts as an inhibitor of microbial growth. The mode of action of cinnamaldehyde is concentration-depended (Shreaz et al., 2016). At low concentrations, cinnamaldehyde interferes the cytokinesis interaction. Cinnamaldehyde has been found to inhibit FtsZ protein which regulates the cell division, thus, the cells grow without dividing (Domadia, Swarup, Bhunia, Sivaraman, & Dasgupta, 2007). At sub-lethal concentrations, cinnamaldehyde acts as a membrane bound ATPase inhibitor when it enters the periplasm. The inhibition of the membrane bound ATPase, which is involved in ATP synthesis and pH regulation, will cause the depletion of ATP and affect the protons and ions transportation (Gill & Holley, 2006; Shreaz et al., 2016; Zheng & Ramirez, 2000). At lethal concentrations, cinnamaldehyde interacts with cell membrane and even perturbs it. For bacteria, one study has shown that cinnamaldehyde at high concentrations altered the fatty acids composition of microbial cells of *Escherichia coli* and *Salmonella* (Di Pasqua, Hoskins, Betts, & Mauriello, 2006). In the test on *S. cerevisiae*, the mechanism of cinnamaldehyde was acting as an inhibitor of cell wall synthesising enzymes (Bang, Lee, Park, & Rhee, 2014). In the tests on molds (*A. fumigatus*), it was speculated that the penetration of EO may cause the accumulation of polysaccharides and lead to the depressed conidial and mycelial growth rate (Khan & Ahmad, 2011).

Carvacrol is the major constituent of oregano EO. Evidence indicated that carvacrol can increase the fluidity and permeability of the cell membrane. The cell changes its membrane composition in response to the exposure to carvacrol, which increases the fluidity of membrane. The increased fluidity enhances permeability for cations like H^+ and K^+, resulting in the dissipation of ion gradients and impairment of cell metabolism (Ultee, Kets, & Smid, 1999). There is also limited evidence showing that carvacrol may also interact with membrane enzymes and proteins (Hyldgaard et al., 2012).

Thymol, which shares similar structure features with carvacrol, is the major constituent of thyme EO. The mode of action of thymol is similar to carvacrol, which involves interactions with membrane phospholipids, proteins and intracellular targets. Thymol can affect the membrane permeability which causes a disruptive action on the cytoplasmic membrane. The interaction with proteins and intracellular targets prevents the recovery of cell (Hyldgaard et al., 2012). Carvacrol and thymol have similar mechanisms against fungi. One study indicated that natural isopropyl cresols like carvacrol and thymol impaired the ergosterol biosynthesis and disrupted the membrane integrity in *Candida* strains (Ahmad et al., 2011). Carvacrol or thymol also disrupted Ca^{2+} and H^{+} homeostasis and affected gene transcription (Rao, Zhang, Muend, & Rao, 2010).

Eugenol is the major active compound in clove EO. Eugenol causes non-specific permeabilization of membrane which increases the transport of K^{+} and ATP. Furthermore, the interaction between eugenol and membrane protein inhibits the enzymes involved in cell metabolism and recovery (Hyldgaard et al., 2012). The mechanism of the antifungal effect of eugenol is not fully understood. It was reported that eugenol is involved in damaging the membrane and cell wall structure (Bennis, Chami, Chami, Bouchikhi, & Remmal, 2004).

Table 2.4 Antimicrobial activity of EOs

Organisms	Essential oil	MIC [a]	Reference
A. flavus	Oregano	400 µg/ml	(Paster et al., 1990)
		17.5 µL/L [b]	(López et al., 2007)
	Thyme	700 µg/ml	(Paster et al., 1990)
		9.35 µg/ml	(Segvic Klaric, Kosalec, Mastelic, Pieckova, & Pepeljnak, 2007)
		175 µL/L [b]	(López et al., 2007)
	Cinnamon	1000 ppm	(Soliman & Badeaa, 2002)
		13.1 µL/L [b]	(López, Sánchez, Batlle, & Nerín, 2005)
		0.05-0.1 mg/mL	(Manso, Cacho-Nerin, Becerril, & Nerín, 2013)
A. niger	Oregano	400 µg/ml	(Paster et al., 1990)
	Thyme	700 µg/ml	(Paster et al., 1990)
		9.35 µg/ml	(Segvic Klaric et al., 2007)
P. chrysogenum	Thyme	19.6 µg/ml	(Segvic Klaric et al., 2007)
S. cerevisiae	Cinnamon	200 ppm	(Kalemba & Kunicka, 2003)
	Clove	400 ppm	(Kalemba & Kunicka, 2003)
B. cereus	Cinnamon	17.5 µL/L [b]	(López et al., 2005, 2007)
	Thyme	131 µL/L [b]	(López et al., 2007)
	Oregano	17.5 µL/L [b]	(López et al., 2005, 2007)
B. subtilis	Cinnamon	1.6 mg/ml	(Prabuseenivasan, Jayakumar, & Ignacimuthu, 2006)
	Clove	3.2 mg/ml	(Prabuseenivasan et al., 2006)

[a] MIC: minimum inhibition concentration; [b] $\mu L_{EO}/L_{headspace}$

2.3.2.2.1 Antimicrobial activity of essential oils

EOs have been screened for their microbial efficacy. The antimicrobial activity of common EOs against some spoilage microorganisms present in bread are summarized in Table 2.4. The units of minimum inhibition concentration (MIC) are not the uniform and even one EO directed the same microorganism gives different MIC values. Therefore, there is a need of a standardized MIC test.

2.3.2.2.2 *In situ* control of microorganisms in bakery products using essential oils

The efficacy of EOs can be investigated in baked goods. There are different techniques for delivery of EOs to bread products. Preservation of bread can be achieved by incorporating EOs in food packaging which allows the slow release of active compounds of EO to the system. One µL/L mustard and 270 µL/L lemongrass oils in an airtight system are effective in preserving rye bread for at least 14 days (Suhr & Nielsen, 2003). Thirty µL of pure clary sage or marjoram oil was added into sealed Petri dishes to preserve the bread slices punched out with circular mold of 90 mm diameter. After 14 days, sage oil inhibited the growth of *A. niger* on wheat bread and the *P. chrysogenum* and *Rhizopus* spp. on rye bread, respectively; in addition, there was no visible growth of *P. chrysogenum* and *Rhizopus* spp. on rye bread treated with marjoram oil (Krisch et al., 2013).

Sachets containing oregano oil were reported to inhibit the growth of yeasts and molds on sliced bread (Passarinho et al., 2014). Active paper containing 6% (w/w) cinnamon oil was effective against sliced bread inoculated with *Rhizopus stolonifer* after 3-day storage, while 4% (w/w) was effective to inhibit the fungal growth on Petri dishes (Rodríguez, Nerín, & Batlle, 2008).

EOs can be incorporated as emulsions into polymers to form antimicrobial films. Edible films from methylcellulose and nanoemulsions of clove bud and oregano oils inhibited the growth of *A. niger* and *Penicillium* spp. *in vitro*, and lessened the growth of yeasts and molds in sliced bread (Otoni,

Pontes, Medeiros, & Soares Nde, 2014). Another study examined that the antimicrobial activity of gliadin film containing 5% cinnamaldehyde preserved the inoculated bread for at least 30 days (Balaguer, Lopez-Carballo, Catala, Gavara, & Hernandez-Munoz, 2013).

The combination of EO with modified atmosphere packaging (MAP) enhances the antimicrobial activity. The growth of bread molds was inhibited on rye bread and wheat bread by using 2 μL mustard oil / slice and 2-3 μL mustard oil / slice combined with MAP (85% CO_2, 1% O_2), respectively (Suhr & Nielsen, 2005).

Direct incorporation of EOs in bakery products by spraying on the bread surface or as a baking ingredient has been examined. It was found that addition of citrus peel EOs into the bread dough affected the sensory properties of bread, and spraying oil on the surface of bread before packaging achieved the maximum efficacy (Rehman et al., 2007). Studies have showed that direct addition of EOs as an ingredient was less effective than expected from *in vitro* tests.

Table 2.5 Application of essential oil as a preservative in bread products

Food	Method	Organisms	Effect	Reference
Rye bread	1 µL/L Mustard and 270 µL/L lemongrass oil added as volatiles in an airtight system	*P. roqueforti* *P. corylophilum* *E. repens* *Endomyces fibuliger*	Inhibition for 14 days	(Suhr & Nielsen, 2003)
Wheat bread	30 µL of clary sage oil in Petri dish	*A. niger*	Inhibition for 14 days	(Krisch et al., 2013)
Rye bread	30 µL of clary sage oil or marjoram oil in Petri dish	*P. chrysogenum* *Rhizopus* spp.	Inhibition for 14 days	(Krisch et al., 2013)
Sliced bread	Sachets containing 5, 10, 15% (v/w) of oregano oil	Yeasts and molds	Inhibition	(Passarinho et al., 2014)
Sliced bread	Active paper packaging containing 6% (w/w) of cinnamon oil	*R. stolonifer*	Inhibition for at least 3 days	(Rodríguez et al., 2008)
Sliced bread	Edible films containing 4% (w/v) clove bud or oregano oils	Yeasts and molds	Inhibition	(Otoni et al., 2014)

Food	Method	Organisms	Effect	Reference
White bread	Gliadin films containing 5% cinnamaldehyde	*P. expansum* *A. niger*	Inhibition for 30 days	(Balaguer et al., 2013)
Rye bread	2 µL Mustard oil / slice combined with MAP	*P. roqueforti* *P. corylophilum* *E. repens* *Endo. fibuliger*	Inhibition for 30 days	(Suhr & Nielsen, 2005)
Wheat bread	2 - 3µL Mustard oil / slice combined with MAP	*P. commune* *P. solitum* *P. polonicum* *A. flavus*	Inhibition for 30 days	(Suhr & Nielsen, 2005)
Wheat bread	Spraying of citrus peel essential oils on the bread surfaces	Bacteria and molds	Inhibition	(Rehman et al., 2007)

2.3.2.2.3 Safety of essential oils

The toxicity of EOs and their major components have been tested on mammalian systems, and no toxicity or low toxicity was shown in most cases (Prakash, Kedia, Kumar Mishra, & Dubey, 2015). EOs and their constituents are also considered as "Generally Reorganized as Safe" (GRAS) by U.S. Code of Federal Regulations (FDA, 2017). The active compounds of EOs, such as thymol, eugenol, cinnamaldehyde, citral, and vanillin, are considered as safe and currently registered by European Commission (EC) to be used as flavourings (Hyldgaard et al., 2012). In Canada, EOs are permitted additives by Health Canada (HC, 2018).

2.4 Conclusion

Mold contamination of bread products during post-baking is the biggest concern to bakery industries. Due to customers' demand of "clean" label of the products, the industries are seeking natural preservatives to take place of the traditional preservatives. Through the literature review, it is known that EOs are effective antimicrobials against the microorganisms that cause bread spoilage. There is a possibility of applying EOs to inhibit the growth of fungi and extend the shelf life of bakery products. Therefore, the project aimed at developing an EO-based active packaging to prolong the shelf life of bread products.

Chapter 3 Materials and Methods

This chapter will outline the materials and methods employed to evaluate the antimicrobial activities of selected EOs and the effectiveness of sachets containing EO for retarding bread mold growth *in vitro* and *in situ*.

3.1 List of EOs and extracted bioactive compounds

The EOs used in the study were provided from different sources and are listed in Table 3.1. All the EOs and extracted bioactive compounds are labelled with the first three letters of its name and the assigned number, except cinnamaldehyde was labeled as CIND-01.

3.2 Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy analysis of EOs and extracted bioactive compounds

The spectra of all the EOs and extracted bioactive compounds were collected by ATR-FTIR to create a database. The diamond crystal (2mm) of ATR-FTIR spectrometer (ML 5500 ATR FTIR spectrometer, Agilent Technologies, Santa Clara, California, USA) was cleaned with ethanol and then air-dried. After that, the background spectrum was taken. Ten µL of the liquid of EO (or extracted bioactive compounds) was added on the diamond crystal and spectrum of the oil was collected immediately. The software used for spectral acquisition was Cognisolve[1] software (Cognisolve Inc., Montréal, Canada). Each spectrum was acquired in the 400-4000 cm^{-1} range by co-addition of 64 scans at a resolution of 8 cm^{-1} at room temperature.

Table 3.1 Essential oils and pure compounds used in the experiment

Manufacture	Essential oil	Cat #	Lot #	Label
Aldrich	Carvacrol (pure component)	W224502	MKCB9457	CAR-01
Aldrich	Eugenol (pure component)	W246700	MKBZ8578V	EUG-01
Aldrich	Cinnamaldehyde (pure component)	W228613	MKBX8146V	CIND-01
Aliksir	Cinnamon	19345	B-CICASCHN08P	CIN-01
Aliksir	Cinnamon	-	B-CIVE E MDG08P	CIN-02
BSA	Cinnamon	750046	217012036	CIN-03
BSA	Cinnamon	750427	217011045	CIN-04
Atoka	Cranberry	51790		CRA-01
Aliksir	Cypress	19573	B-CUSEESP12Q	CYP-01
BSA	Oregano	750422	215122241	ORE-01
Novotaste	Oregano	-	-	ORE-02
BSA	Clove	750019	217022164	CLO-01
Aldrich	Thyme	W306509		THY-01
BSA	Piment	750400	217010747	PIM-01
BSA	Garlic	750403	216110573	GAR-01
Novotaste	Garlic	-	-	GAR-02
Novotaste	Garlic	-	-	GAR-03
New Directions	Chili	-	11196-A09-2	CHI-01
Nascent	Cedar	-		CED-01
Aliksir	Rosemary	17817	B-ROOFMAR02P	ROS-01
Aliksir	Pin	18027	B-PISTCAN11P	PIN-01
BSA	Sage	750014	217012241	SAG-01
BSA	Onion	750092	217011332	ONI-01

3.3 Chemicals and media

Potato dextrose agar (PDA) and potato dextrose broth media were obtained from DIFCO™ Becton, Dickinson and Company, MD 21152 USA. Sodium chloride (NaCl) was obtained from Fischer Scientific, USA.

3.4 Fungal isolation and identification

3.4.1 Fungal isolation

Commercial breads were purchased locally and placed on the lab bench at room temperature (22 °C) until visible growth of molds was observed. Mold colonies were picked from the moldy bread and streaked onto the surface of PDA plates. The plates were incubated for 2-3 days at 28 °C. Single-colony isolates were transferred onto PDA plates and incubated under the same conditions mentioned above to check purity and identity. Plates were stored at 4 ºC until use.

3.4.2 Fungal identification

Based on their morphology, three different molds were isolated from moldy bread and transferred onto PDA plates. The plates were incubated for 3 days at 28 °C. The fungal identification was carried out at the Laboratoire de Santé Publique du Québec (LSPQ) using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). To extract proteins for MALDI-TOF MS, a circle of 1 to 2 cm diameter of molds was collected using wet swab and then suspended into a 2 mL tube containing 900 µL of 70% ethanol. Each tube was vortexed for 5 minutes and then centrifuged at 10000 to 14000 g for 2 minutes. After the supernatant was discarded, the pellet was resuspended in 40 µL of 70% formic acid and 40 µL of acetonitrile. The tube was vortexed again for 5 minutes and centrifuged at 10000 to 14000 g for 2 minutes. One µL of supernatant from each tube was spotted in duplicate onto a conductive metallic target plate. After the samples had dried, 1 µL of matrix solution (a-cyano-4-hydroxycinnamic acid) was pipetted onto each of the samples. The protein mass fingerprints were obtained using the MALDI-TOF Vitek MS™ system (bioMérieux) and identified using the Vitek MS™ database.

3.5 Antifungal activity tests

3.5.1 Fungal inoculum preparation

Three fungal isolates were transferred onto fresh PDA plates separately. Fungal conidia were harvested with a sterilized loop and transferred to spore suspension buffer which contained 9 ml distilled water supplemented with 0.1% Tween-80. The concentration of suspension of each fungal isolate was adjusted to 10^5 spores/mL by using the haemocytometer under the light microscope. Final concentration was approximately 10^4 spores/mL produced by a 10-fold dilution of the stock into 0.9% sterile NaCl (Ma, 2012).

3.5.2 Disc diffusion assay

Disc diffusion assays were carried out on EOs CAR-01, ORE-01, ORE-02, CIN-03, CLO-01, and THY-01. The surface of PDA plates was spread with a 0.1 mL of suspension containing 10^4 spores/mL of fungal strain. Pre-sterilized filter paper discs (5 mm in diameter, Whatman no. 1) were impregnated with three different volumes (2, 4 and 6 µL per disc) of neat EO and placed on centre of the inoculated plates. The plates were sealed by Parafilm and incubated at 28 ºC for 14 days. Discs with three different volumes (2, 4 and 6 µL per disc) of distilled water were used as the control. The test was done in triplicate. The antifungal activity was assessed by measuring the diameter of the mycelia inhibition zone (MIZ) (Boukhatem, Ferhat, Kameli, Saidi, & Kebir, 2014). MIZ is defined as an area around the disc where there is no visible mold growth. Diameter was determined by averaging the three measurements.

3.5.3 Vapor diffusion assay

Antifungal activity of EOs and the extracted bioactive compounds in the vapor phase was measured by vapor diffusion method. CAR-01, ORE-01, ORE-02, CIN-03, CLO-01, and THY-01

were selected to perform this test. The surface of PDA plates was inoculated with 0.1 mL of suspension containing 10^4 spores/mL of fungal strain. Pre-sterilized filter paper discs (5 mm in diameter, Whatman no. 1) were impregnated with three different volumes (2, 4 and 6 µL per disc) of EO and laid on the inside surface of the upper plate lid. The plates were sealed by Parafilm and incubated inversely at 28 ºC for 14 days. Discs with three different volumes (2, 4 and 6 µL per disc) of distilled water were used as the control. The test was performed in triplicate. The effectiveness of EO against molds was calculated by measuring the diameter of the MIZ (Boukhatem et al., 2014). Diameter was determined by averaging the three measurements.

3.5.4 Agar dilution assay

MIC values of EOs and their bioactive constituents against fungal isolates was studied by the agar dilution method (Gulluce et al., 2003; Sahan, 2011). All the EOs and their bioactive constituents were evaluated by this method. The EOs or constituents were added aseptically to the pre-sterilized molten PDA medium at certain volumes to produce the concentration range of 7.8-1000 µL/L (7.8-1000 ppm). After vortexing, the PDA medium containing the EO was poured into blank Petri dishes. The plates were spot inoculated with 5µl (10^4 spore/ml) of each fungal isolate. The PDA plates without EOs were considered as the control. The inoculated plates were sealed with Parafilm and incubated at 28°C for 7 days. At the end of incubation period, the plates were evaluated for presence or absence of fungal growth. MIC values were determined as the lowest concentration of the EO where there was no visible growth of mold. Plates showing no growth were sub-cultured on new PDA plates and incubated at 28°C for 5 days to determine if the inhibition was reversible or permanent, minimal fungicidal concentration (MFC) was determined as the lowest concentration of EO where no growth occurred on the plates. Each test was carried out in triplicate.

3.6 EO-containing sachet elaboration

Ziploc® sandwich bags (1 mil, 17.7 × 20.3 cm, polyethylene) were bought from a local store. The sachet was made by heat sealing two layers (5 × 5 cm) cut from Ziploc ® sandwich bags by a vacuum sealing machine (FoodSaver® Countertop FM2000 Vacuum Sealing System). The EO-containing sachet was prepared by adding certain amount of liquid EO or constituent.

3.7 Efficacy of EO-containing sachet against mold growth *in vitro*

The cocktail of the three fungal isolates suspension was prepared by equally mixing three fungal isolate suspensions of 10^4 spores/mL which were prepared according to section 3.4.1.

EO-containing sachets were prepared as mentioned in section 3.6. CIN-03 and CAR-01 were chosen to be incorporated into a sachet. Sachets containing 1, 1.5, 2, 2.5, 5, 10, or 100 µL of CIN-03 or CAR-01 were used to perform this test.

A volume of 0.1 mL of cocktail (10^4 spores/mL) was spread onto the PDA plates. A sachet containing a fixed amount of EO was attached with a double-sided tape to the inside surface of the upper plate lid in each plate. The plates were sealed by Parafilm and incubated for 7 days at 28 °C. After incubation, fungal growth was evaluated (Passarinho et al., 2014). The test was operated in triplicate. The plates treated with empty sachets were used as the control.

3.8 Efficacy of EO-containing sachet against mold growth on bread

3.8.1 Assay in sealed jar system

Preservative-free sliced white bread was bought from a local store. Bread slices were punched into cylindrical shape by using a sterilized cork borer (inner diameter of 1.0 cm). Each bread plug was spot inoculated with 5 µL of cocktail of fungal suspension of 10^4 spores/mL which was prepared

according to section 3.7. A total of 20 inoculated bread plugs were placed in 1-L sterilized Mason jar in four rows of five plugs (Ma, 2012). Sachet containing 25, 50, 100, or 1000 µL of CAR-01 or CIN-03 were prepared in advance. Each sachet was fixed to the inside wall of the jar with a double-sided tape in one jar. Jars containing inoculated bread plugs with empty sachets were served as the control. Forty bread plugs in two jars were considered as one treatment and each treatment was done in triplicate.

The effect of exposure time and EO dose against growth of the mold on bread were assessed by incubating bread plugs in the sealed jar for 0, 3, 5, 7 and 14 days at room temperature (22 °C). After fixed exposure time, 4 bread plugs were aseptically sampled, added to 0.9% NaCl solutions and homogenized for 30s. Serial 10-fold dilutions were performed to enumerate the counts of bread molds (Downes & ITO, 2001). At the same time, after 0, 3, 5, 7 and 14 days of incubation, 24 bread plugs were randomly selected with a sterile tweezer from each treatment and transferred to two PDA plates (12 plugs/ plate) for a 7-day incubation at room temperature (22 °C) to recover the injured molds. The fungistatic concentration of EO was determined by the lack of visible fungal growth in sealed jars. The fungicidal concentration of EO was recognized by the absence of mold growth in both the jars and PDA plates (Ma, 2012).

3.8.2 Assay in plastic bags

Sliced whole wheat bread was provided from a bakery industry. Each bread slice was inoculated at three points on the surface with a 5 µL cocktail of fungal suspension of 10^4 spores/mL which was prepared according to section 3.7. Each slice was packaged with two sachets containing 100, 125, 250, 500, or 1000 µL of CIN-03 in a Ziploc® freezer bag (17.7 × 18.8cm). The two sachets were placed on both sides of onto the centre of the bread slice. Bread slices treated with empty

sachets were served as the control. All the slices were stored at room temperature (22 °C) for 14 days. The signs of fungal growth on bread slices were checked daily. The test was done in triplicate.

3.8.3 Assay in bread package

Sliced whole wheat bread was provided from a bakery industry. Each bread loaf was spot inoculated on three different sides with 5 µL of cocktail of fungal suspension of 10^4 spores/mL which was prepared according to section 3.7. Two sachets containing 125, 250, 500, or 1000 µL of CIN-03 was added into the bread packaging on two sides. The positions of inoculation and sachets in the bread package are showed in Figure 3.1. Bread loaves packaged with empty sachets were considered as the control. All the bread loaves were stored at room temperature (22 °C) for 14 days. Signs of fungal growth were monitored daily. The test was done in triplicate.

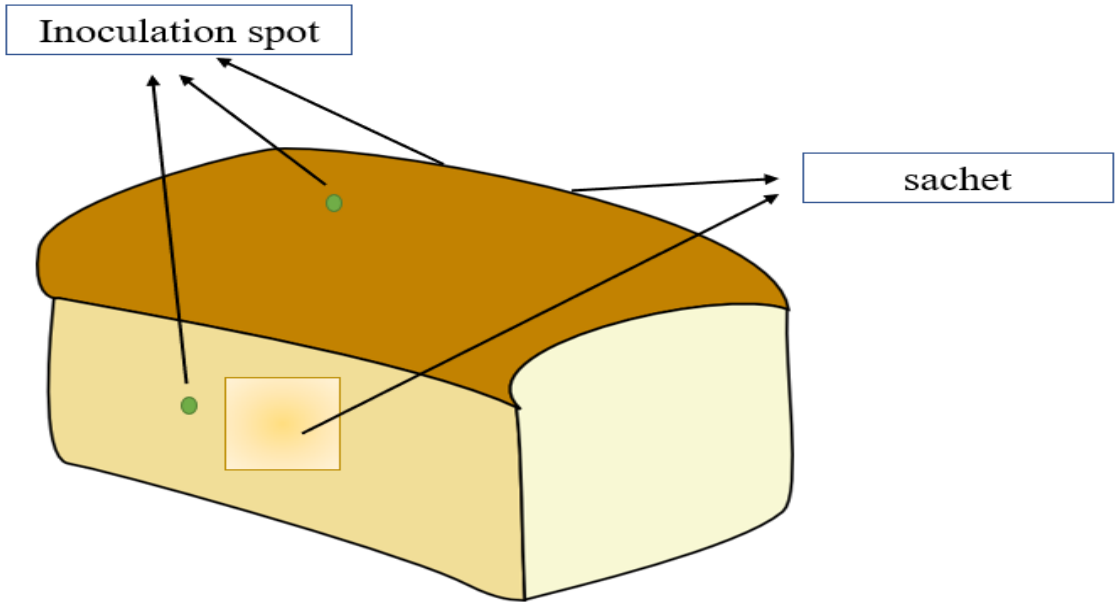


Figure 3.1 Packaged bread loaf system used to test the antifungal activity of a sachet containing CIN-03 on the growth of *A. flavus* and *Penicillium* spp.

3.9 Statistical analysis

The experiment was performed in a randomized mode. The data were submitted to analysis of variance, followed by Tukey's test at the 5% of probability level. SPSS commercial software (IBM SPSS Statistics 23.0) was used to perform statistical analysis.

Chapter 4 Results and Discussion

4.1 ATR-FTIR analysis of EOs and bioactive extracts of EOs

The ATR-FTIR spectra of EOs and pure bioactive extracts of EOs are shown in Figure 4.1 and 4.2. The ATR-FTIR spectra characteristic fingerprint peaks for EOs and pure bioactive compounds are observed in the infrared spectral range between 1800 and 700 cm^{-1}. In Figure 4.1, the characteristic peaks of cinnamaldehyde (CIND-01) were at 1679 cm^{-1} and 1626 cm^{-1}, which corresponds to the stretching vibration of the aldehyde carbonyl (C=O) and vibration of the unsaturated benzene ring, respectively (Li, Kong, & Wu, 2013; Wen et al., 2016). The spectra of EOs CIN-01, CIN-02 and CIN-03 were quite similar to the spectrum of CIND-01 and they had the intense characteristic peaks of cinnamaldehyde, indicating significant presence of cinnamaldehyde in these cinnamon oils. Comparing the absorbance of the peaks at 1679 cm^{-1} and 1626 cm^{-1} of these three cinnamon EOs, it was found that CIN-01 and CIN-03 had higher absorbance values than that of CIN-02. Since absorbance values are proportional to the concertation of a compound, it can be concluded that CIN-01 and CIN-03 possessed higher concentrations of cinnamaldehyde than did CIN-02. The spectrum of CIN-04 was more similar to the spectrum of eugenol (EUG-01) instead of that of CIND-01, indicating that the major compound of CIN-04 was eugenol. The signature IR peaks of eugenol (EUG-01) were at 1638 cm^{-1}, 1609 cm^{-1}, 1514 cm^{-1}, and 1267 cm^{-1} (Chowdhry, Ryall, Dines, & Mendham, 2015; Shengfeng et al., 2015). The peaks at 1638 cm^{-1}, 1609 cm^{-1} and 1514 cm^{-1} were assigned to C=C stretching. The peak at 1267 cm^{-1} was characteristic of C-O stretching (Chowdhry et al., 2015). The chemical composition of cinnamon EOs depends on the part of the plant from which they are extracted and the extraction method. Cinnamaldehyde is the main component of bark cinnamon EO, while eugenol is the major compound of cinnamon leaf EO

(Nabavi et al., 2015), which was in accordance with their spectra. Therefore, CIN-01, CIN-02 and CIN-03 are extracted from bark cinnamon EOs and CIN-04 is extracted from cinnamon leaves. The spectrum of CLO-01 was also similar to the spectrum of EUG-01, indicating that the major component of CLO-01 is eugenol. These findings illustrated the potential of ATR-FTIR spectroscopy for differentiation of EOs and identification of the relative amounts of the major component of EOs.

The infrared spectra of CAR-01, ORE-01, ORE-02 and THY-01 were very similar (Figure 4.2). The major component of thyme EO is thymol which has a similar chemical structure to carvacrol, thus leading to the similar ATR-FTIR spectra of THY-01 with CAR-01 and oregano oils. The typical peaks in the spectrum of carvacrol included bands at 1419 cm^{-1} due to C-C ring stretching, 1223 cm^{-1} due to C-O-H bending mode, and 811 cm^{-1} due to aromatic C-H bending (Pelissari, Grossmann, Yamashita, & Pineda, 2009).

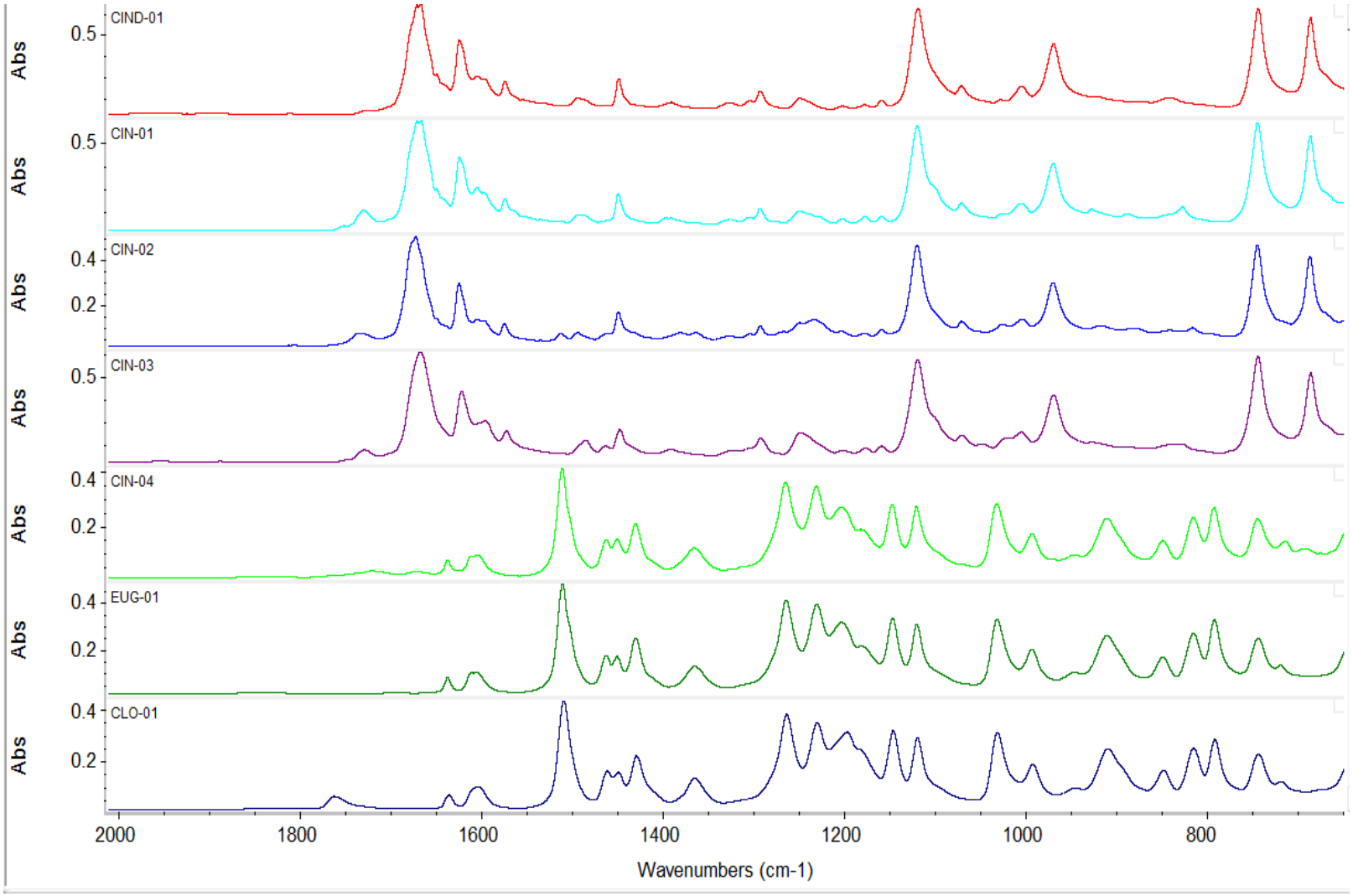


Figure 4.1 ATR-FTIR spectra of CIND-01, CIN-01, CIN-02, CIN-03, CIN-04, EUG-01 and CLO-01

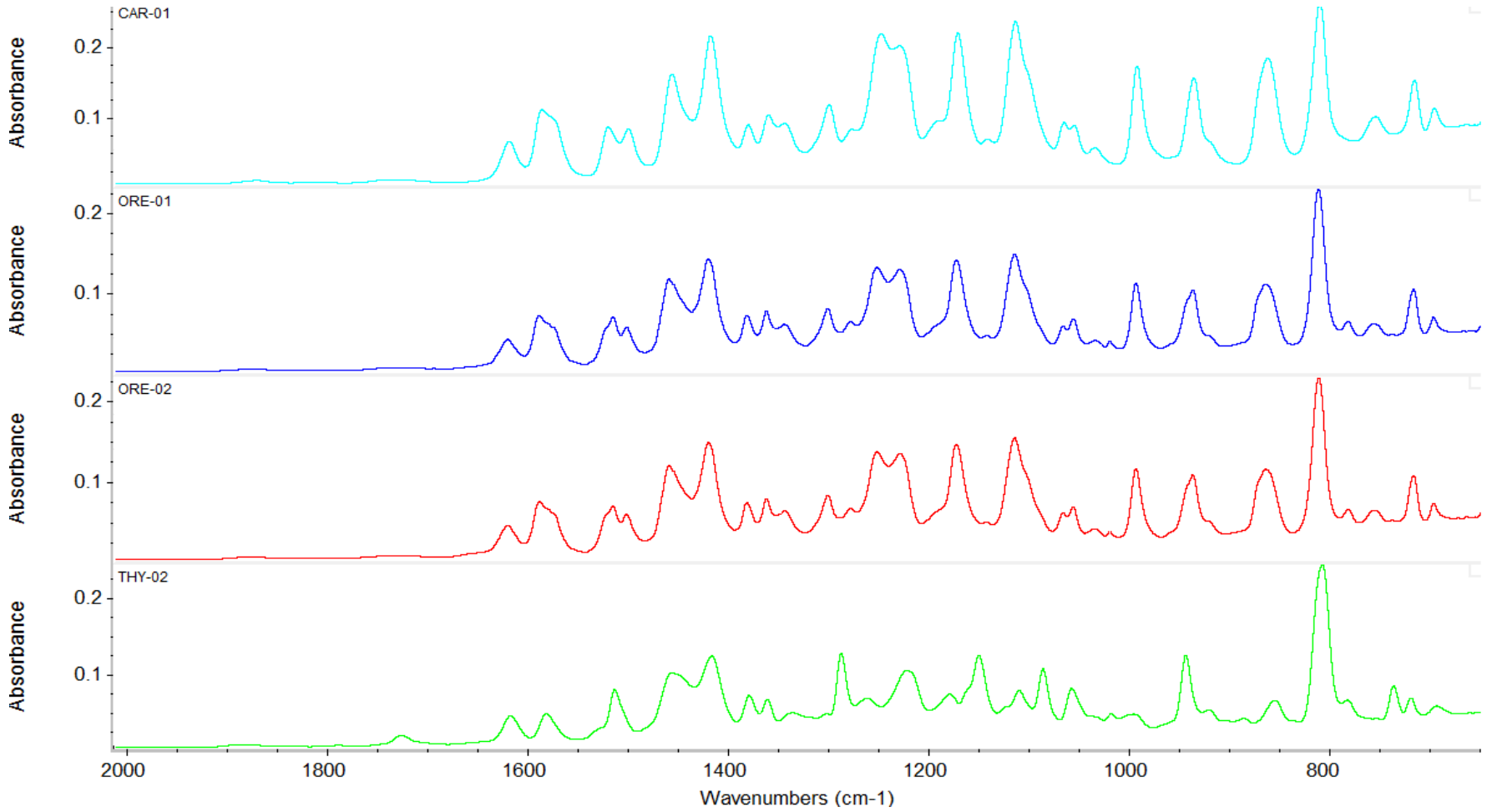


Figure 4.2 ATR-FTIR spectra of CAR-01, ORE-01, ORE-02 and THY-01

4.2 Fungal identification

The pure fungal culture was sent to LSPQ for identification by using MALDI-TOF MS. Three isolates from moldy bread were identified as *P. roqueforti*, *Penicillium* spp., and *Aspergillus flavus (A. flavus)*, as shown in the Table 4.1. The colony morphology of three strains is shown in Figure 4.3.

About 90% of wheat bread spoilage was caused by *Penicillium* spp. (Legan & Yoysey, 1991). The predominance of *Penicillium* spp. may be due to their abilities to grow over wide range of temperatures and water activities (Magan et al., 2012). *P. roqueforti* is one of the common species of *Penicillium* found in bread products, because it is resistant to organic acid and capable of growing under conditions of low pH. *P. roqueforti* can produce PR-toxin which is a toxic fungal metabolite (K. F. Nielsen, Sumarah, Frisvad, & Miller, 2006).

A. flavus is one of the most toxigenic contaminant found in cereals, nuts and bakery products. During secondary metabolism, *A. flavus* can produce aflatoxins which are potent hepatocarcinogens to animals (Gourama & Bullerman, 1995).

Table 4.1 Fungal isolates from moldy bread identified by MALDI-TOF MS

Culture #	Organism name	Confidence value
A_1P	*A. flavus*	99%
A_2P	*P. roqueforti*	99%
AP	*Penicillium* spp.	99%

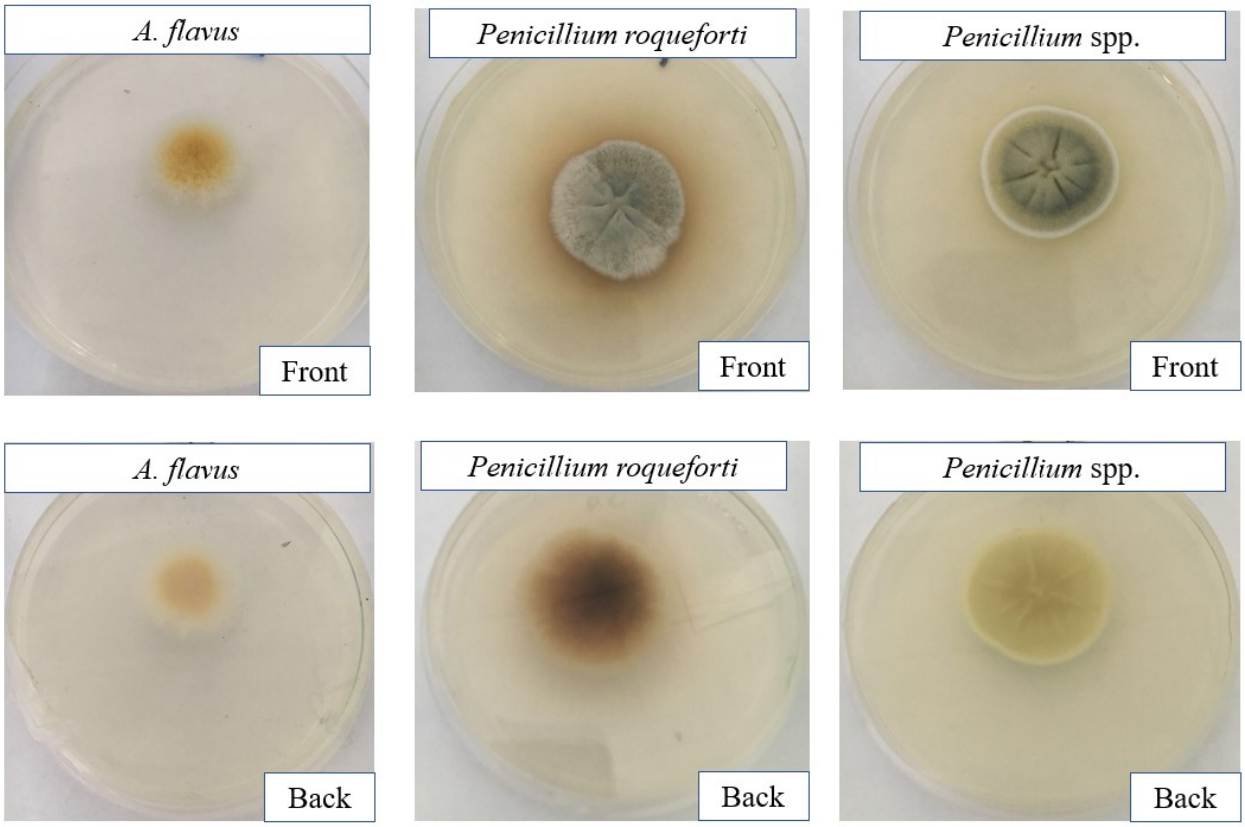


Figure 4.3 Colonies of three strains isolated from moldy bread were cultured on the PDA plates

4.3 Antifungal activity of EOs and their major constituents

The results of antifungal activity of EOs and their major constituents evaluated by the disc diffusion method and vapor diffusion method are shown in Figures 4.4-4.9. The data of diameter of MIZ are the mean of the three replicates and the standard deviations of measurements are expressed as error bars. In the cases where no error bars are plotted, the values for the triplicates were in agreement within 3 %.

In the disc diffusion test, the diameter of MIZ decreased over time due to the recovery of the molds, except CAR-01 (4 or 6 µL), ORE-01 (6 µL), ORE-02 (6 µL) or THY-01 (6 µL) which was found to inhibit the *P. roqueforti* completely. Six µL of CAR-01, ORE-01, or ORE-02 was also

100 % effective against the other *Penicillium* spp. In Figure 4.4, *A. flavus* showed less sensitivity to all EOs at a dose of 2 µL, but compared to the control, 2 µL of the EOs inhibited the growth of *A. flavus* slightly ($p < 0.05$). Statistical analysis also showed that the effect of 4 µL and 6 µL of EO against *A. flavus* were not significantly different at day 14 ($p > 0.05$). At a dose of 6 µL, CAR-01 and ORE-02 achieved a greater inhibition zone than other EOs ($p < 0.05$). In Figure 4.5, CAR-01, ORE-01, ORE-02, or THY-01 at a dose of 4 µL or 6 µL inhibited the growth of *P. roqueforti* completely in the first 7 days, but the mold treated with 4 µL of ORE-01, ORE-02 or THY-01 recovered at day 14. Similar activity of ORE-01, ORE-02 or THY-01 against *P. roqueforti* was observed and attributed to the structure similarities of the main active constituents. CIN-03 also inhibited the growth of *P. roqueforti* effectively. With 6 µL of CIN-03 added to the filter paper, the diameters of MIZ in the first 7 days were not significantly different ($p > 0.05$). In Figure 4.6, all EOs with higher doses exhibited stronger inhibitory effect against *Penicillium* spp. CAR-01 as a pure component of EO, achieved a larger diameter of MIZ than EOs at lower doses.

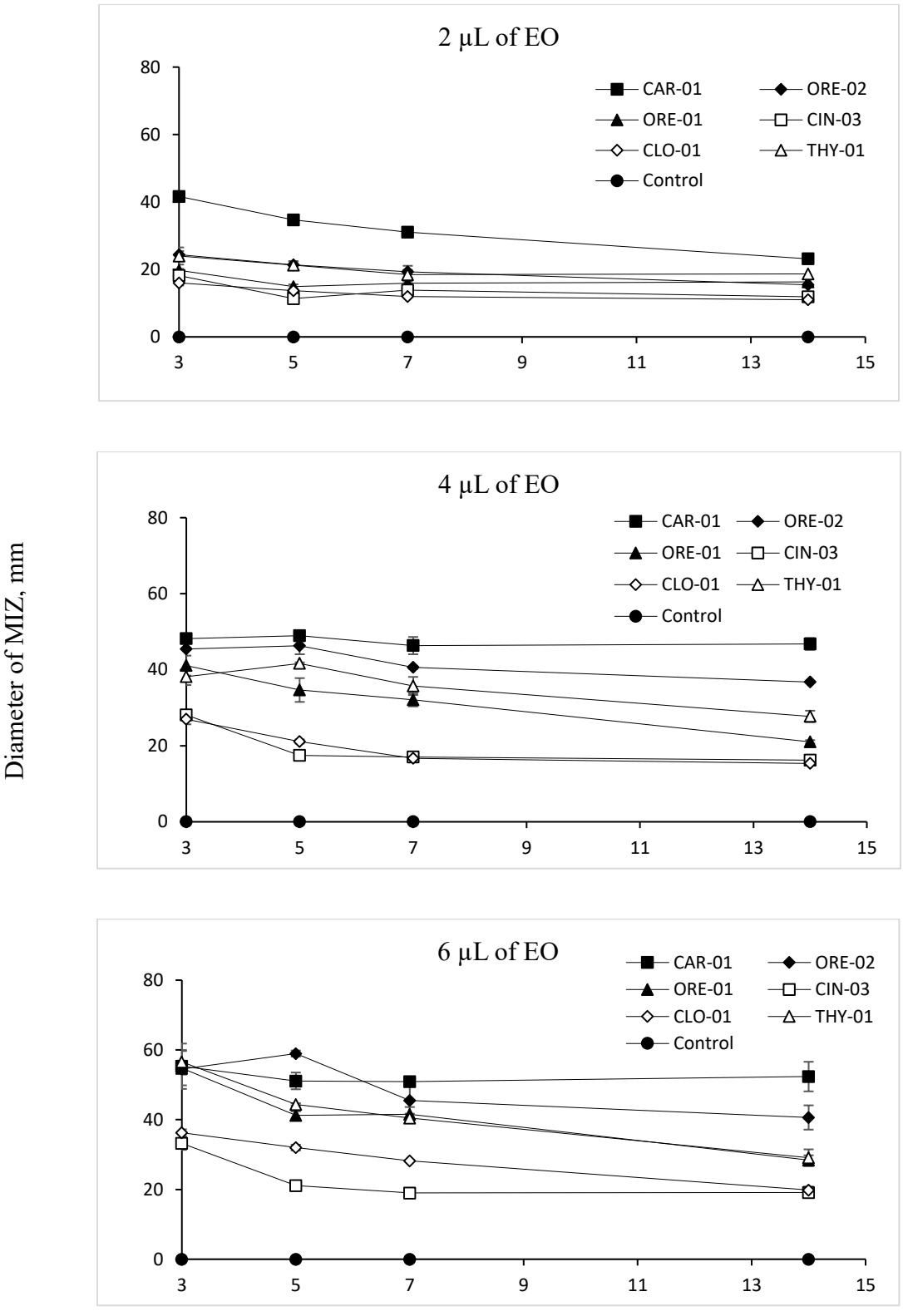


Figure 4.4 Effect of EOs against the *A. flavus* using disc diffusion method

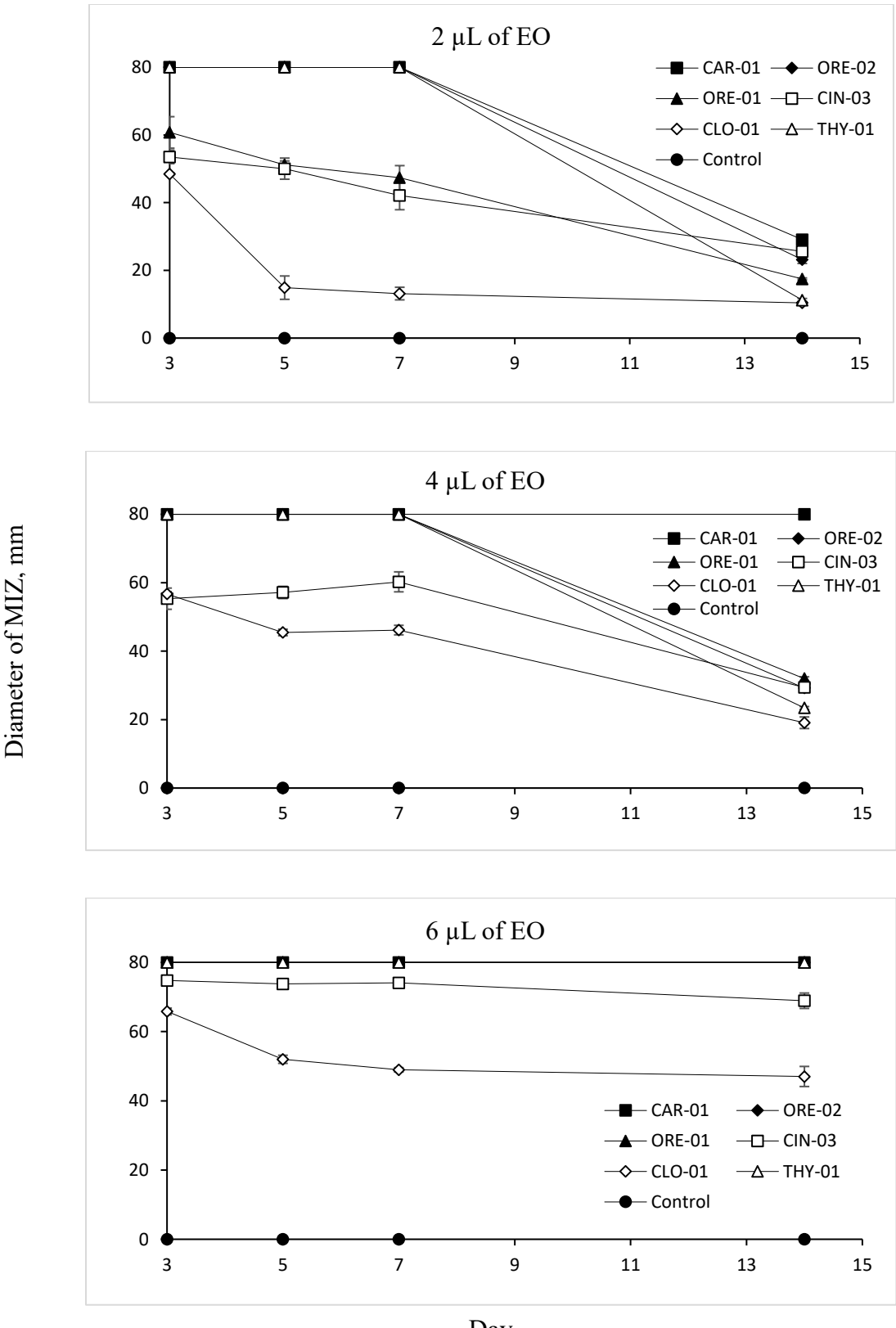


Figure 4.5 Effect of EOs against the *P. roqueforti* using disc diffusion method

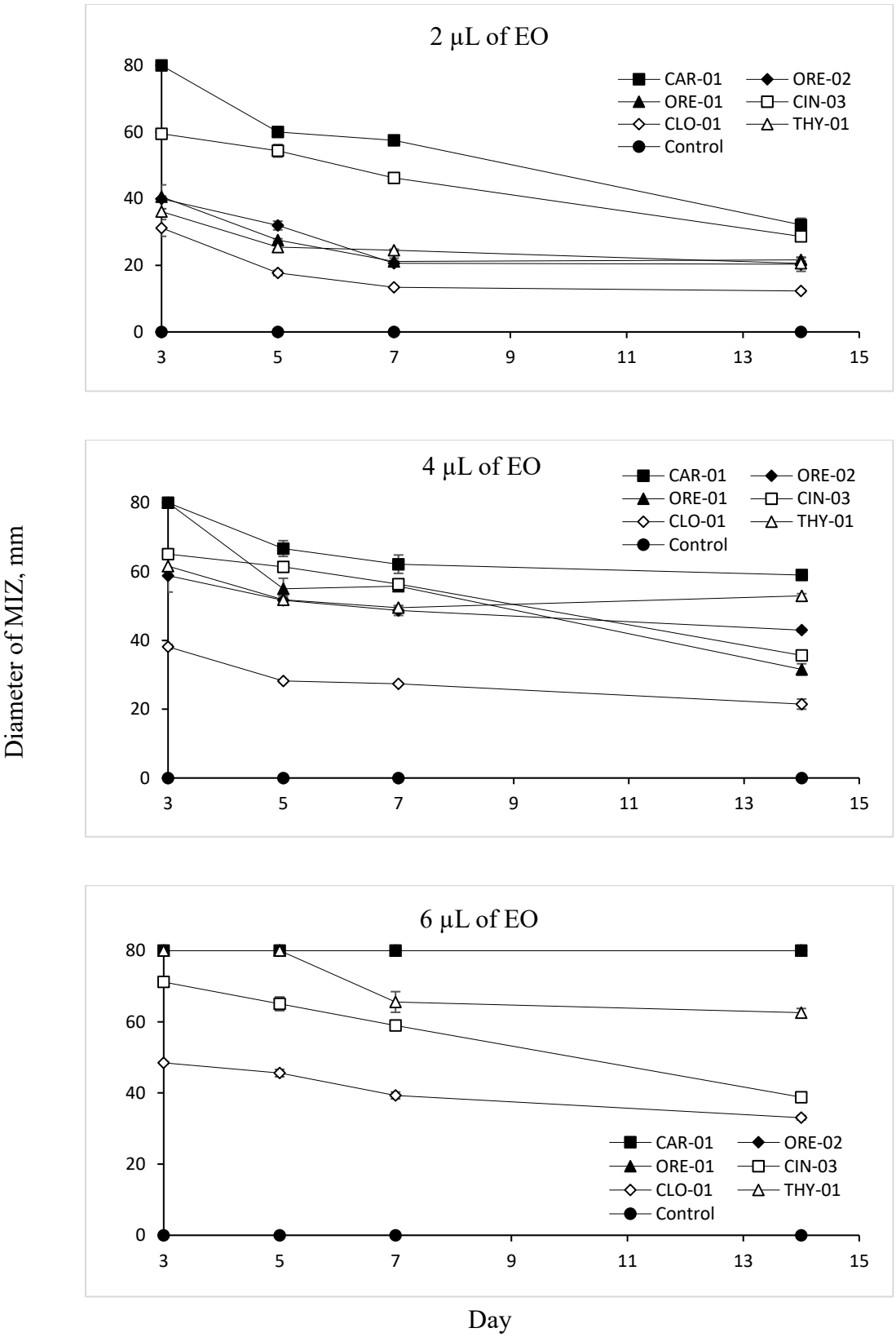


Figure 4.6 Effect of EOs against the *Penicillium* spp. using disc diffusion method

The results of antifungal activity of EOs evaluated by the vapor diffusion method are shown in Figures 4.7, 4.8 and 4.9. In the vapor diffusion test, the inhibitory effect of EOs was relatively weak compared to the disc diffusion method, which may due to the different evaluation methods applied. However, CIN-03 presented stronger antifungal activity against *P. roqueforti*. At all tested doses, CIN-03 achieved complete inhibition of *P. roqueforti* for 14 days. In Figure 4.7, *A. flavus* showed resistance to EOs. At a dose of 2 μL, only CIN-03 presented inhibitory effect on day 14, and the plates treated with other 4 EOs including ORE-01, ORE-02, CLO-01, and THY-01 showed no inhibition zone after 3 days. At a dose of 4 μL or 6 μL, CAR-01 achieved a larger inhibition zone in first 7 days, but at day 14, there was no significant difference of diameter of MIZ compared with that of plates treated with other EOs ($p > 0.05$). In Figure 4.8, *P. roqueforti* showed great sensitivity to CIN-03 and CAR-01, while THY-01 at a dose of 4 or 6 μL also inhibited its growth completely in the first 7 days. The plates treated with 2 μL of ORE-01, ORE-02, CLO-01 or THY-01 had no inhibition zone at day 14. With the higher dose of 4 μL, only ORE-02, CIN-03 and CAR-01 inhibited the growth of mold throughout the incubation period. When applying 6 μL of EOs, all EOs except CLO-01 exhibited marked inhibition compared to the control ($p < 0.05$). In Figure 4.9, CLO-01 also showed weak inhibitory effect against *Penicillium* spp. No inhibition zone was observed after 5-day treatment with 4 μL of CLO-01 and 7-day treatment with 6 μL of CLO-01. The antifungal activities of ORE-01, ORE-02, THY-01, and CAR-01 at a dose of 4 μL were not significantly different at day 14 ($p > 0.05$). Both CAR-01 and CIN-03 depressed the growth of *Penicillium* spp. effectively compared to other EOs, but the MIZ shrunk during the incubation period, indicating that they had fungistatic effects rather than fungicidal effects.

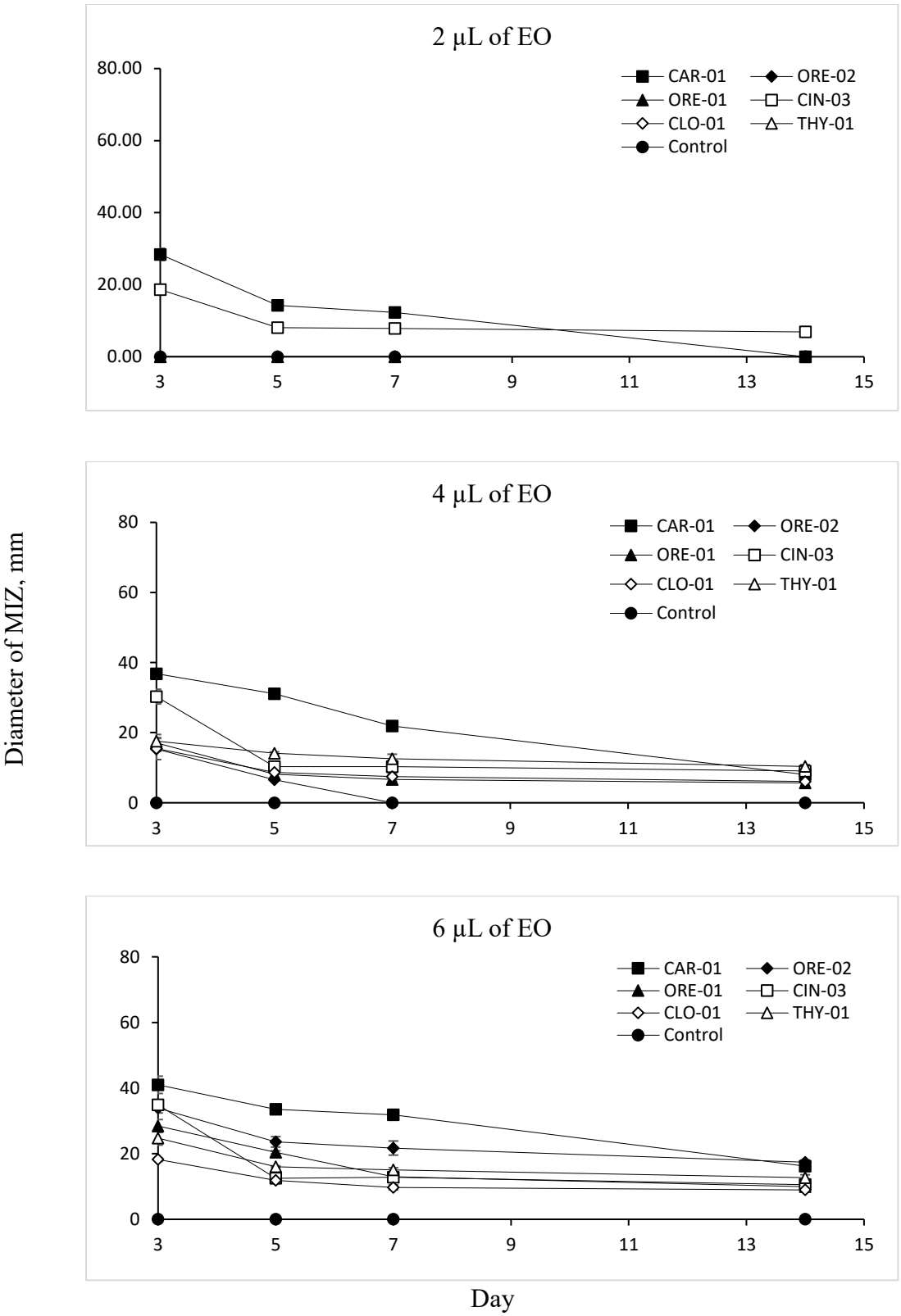


Figure 4.7 Effect of EOs against the *A. flavus* using vapor diffusion method

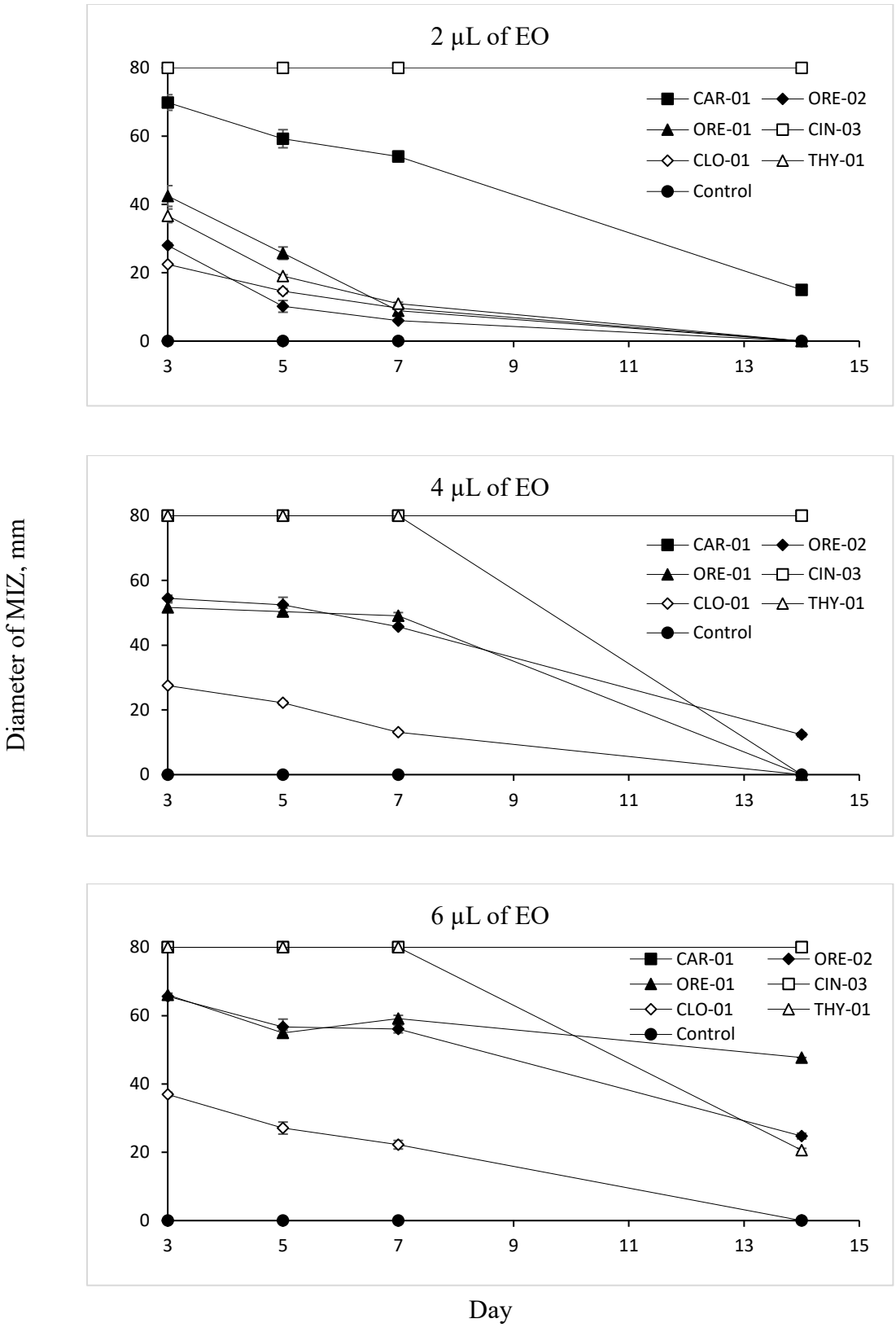


Figure 4.8 Effect of EOs against the *P. roqueforti* using vapor diffusion method

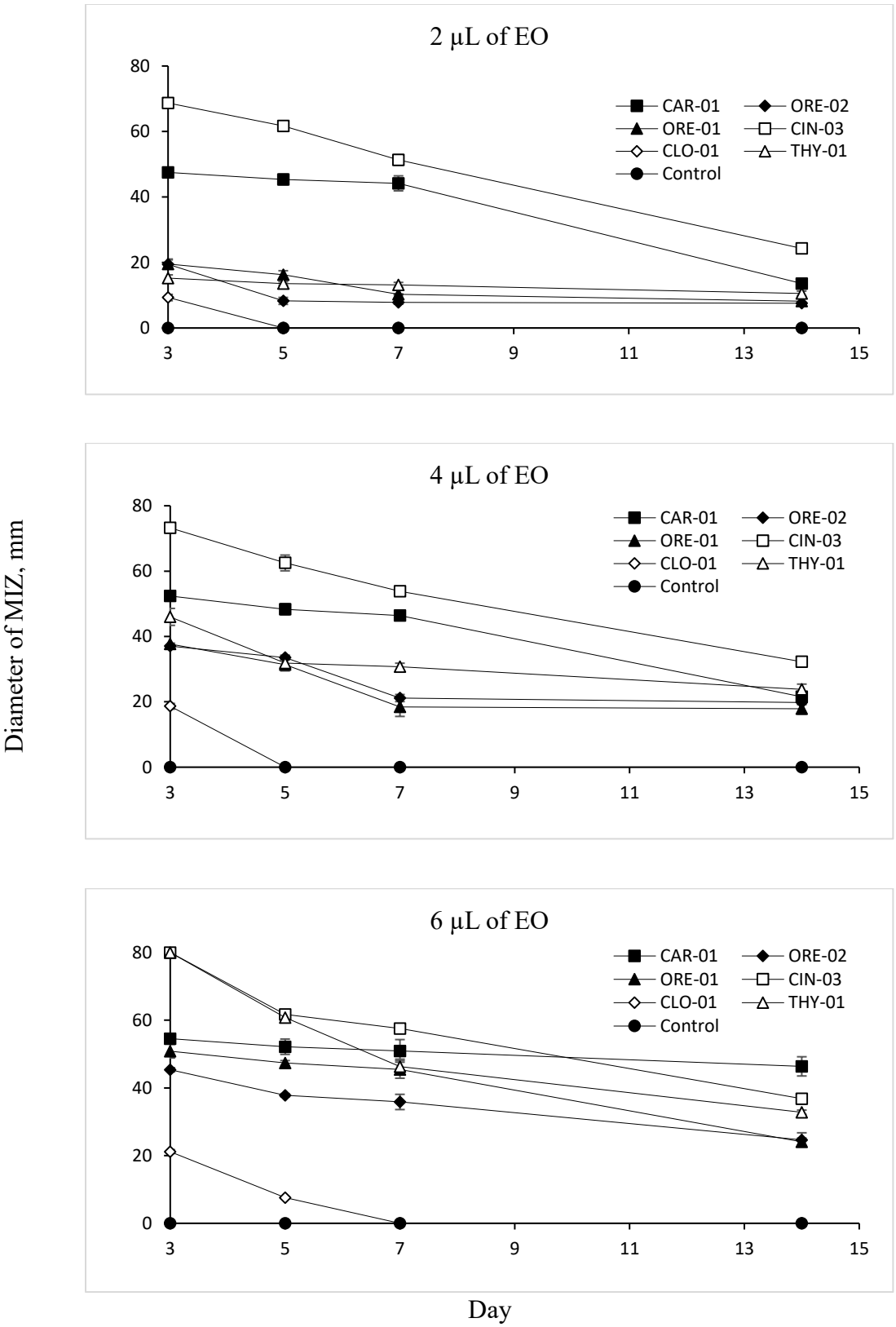


Figure 4.9 Effect of EOs against the *Penicillium* spp. using vapor diffusion method

The antimicrobial activity of EOs has been demonstrated by numerous authors. Antifungal effect of cinnamon EO and clove EO was investigated by disc diffusion and gas diffusion tests in one very recent study (Ju et al., 2018). These authors showed that cinnamon EO exhibited a strong inhibitory effect against *Aspergillus* spp. and *Penicillium* spp. Additionally, all tested molds were sensitive to clove EO, but in gas diffusion tests, clove EO had weaker inhibitory effect. Our results also revealed the weaker inhibitory effect of clove EO against molds in vapour diffusion test. In another study, cinnamon EO was proven to inhibit the growth of *Aspergillus* spp. and *Penicillium* spp., and the diameter of inhibition zone increased as the concentration of EO increased (Xing, Li, Xu, Yun, & Lu, 2010). Our results also showed that cinnamon EO had enhanced inhibitory effect as its volume increased from 2 to 6 μL.

The MIC values of EOs against fungi isolates tested by agar dilution method are presented in Table 4.2. The lower MIC of the EO reflects a stronger inhibitory effect against molds. It may be noted that the MIC and MFC values are generally higher for *A. flavus* than for the *Penicillium* spp., again indicating the greater resistance of *A. flavus* to the inhibitory effects of cinnamon and oregano oils. An additional 10 EOs including GAR-01, GAR-03, CRA-01, CYP-01, ONI-01, CHI-01, SAG-01, CED-01, ROS-01 and PIN-01 were tested in this study. Our results showed that the MIC and MFC values obtained with these 10 EOs were higher than 1000 ppm, suggesting that these compounds were ineffective in inhibiting the growth of the tested isolates.

The top three most effective EOs were CIN-01, CIN-02 and CIN-03 of which at concentrations less than or equal to 125 ppm caused complete inhibition of the growth of all test isolates. CIND-01 was found as effective as these three effective cinnamon oils. CIN-04 was determined to be not as effective as these three cinnamon EOs, we attribute to the presence of a different major active compound in CIN-04. As mentioned in section 4.1, from the ATR-FTIR spectra in Figure 4.1, it

was known that the main active compound in CIN-01, CIN-02 and CIN-03 was cinnamaldehyde. The spectrum of CIN-04 showed that CIN-04 contained low levels of cinnamaldehyde but a higher level of eugenol. This observation can explain why the MIC values of CIN-04 were close to that of EUG-01. The antifungal activity of ORE-01, ORE-02 and THY-01 were similar which is in accordance with the similarity of their infrared spectra (Figure 4.2). The known MIC and MFC values were identical for the same EO or pure bioactive components, which may due to the long exposure to the EO or pure component during the incubation. MFC values indicated that EOs had fungicidal effects against molds.

The MIC values of EOs against *Aspergillu*s spp. and *Penicillium* spp. have been studied by many other authors (López et al., 2007; Manso et al., 2013; Paster et al., 1990; Segvic Klaric et al., 2007; Soliman & Badeaa, 2002; Suhr & Nielsen, 2003; Xing et al., 2010). For example, thyme and cinnamon oils at a concentration of 500 ppm or less were reported to inhibit spore germination of *Aspergillus* spp. completely (Soliman & Badeaa, 2002). The MIC of cinnamon oil against *A. flavus* and *P. expansum* was 1600 ppm (Xing et al., 2010). In our experiment, the cinnamon and thyme oils have the values of MIC less than or equal to 500 ppm against *Aspergillus* spp. However, the differences in the MIC values are expected due to natural variability of EOs.

In conclusion, the CIN-01, CIN-03, CIND-01, CIN-02 and CAR-01 were found to inhibit the fungal growth effectively. CIN-03 and CAR-01 were chosen for the later experiments as they possessed the lowest MIC and MFC values against bread molds. They also expressed stronger inhibitory effect compared to other EOs in the vapor diffusion test indicating their higher volatility, which is a very significant property in developing EO-based active packaging.

Table 4.2 MIC Values of EOs and pure compounds against three fungal isolated tested in agar dilution assay

	ppm	*A. flavus*	*P. roqueforti*	*Penicillium* spp.
CIN-03	MIC	125	31	62
	MFC	125	31	62
CIN-01	MIC	125	31	62
	MFC	125	31	62
CIND-01	MIC	125	31	62
	MFC	125	31	62
CIN-02	MIC	125	62	125
	MFC	125	62	125
CAR-01	MIC	250	62	125
	MFC	250	62	125
ORE-02	MIC	500	125	250
	MFC	500	125	250
ORE-01	MIC	500	250	250
	MFC	500	250	250
THY-01	MIC	500	250	250
	MFC	500	250	250
EUG-01	MIC	500	500	500
	MFC	500	500	500
CLO-01	MIC	500	500	500
	MFC	500	500	500
CIN-04	MIC	1000	250	500
	MFC	1000	250	500
PIM-01	MIC	1000	250	250
	MFC	1000	250	250
GAR-02	MIC	>1000	500	500
	MFC	>1000	500	500
GAR-03	MIC	>1000	1000	1000
	MFC	>1000	1000	1000
GAR-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000
CRA-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000
CYP-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000
ONI-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000
CHI-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000

	ppm	*A. flavus*	*P. roqueforti*	*Penicillium* spp.
SAG-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000
CED-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000
ROS-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000
PIN-01	MIC	>1000	>1000	>1000
	MFC	>1000	>1000	>1000

4.4 Efficacy of EO-containing sachet against mold growth on PDA plates

The sachets containing CIN-03 or CAR-01 released volatile compounds that inhibited the growth of molds on PDA plates. Table 4.3 summarizes the antifungal activity against *A. flavus* and *Penicillium* spp. by EO-containing sachets. The antifungal activity varied depending on the volume of active compounds in the sachet and the type of active compound incorporated. CIN-03 at a dose of 2 µL was enough to achieve complete inhibition of mold growth, while 5 µL of CAR-01 in the sachet was effective. Thus, CIN-03 was found to be more effective than CAR-01 when incorporated in the sachet.

The antimicrobial activity of bark cinnamon oil is attributed to its major component, cinnamaldehyde which is an active inhibitor of bacteria (Chang, Chen, & Chang, 2001; Friedman, Henika, & Mandrell, 2002), yeasts and filamentous molds (Lee, Cheng, & Chang, 2005; Taguchi et al., 2010). Moreover, cinnamon EO and cinnamaldehyde vapors were reported to be effective against microorganisms. Cinnamaldehyde administrated as gas was found to retard the hyphal elongation of *A. niger* (Matsuoka, Li, Takekawa, & Teraoka, 1990). Vapor released from polyethylene terephthalate films containing 4% of cinnamon EO fully inhibited the growth of *A. flavus in vitro* (Manso et al., 2013). These studies encourage the application of active packaging of cinnamon EO and its active compound that allows their volatilization.

Table 4.3 Antifungal activity of sachets containing CIN-03 or CAR-01 against mixed molds *in vitro*

Volume of active compounds	Fungal growth [a]	
	CAR-01	CIN-03
1 µL	+	+
1.5 µL	+	+
2 µL	+	–
2.5 µL	+	–
5 µL	–	–
10 µL	–	–
100 µL	–	–

[a] – absence of fungal growth; + presence of fungal growth

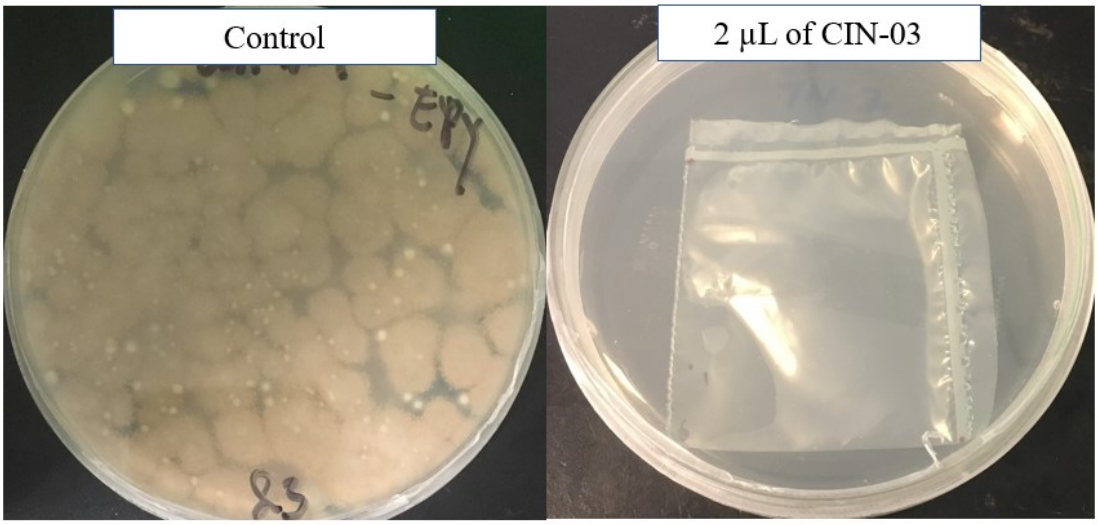


Figure 4.10 Antifungal effect of sachets containing EO against mixed isolates of *A. flavus* and *Penicillium* spp. *in vitro*

4.5 Efficacy of EO-containing sachet against mold growth on bread

4.5.1 Inhibiting mold growth in sealed jar system

The antimicrobial activities of the EO-containing sachets were evaluated by counts of molds on the bread plugs which exposed to sachets throughout the storage. Results presented show that sachet containing CIN-03 effectively suppressed the growth of *A. flavus* and *Penicillium* spp. in the jar (Figure 4.11). The visual fungal growth appeared on the surface of bread plugs on 3rd day of post incubation, while no fungal growth was visually observed on the plugs that were treated with 50 µL of CIN-03.

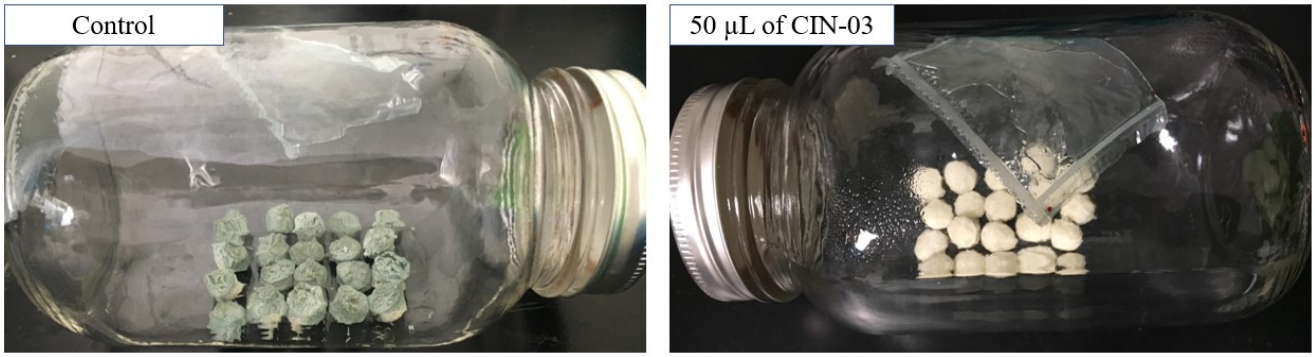


Figure 4.11 Sachet containing CIN-03 against the growth of *A. flavus* and *Penicillium* spp. on the surface of bread plugs in the sealed jar system. The pictures were taken on day 14 of storage

The counts of molds throughout the storage of bread plugs treated by EO-containing sachet were illustrated in Figure 4.12 and 4.13. At the beginning of the incubation (day 0), the counts of molds in both figures did not differ among all different treatments and the control ($p > 0.05$). In Figure 4.12, there was a trend of increasing counts of molds (denoted as log CFU per gram) during storage regardless of the volume of CAR-01 in the sachets. At day 3 and day 5, the resulting counts from all treatments were not significantly different ($p > 0.05$). After 7 days, the counts of molds which treated by sachet containing 50, 100 and 1000 µL of CAR-01 were found to be different from the

counts of the control ($p < 0.05$). At the end of the storage, only treatments with 100 and 1000 µL of CAR-01 decreased the growth of molds ($p < 0.05$).

In Figure 4.13, after 3 days of storage, the EO-free treatment allowed greater growth of molds when compared to the treatments where CIN-03 was included ($p < 0.05$). There were no detected molds on the bread plugs treated by sachet containing 50, 100 or 1000 µL of CIN-03, indicating that these three types of sachets were effective to completely inhibit the growth of molds throughout the storage. The required volume of EO to achieve complete inhibition via *in situ* tests was much higher than that with *in vitro* tests, because interaction between the EO and fat and protein contents of the bread may decrease the concentration of active volatile compounds (Ma, 2012). The sachet containing 25 µL of CIN-03 had a marked inhibitory effect against the mold growth on the bread plugs. At day 14, there was a 2.5-log mold reduction of counts of molds on bread treated by 25 µL of CIN-03 compared to the counts in the control.

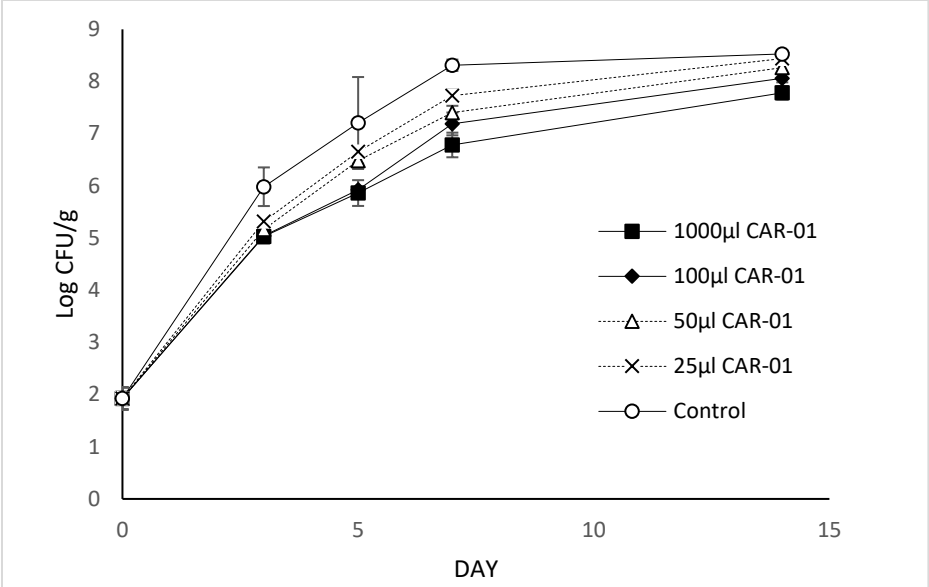


Figure 4.12 Growth behaviour of *A. flavus* and *Penicillium* spp. on bread plugs treated with sachets containing 0, 25, 50, 100, 1000 µL of CAR-01 during 14 days of storage

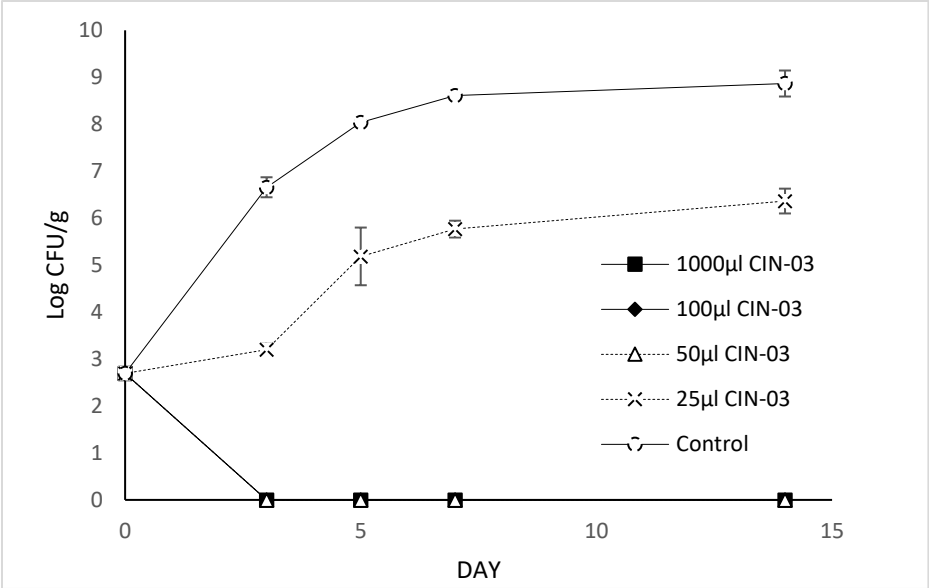


Figure 4.13 Growth behaviour of *A. flavus* and *Penicillium* spp. on bread plugs treated with sachets containing 0, 25, 50, 100, 1000 µL of CIN-03 during 14 days of storage

The above results of enumeration were supported by growth recovery experiment. Figure 4.14, 4.15, 4.16, and 4.17 indicated that spores of molds on the bread plugs which had been pre-exposed to CAR-01 in the sachet could recover their ability to propagate and grow. For the bread plugs which were pre-exposed to the CAR-01 for 7 days or less, the percent incidence mold growth of was negatively correlated to the volume of CAR-01 in the sachet. After 14-day pre-exposure, all the CAR-01 treatments showed the 100% recovery of molds, which may due to that the spores had adapted to the stressed conditions and overcome the inhibitory effect of CAR-01 and finally survived successfully.

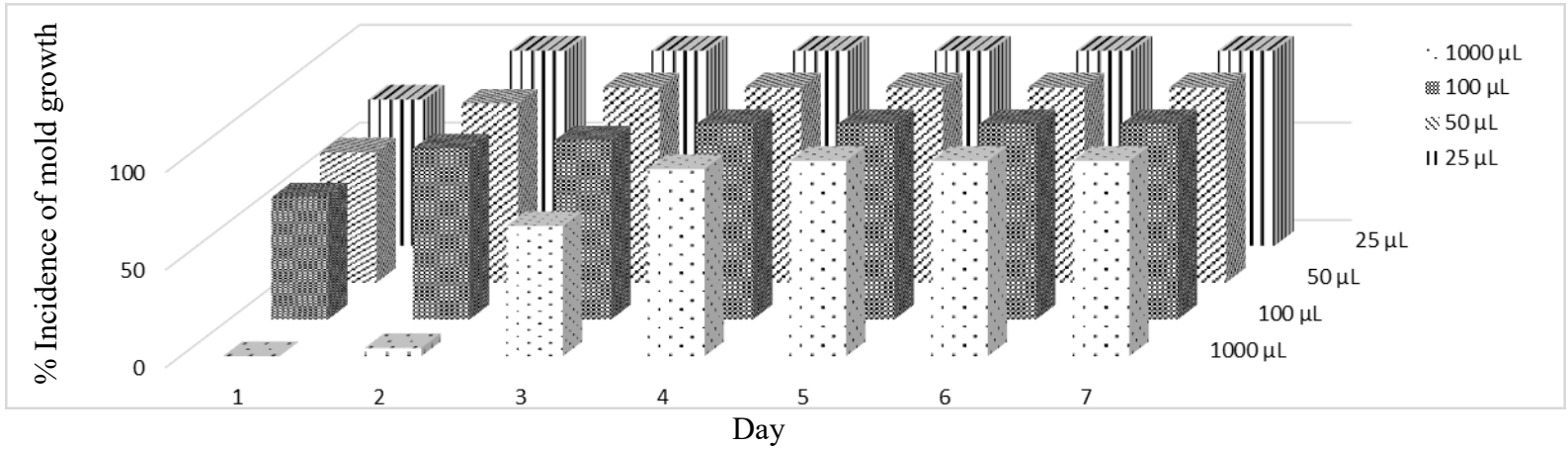


Figure 4.14 Effect of sachet containing CAR-01 on the growth of recovered *A. flavus* and *Penicillium* spp. on bread plugs pre-exposed to CAR-01 for 3 days

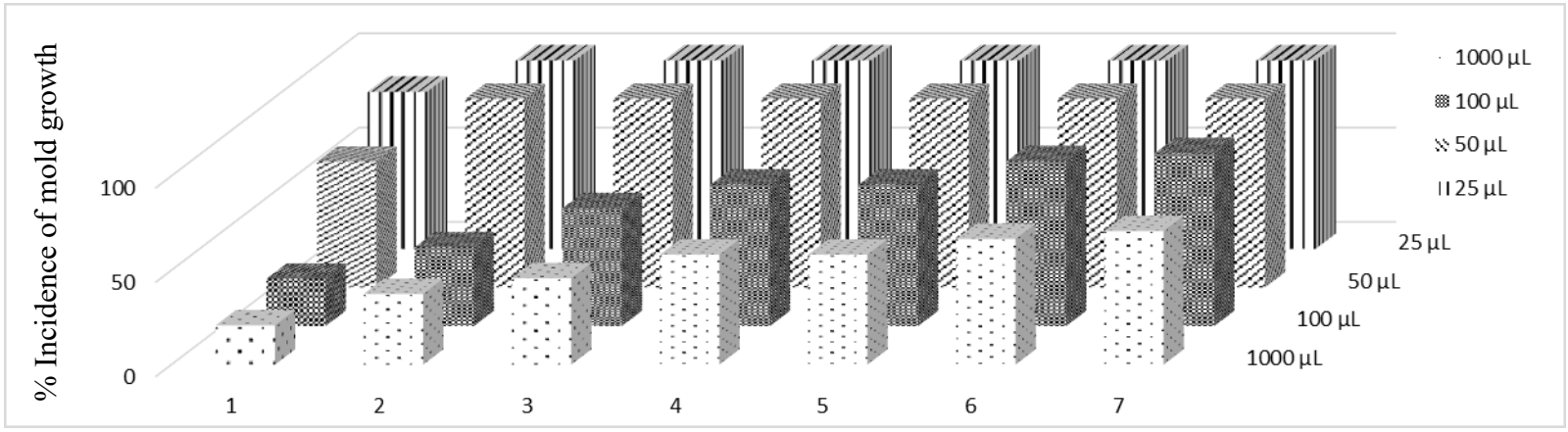


Figure 4.15 Effect of sachet containing CAR-01 on the growth of recovered *A. flavus* and *Penicillium* spp. on bread plugs pre-exposed to CAR-01 for 5 days

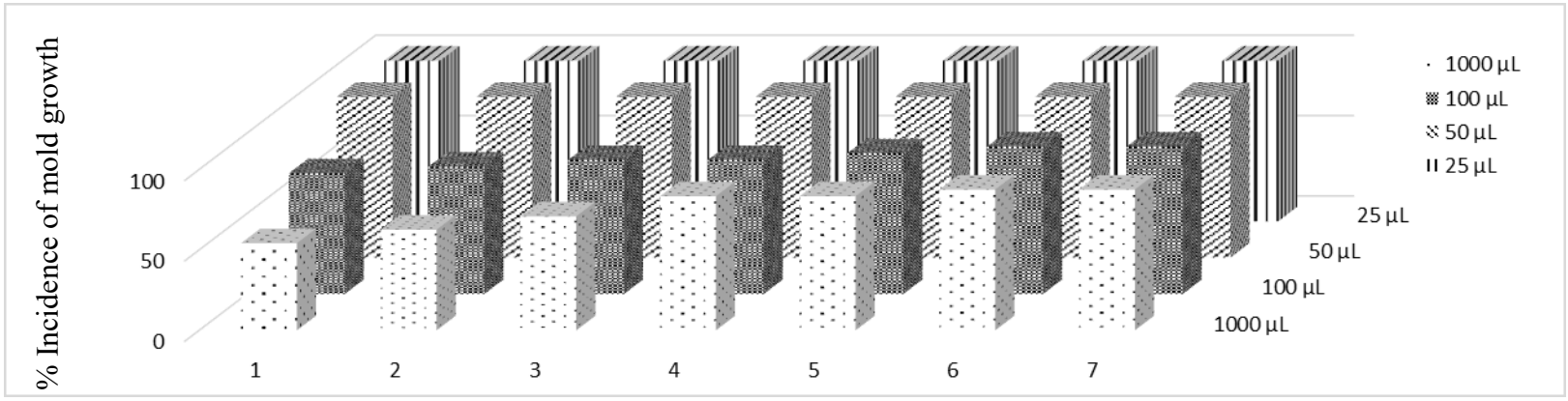


Figure 4.16 Effect of sachet containing CAR-01 on the growth of recovered *A. flavus* and *Penicillium* spp. on bread plugs pre-exposed to CAR-01 for 7 days

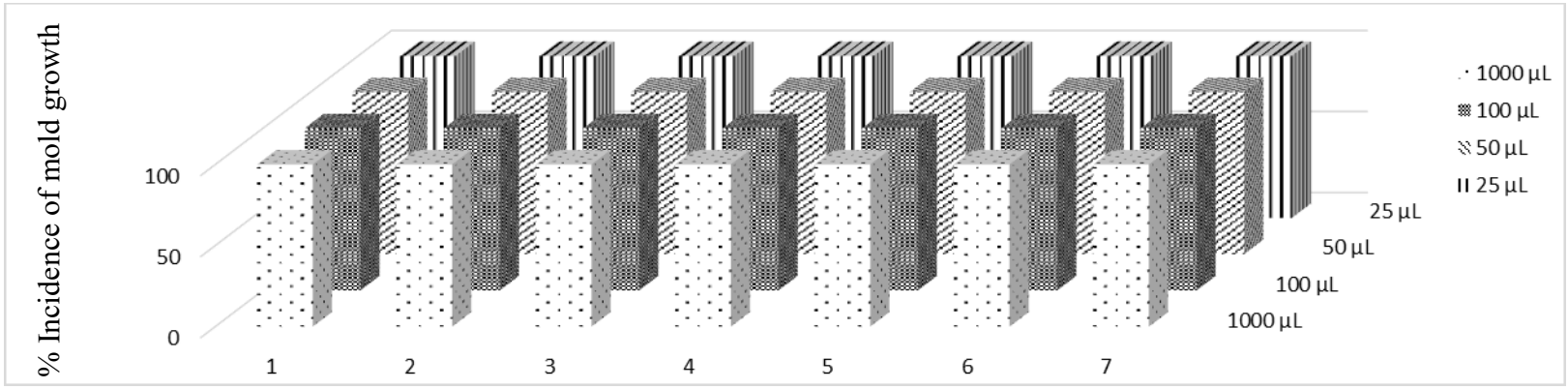


Figure 4.17 Effect of sachet containing CAR-01 on the growth of recovered *A. flavus* and *Penicillium* spp. on bread plugs pre-exposed to CAR-01 for 14 days

The absence of mold growth after the treatments of bread plugs with sachets containing 50, 100 or 1000 µL of CIN-03 shown in Figure 4.13 is consistent with the results in Figures 4.18, 4.9, 4.20, and 4.21 showing that fungal spores pre-exposed to sachets containing 50, 100 or 1000 µL of CIN-03 could not recover their ability to grow. This suggests that the antimicrobial effects are fungicidal when the volume of CIN-03 in the sachets is 50 µL or higher in the controlled jar system. Spores pre-exposed to sachets containing 25 µL of CIN-03 could recover, indicating that doses at 25 µL of CIN-03 have fungistatic effect against molds on the bread plugs.

Overall, the results demonstrated that the sachet containing CIN-03 was much more effective than sachet containing CAR-01 in the jar system. At the end of the storage, sachets containing 100 or 1000 µL of CAR-01 could only achieve less than 1-log reduction of counts of molds, while 25 µL of CIN-03 had fungistatic effect decreasing the growth of molds by 2.5 logs and 50 µL or higher levels of CIN-03 could be lethal to mold spores during the entire experiment period. The antimicrobial activity of EOs and their constituents in vapor phase were studied by other studies. The MIC of cinnamon oil was 17.5-52.4 µL/L against gram positive bacteria, 17.5-131 µL/L against gram negative bacteria, and 8.7-13.1 µL/L against fungi, while the MIC of carvacrol was 10.9-21.8 µL/L for bacteria and fungi (López et al., 2007). In one study, inhibitory effect of volatile EOs against mold growth on bread was also tested in an airtight glass jar (Suhr & Nielsen, 2003). It was found that mustard and lemongrass oils were efficient in inhibiting fungal growth on rye bread. Cinnamon oil in levels of 270 µL/L in the air phase could only achieve 20% inhibition of *P. roqueforti* and 60% inhibition of *A. flavus*, respectively (Suhr & Nielsen, 2003). Differences in the compositions of bread, surface microenvironment and the use of EO may cause the variations of results from the previous studies.

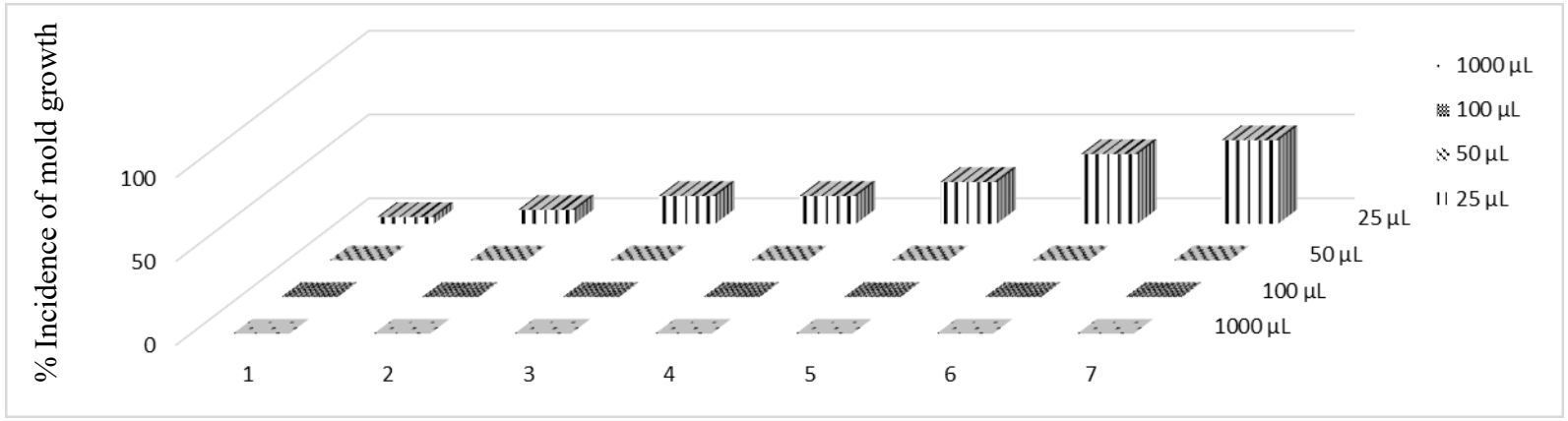


Figure 4.18 Effect of sachet containing CIN-03 on the growth of recovered *A. flavus* and *Penicillium* spp. on bread plugs pre-exposed to CIN-03 for 3 days

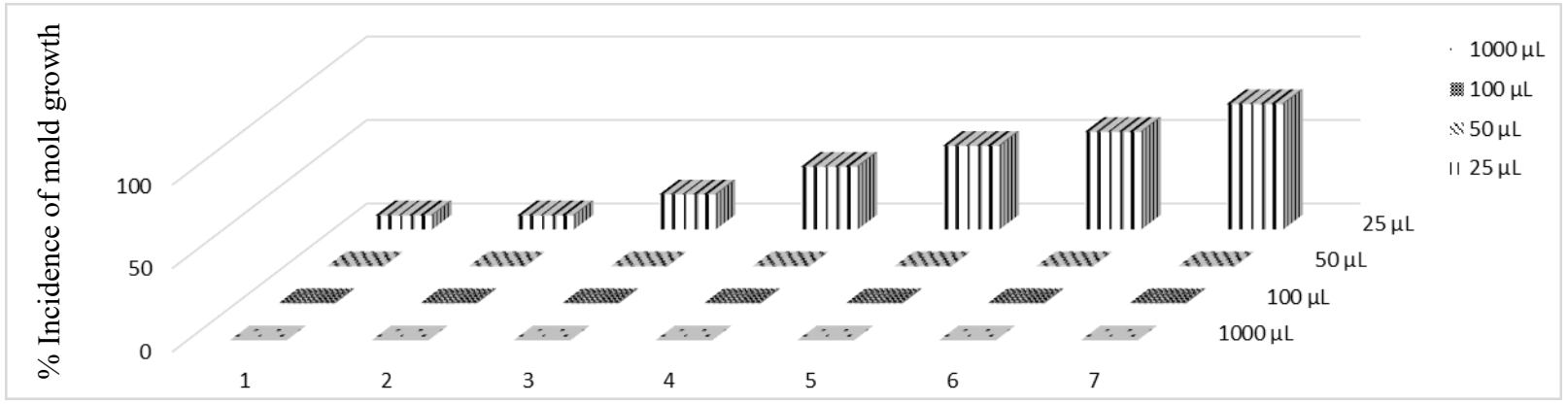


Figure 4.19 Effect of sachet containing CIN-03 on the growth of recovered *A. flavus* and *Penicillium* spp. on bread plugs pre-exposed to CIN-03 for 5 days

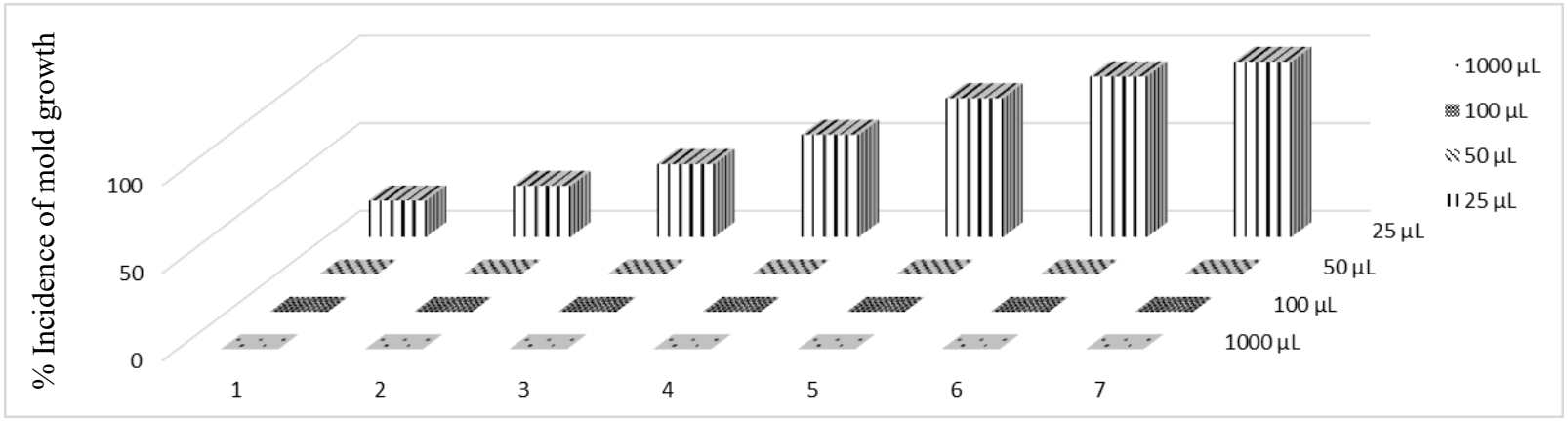


Figure 4.20 Effect of sachet containing CIN-03 on the growth of recovered *A. flavus* and *Penicillium* spp. on bread plugs pre-exposed to CIN-03 for 7 days

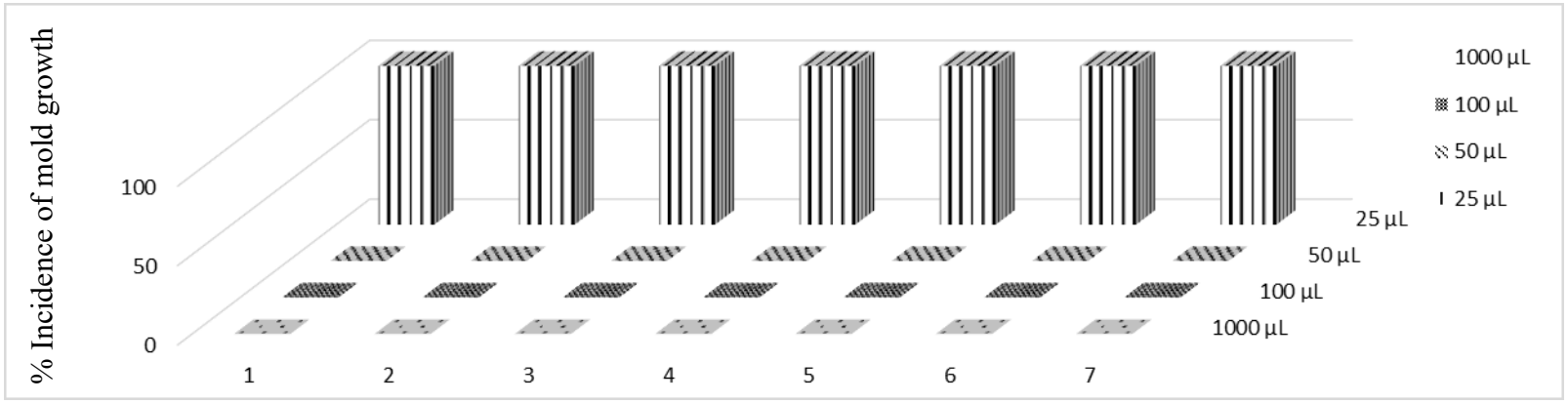


Figure 4.21 Effect of sachet containing CIN-03 on the growth of recovered *A. flavus* and *Penicillium* spp. on bread plugs pre-exposed to CIN-03 for 14 days

4.5.2 Inhibiting mold growth in plastic bags

Since CAR-01 vapor did not inhibit fungal growth on the bread plugs in the previous tests, only CIN-03 was used in the following experiments. The fungal growth on bread slices packaged with sachets containing CIN-03 are summarized in Table 4.4. The results show sachets containing CIN-03 were effective in retarding fungal mold on bread slice effectively. After 3 days of storage, white hyphae appeared on the control samples. For the bread slices packaged with EO sachets, 100 µL treatment and 125 µL treatment delayed the fungal growth for 3 ± 1 days and 8 ± 1 days, respectively. For both treatments, the inhibitory effect was fungistatic. There was no sign of growth on the bread slices treated by sachets containing 250, 500, or 1000 µL of CIN-03 throughout the storage period. In fact, these bread slices were kept in the lab for more than 30 days with no visible fungal growth detected, indicating that sachets containing 250, 500, or 1000 µL of CIN-03 showed fungicidal activity. This could be attributed to the higher concentration of the volatile active compounds. The partition equilibrium was reached between the sachet and the atmosphere in the package. Therefore, if the volume of CIN-03 in the sachet increased, the concentration of active compounds in the atmosphere also increased to reach equilibrium.

Other investigations were carried out to study the application of EOs to extend the shelf life of bakery slices. The shelf life of sliced bread was extend from 3 to 10 days by applying active paper packaging containing cinnamon oil (Rodríguez et al., 2008). One study found that sachets containing oregano oil was effective in retarding fungal growth on the surface of sliced bread (Passarinho et al., 2014). Patches containing mustard oil could achieve complete inhibition of fungal growth on bread slices for more than 90 days (Ma, 2012). Different types EOs, concentration of EOs, various application methods and composition of food matrix play significant roles in active packaging system.

Table 4.4 Inhibitory effect of sachet containing CIN-03 on the growth of *A. flavus* and *Penicillium* spp. on whole wheat bread slices in active packaging

Volume of EO in sachet (µL)	Growth delay days	Fungal growth [a]	
		Day 7	Day 14
100	3	+	+++
125	8 ± 1	–	++
250	> 27	–	–
500	> 27	–	–
1000	> 27	–	–

[a] – absence of fungal growth; + fungal growth on < 25% of the surface, ++ fungal growth on around 50% of surface, +++ fungal growth on > 75% of the surface

Figure 4.22 Sachet containing CIN-03 against the growth of *A. flavus* and *Penicillium* spp. on the surface of bread plugs in the plastic bags. The pictures were taken on day 14th of storage

4.5.3 Inhibiting mold growth in packaged bread

Results in the Table 4.5 indicated that CIN-03 incorporated in sachet format was able to delay the fungal growth and prolong the shelf life of inoculated bread loaf. Visible growth was found on bread loaves which were not packaged with EO-containing sachet on day 3. Sachets containing 125 µL of CIN-03 could delay the fungal growth for 2 days. The mold growth was observed on bread loaves treated by sachets containing 250, 500, or 1000 µL of CIN-03 on day 7. All the treatments showed the fungistatic activity. It was observed that the visible growth started to appear on the top side of the bread loaves, at which the inoculation spot was far from the sachets compared to the other two inoculation spots. Then filamentous molds grew massively on the top side and spread to the other sides of bread loaf. At the end of storage period, there was a clear inhibition zone around the sachet, which indicating that sachet still had inhibitory effect against mold growth (Figure 4.23). Compared to the previous tests in the plastic bags, the sachets had limited antifungal activity in the bread package which may due to the larger headspace and larger surface area of the bread loaf.

The shelf life extension of bread loaf by using EOs was also studied by others. Ma (2012) tested the antimicrobial activity of patches but incorporated with mustard oil. The use of mustard-based active packaging increased the shelf life of bread loaf from 1 to 15 days. In one study, active packaging with cinnamon EO protected bread for two months, while active packaging combined with MAP could achieve absence of mold growth on bread for more than 90 days (Gutiérrez, Batlle, Andújar, Sánchez, & Nerín, 2011). Bread products used in these studies had no inoculation of microorganisms as in the commercial situations. However, the bread loaves used in the project contained preservatives such as calcium propionate, vinegar etc., thus, the loaves were inoculated

with a high load of fungal suspensions, which may lessen the antimicrobial activity of active packaging compared to other studies.

Table 4.5 Inhibitory effect of sachet containing CIN-03 on the growth of *A. flavus* and *Penicillium* spp. on whole wheat bread loves in active packaging

Volume of EO in sachet (µL)	Growth delay days	Fungal growth [a]	
		Day 7	Day 14
125	2	+	+++
250	3	+	+++
500	3	+	++
1000	3	+	++

[a] + fungal growth on < 25% of the surface, ++ fungal growth on around 50% of surface, +++ fungal growth on > 75% of the surface

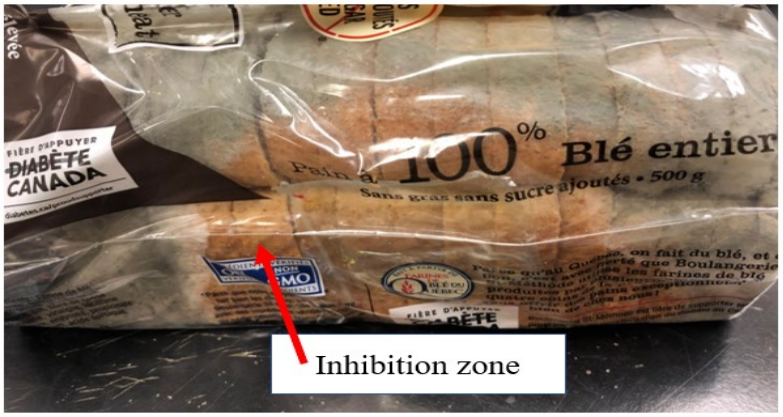


Figure 4.23 Whole wheat bread loaf packaged with sachets containing 250 µL of CIN-03. The picture was taken on day 14th of storage

Chapter 5 General Conclusion

Recently, interest in the applications of natural preservatives has increased due to consumers' demand of safe products with "clean" labels. EO, extracted from plants, is considered to be a possible replacement of traditional preservatives (propionates, sorbic acids and sorbate, etc.) used in bakery products. Chapter 2 (literature review) reviewed the microbial spoilage of bread and different methods to preserve bread products. It was revealed that EOs and their constituents have broad spectrum antimicrobial activity against microorganisms and some of them were reported to have marked effects (Hyldgaard et al., 2012; Krisch et al., 2011; López et al., 2007; Mendonca et al., 2018). Moreover, many studies have been done to investigate the effectiveness of EO against bread *in situ*, and it was found out that EOs can be applied directly or combined with AP or MAP (Krisch et al., 2013; Otoni et al., 2014; Passarinho et al., 2014; Rehman et al., 2007; Rodríguez et al., 2008; Suhr & Nielsen, 2003, 2005).

In this project, the difference in the chemical composition of EOs was elucidated by ATR-FTIR spectroscopy. The effect of selected EOs and their pure bioactive compounds and various methods of exposure of the mold to EOs in retarding the growth of mold, *A. flavus* and *Penicillium* spp., isolated from moldy bread was investigated. The results obtained from experiments are summarized as follows:

1. ATR-FTIR analysis of EOs and their pure bioactive compounds

The spectra of CIN-01, CIN-02 and CIN-03 were quite similar to the spectrum of CIND-01. They all had characteristic peaks at 1679 cm^{-1} and 1626 cm^{-1}. The signature peaks of CIN-04, CLO-01 and EUG-01 were at 1638 cm^{-1}, 1609 cm^{-1}, 1514 cm^{-1}, and 1267 cm^{-1}. It was found that CIN-04 was cinnamon leaf EO of which the major component was eugenol, while the other three cinnamon

oils were bark cinnamon EOs and their major component was cinnamaldehyde. The spectra of CAR-01, ORE-01, ORE-02 and THY-01 were very similar due to the similar structure of carvacrol and thymol. This work demonstrated the utility of ATR-FTIR spectroscopy in the identification and differentiation of EOs based on differences in their chemical composition.

2. Bread molds and antifungal activity of EOs and their constituents

A. flavus, *P. roqueforti* and *Penicillium* spp. were isolated from moldy bread. It was found that *A. flavus* was most resistant to all EOs, while *P. roqueforti* was the most sensitive mold. In the disc diffusion method, CAR-01, ORE-02 and ORE-03 expressed marked inhibitory effects compared to other EOs. In the vapor diffusion method, CIN-03 with any tested doses achieved complete inhibition of *P. roqueforti.* CAR-01 and CIN-03 presented stronger inhibitory effect than other EOs against *A. flavus* and *Penicillium* spp., indicating that CAR-01 and CIN-03 had higher volatility than other EOs. In the agar dilution method, CIN-01, CIN-03 and CIND-01 were reported to have the lowest MIC values against bread molds. The MFC values indicted the fungicidal effect of EOs against bread molds.

3. Efficacy of EO-containing sachet against mold growth *in vitro* and *in situ*

When applying EO-containing sachets to PDA plates inoculated with mixed bread molds, CIN-03 at a dose of 2 μL and 5 μL of CAR-01 achieved complete inhibition of mold growth.

In the sealed jar system, sachets containing 100 or 1000 μL of CAR-01 had limited inhibitory effect against growth of mold on bread plugs, and sachets containing 25 μL of CIN-03 achieved a 2.5-log mold reduction, suggesting these treatments had fungistatic effects against growth of molds on the bread plugs. Additionally, sachets containing 50, 100 or 1000 μL of CIN-03 suppressed the

growth of mold completely and there was no recovery of spores, indicating that antimicrobial effects were fungicidal when the level of CIN-03 was 50 μL or higher in the controlled jar system.

In the plastic bag system, inoculated bread slices were treated with sachets containing CIN-03. Hundred μL treatment and 125 μL treatment delayed the fungal growth for 3 ± 1 days and 8 ± 1 days, respectively. There was no sign of mold growth on the bread slices treated with sachets containing 250, 500, or 1000 μL of CIN-03 throughout the storage period.

In the bread package system, sign of mold growth was observed on the bread loaves packaged without EO-containing sachets on day 3. Sachets containing 250, 500, or 1000 μL of CIN-03 were able to delay the growth of molds on inoculated bread loaves for 3 days.

In conclusion, this study identified CIN-03 had marked antifungal activity *in vitro* and *in situ*, compared to other EOs or pure active compounds. The sachet containing CIN-03 could inhibit the fungal growth and prolong the shelf life of bread products. In the literature, EOs (mustard oil, oregano oil, etc.) incorporated in active paper, edible films or combined with MAP were found to preserve bread products effectively. This project reported the first use of a plastic sachet containing cinnamon oil for preservation of bread products. Overall, these findings are beneficial to bakery industries and their customers. The application of sachet containing CIN-03 in bakery products should be investigated as an alternative to the addition of chemical preservatives and allow industries to have “clean” labels of their products, and also customers can have safe and natural foods to consume.

However, the EOs, as extracts from natural plants, have strong odor that may affect the organoleptic properties of the products. Therefore, further investigations are needed to determine the negative impact of EO-containing sachet on sensory properties of bread products. Also, lot-to-

lot variability the chemical composition of each EO may result in differences in their antimicrobial activity, thus the standardization of composition of EOs would be need for effective application of EO.

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