INVESTIGATION OF THE EFFECTIVENESS OF SELECTED ESSENTIAL OILS AND ENRICHED VARIANTS AS FUNCTIONAL INGREDIENTS, AND OF THEIR ENZYMATIC MODIFICATION

By

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ABSTRACT

The antioxidant capacity of 38 essential oils (EOs) and their enrichments were evaluated using two *in-vitro* methods: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the Oxygen Radical Absorption Capacity (ORAC) assays. Phenols were found to significantly contribute ($p < 0.05$) to the synergistic effects within EOs in both assays. Combining EOs with polyphenols (PPs) can lead to synergistic effects, decreasing the quantity required to extend shelf life. These effects were investigated using a multiple-method approach where 6 selected EOs were enriched with 8 individual PPs, 4 mixtures of major plant extract PPs, and 4 crude plant extracts. As the number of advantageous functional groups increased in individual PPs, the antioxidant capacity of their enrichments with EOs increased proportionally. As such, the phenolic acid that best improved the overall antioxidant capacities of EOs was rosmarinic acid, whereas *p*-coumaric acid improved them the least. Furthermore, the most effective flavonoid was quercetin, whereas rutin hydrate was the least effective. Enrichments with PP mixes and crude plant extracts showed synergistic and additive effects in the DPPH assay. In the ORAC assay, the latter enrichment showed an increase in additive effects and a decrease in antagonistic effects. These combinatorial effects could partly be explained by the possible formation of stable intermolecular complexes that subsequently enable the regeneration of certain antioxidants. These findings suggest that EOs interact with either the major compounds or both major and minor compounds of plant extracts.

The reaction capacities of 3 terpenes (eugenol, thymol, geraniol) with vinyl acetate (VA), vinyl propionate (VP), and commercial lipases from *Candida rugosa* and *Candida antarctica* (B fraction) (Novozym® 435) were studied. Phenolic terpenes did not participate in the transesterification reaction. Geraniol was successfully modified in toluene and solvent-free in lemon oil, with bioconversion yields as high as 100 and 88%, respectively. Modified and unmodified lemon oil emulsions exhibited the same cytotoxic behavior. Production of IL-8 was significantly ($p < 0.05$) increased in lipopolysaccharide (LPS)-stimulated U-937 cells but showed a significant ($p < 0.05$) decrease in the presence of unmodified and modified lemon oil emulsions, as well as geraniol emulsions. The anti-inflammatory activity of the oils was not dose-dependent and could potentially be attributed to a plateau in activity in either of the oils. The modification of oil did not affect the response to the anti-inflammatory activity test. These findings suggest that solvent-free modification of terpene alcohols in EOs is possible. This method could, therefore, be considered to modify EOs to be used in food products.

RÉSUMÉ

La capacité antioxydante de 38 huiles essentielles (EOs) et leurs enrichissements ont été évaluées en utilisant deux méthodes *in vitro* : les tests 2,2-diphényl-1-picrylhydrazyl (DPPH) et la capacité d'absorption des radicaux libres (ORAC). Il a été constaté que les phénols contribuent de manière significative ($p < 0,05$) aux effets synergiques au sein des EOs dans les analyses DPPH et ORAC. La combinaison des EOs avec les polyphénols (PP) peut également entraîner des effets synergiques, ce qui permet une diminution de la quantité nécessaire pour prolonger la durée de conservation. Ces effets ont été étudiés en utilisant une approche à méthodes multiples où 6 EOs sélectionnées ont été enrichies avec 8 PPs individuels, 4 mélanges de PPs majeures provenant d'extraits de plantes et 4 extraits de plantes brutes. À mesure que le nombre de groupes fonctionnels avantageux augmentait dans les PP individuelles, la capacité antioxydante de leurs enrichissements avec des EOs augmentait proportionnellement. Ainsi, l'acide phénolique qui améliorait le mieux les capacités antioxydantes globales des EOs était l'acide rosmarinique, tandis que l'acide *p*-coumarique les améliorait le moins. En outre, le flavonoïde le plus efficace était la quercétine, alors que l'hydrate de rutine a été le moins efficace. Les enrichissements avec des mélanges de PPs majeurs d'extraits de plantes, ainsi que des extraits de plantes bruts, ont montré des effets synergiques et additifs dans le test DPPH. Dans le test ORAC, ce dernier enrichissement a montré une augmentation des effets additifs et une diminution des effets antagonistes. Ces effets combinatoires pourraient être expliqués en partie par la formation possible de complexes intermoléculaires stables qui permettent ensuite la régénération de certains antioxydants. Ces résultats suggèrent que les effets combinatoires entre les EOs et les extraits de plantes peuvent être attribués aux interactions des EOs avec soit les composés majeurs ou les composés majeurs et mineurs des extraits de plantes.

Les capacités de réaction de 3 terpènes (eugénol, thymol, géraniol) avec des esters vinyliques (acétate de vinyle (VA), propionate de vinyle (VP)) et des lipases commerciales de *Candida rugosa* et *Candida antarctica* (fraction B) (Novozym® 435) ont été étudiées. On a constaté que les terpènes phénoliques n'avaient pas participé à la réaction de transestérification et n'étaient donc pas modifiés, ce qui pourrait être attribué à la position du groupe hydroxyle sur la fraction phényle. Le géraniol a été modifié avec succès dans du toluène et dans un milieu sans solvant en utilisant de l'huile de citron comme milieu réactionnel, avec des rendements de bioconversion atteignant respectivement 100 % et 88 %. Les émulsions d'huile de citron modifiée et non modifiée ont

présenté des activités cytotoxiques similaires. La production d'IL-8 a augmenté de manière significative ($p < 0,05$) dans les cellules U-937 stimulées par les lipopolysaccharides (LPS), mais a diminué de manière significative ($p < 0,05$) en présence d'émulsions d'huile de citron modifiée et non modifiée, ainsi que d'émulsions de géraniol. L'activité anti-inflammatoire des huiles n'était pas dose-dépendante et pourrait potentiellement être attribuée à un plateau d'activité dans les huiles. La modification de l'huile de citron n'a pas affecté la réponse au test d'activité anti-inflammatoire. Ces résultats suggèrent qu'une modification sans solvant des alcools terpéniques dans les EOs est possible. Cette méthode pourrait donc être considérée pour modifier les EOs destinés à être utilisés dans les produits alimentaires.

CONTRIBUTION OF AUTHORS

This thesis consists of the three following chapters:

Chapter I provides a comprehensive review of the literature on the biological, immunomodulatory, techno-functional properties of EOs. A review of the applications and modifications of EOs was also provided.

Chapter II assesses the antioxidant capacity of 38 EOs, as well as their enrichments with individual polyphenols, mixtures of polyphenols, and crude plant extracts. This chapter also explores the combinatorial effects between the selected EOs and their enrichments to understand the interactions between their components.

Chapter III investigates the lipase-catalyzed transesterification of selected terpenes with vinyl esters. This chapter also evaluates the cytotoxicity and anti-inflammatory properties of modified and unmodified lemon oil.

Connecting statements are also included to provide a succinct summary of each chapter and to introduce the next one.

Marina Minh Nguyen, the author, was responsible for the experimental work and the writing of the thesis.

Dr. Salwa Karboune, the MSc student's supervisor, guided all the research and critically revised the thesis prior to its submission.

Dr. Nastaran Khodaei analyzed and characterized the EOs by GC-MS.

Dr. Asma Mdimagh conducted statistical analyses and provided PCA graphs and heatmaps for Chapter II.

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LIST OF ABBREVIATIONS

AKT:	Protein kinase B
AMPK:	5' adenosine monophosphate-activated protein kinase
ANOVA:	Analysis of variance
BHA:	Butylated hydroxyanisole
BHT:	Butylated Hydroxytoluene
C4CAPP:	Cinnamon (HE-CAN-04) essential oil enriched with chlorogenic acid
C4CPP:	Cinnamon (HE-CAN-04) essential oil enriched with catechin
C4EPP:	Cinnamon (HE-CAN-04) essential oil enriched with epicatechin
C4FAPP:	Cinnamon (HE-CAN-04) essential oil enriched with ferulic acid
C4GT1CE:	Cinnamon (HE-CAN-04) essential oil enriched with crude green tea (EX-THE-01) extract
C4GT1PM:	Cinnamon (HE-CAN-04) essential oil enriched with green tea polyphenol mix
C4P4CE:	Cinnamon (HE-CAN-04) essential oil enriched with crude apple (EX-POM-04) extract
C4P4PM:	Cinnamon (HE-CAN-04) essential oil enriched with apple polyphenol mix
C4PCAPP:	Cinnamon (HE-CAN-04) essential oil enriched with *p*-coumaric acid
C4QPP:	Cinnamon (HE-CAN-04) essential oil enriched with quercetin
C4R4CE:	Cinnamon (HE-CAN-04) essential oil enriched with crude rosemary (EX-ROM-04) extract
C4R4PM:	Cinnamon (HE-CAN-04) essential oil enriched with rosemary polyphenol mix
C4RA1PM:	Cinnamon (HE-CAN-04) essential oil enriched with grape seed polyphenol mix
C4RA1CE:	Cinnamon (HE-CAN-04) essential oil enriched with crude grape seed (EX-RAI-01) extract
C4RAPP:	Cinnamon (HE-CAN-04) essential oil enriched with rosmarinic acid
C4RHPP:	Cinnamon (HE-CAN-04) essential oil enriched with rutin hydrate
C4X:	Cinnamon (HE-CAN-04) essential oil, no enrichment
CL1CAPP:	Clove (HE-CLO-01) essential oil enriched with chlorogenic acid
CL1CPP:	Clove (HE-CLO-01) essential oil enriched with catechin
CL1EPP:	Clove (HE-CLO-01) essential oil enriched with epicatechin
CL1FAPP:	Clove (HE-CLO-01) essential oil enriched with ferulic acid
CL1GT1CE:	Clove (HE-CLO-01) essential oil enriched with crude green tea (EX-THE-01) extract

CL1GT1PM:	Clove (HE-CLO-01) essential oil enriched with green tea polyphenol mix
CL1P4CE:	Clove (HE-CLO-01) essential oil enriched with crude apple (EX-POM-04) extract
CL1P4PM:	Clove (HE-CLO-01) essential oil enriched with apple polyphenol mix
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CL1R4PM:	Clove (HE-CLO-01) essential oil enriched with rosemary polyphenol mix
CL1RA1CE:	Clove (HE-CLO-01) essential oil enriched with crude grape seed (EX-RAI-01) extract
CL1RA1PM:	Clove (HE-CLO-01) essential oil enriched with grape seed polyphenol mix
CL1RAPP:	Clove (HE-CLO-01) essential oil enriched with rosmarinic acid
CL1RHPP:	Clove (HE-CLO-01) essential oil enriched with rutin hydrate
CL1X:	Clove (HE-CLO-01) essential oil, no enrichment
EO:	Essential oil
GC-MS:	Gas Chromatography-Mass Spectrometry
h:	Hours
DPPH:	2,2-diphenyl-1-picrylhydrazyl
ERK:	Extracellular-signal-regulated kinase
HAT:	Hydrogen Atom Transfer
IL:	Interleukin
JNK:	c-Jun N-terminal kinase
L:	Unmodified lemon oil
LG:	Unmodified lemon oil enriched with 5% geraniol
LGVAL:	Lemon oil enriched with 5% geraniol, modified with vinyl acetate. Low bioconversion (~45%).
LGVAH:	Lemon oil enriched with 5% geraniol, modified with vinyl acetate. High bioconversion (~90%).
LGVPL:	Lemon oil enriched with 5% geraniol, modified with vinyl propionate. Low bioconversion (~45%).
LGVPH:	Lemon oil enriched with 5% geraniol, modified with vinyl propionate. High bioconversion (~90%).
LPS:	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

NF-κB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
O3CAPP:	Oregano (HE-ORI-03) essential oil enriched with chlorogenic acid
O3CPP:	Oregano (HE-ORI-03) essential oil enriched with catechin
O3EPP:	Oregano (HE-ORI-03) essential oil enriched with epicatechin
O3FAPP:	Oregano (HE-ORI-03) essential oil enriched with ferulic acid
O3GT1CE:	Oregano (HE-ORI-03) essential oil enriched with crude green tea (EX-THE-01) extract
O3GT1PM:	Oregano (HE-ORI-03) essential oil enriched with green tea polyphenol mix
O3P4CE:	Oregano (HE-ORI-03) essential oil enriched with crude apple (EX-POM-04) extract
O3P4PM:	Oregano (HE-ORI-03) essential oil enriched with apple polyphenol mix
O3PCAPP:	Oregano (HE-ORI-03) essential oil enriched with *p*-coumaric acid
O3QPP:	Oregano (HE-ORI-03) essential oil enriched with quercetin
O3R4CE:	Oregano (HE-ORI-03) essential oil enriched with crude rosemary (EX-ROM-04) extract
O3R4PM:	Oregano (HE-ORI-03) essential oil enriched with rosemary polyphenol mix
O3RA1CE:	Oregano (HE-ORI-03) essential oil enriched with
O3RA1PM:	Oregano (HE-ORI-03) essential oil enriched with grape seed polyphenol mix
O3RAPP:	Oregano (HE-ORI-03) essential oil enriched with rosmarinic acid
O3RHPP:	Oregano (HE-ORI-03) essential oil enriched with rutin hydrate
O3X:	Oregano (HE-ORI-03) essential oil, no enrichment
ORAC:	Oxygen radical absorbance capacity
P1CAPP:	Pimento Berry (HE-PIM-01) essential oil enriched with chlorogenic acid
P1CPP:	Pimento Berry (HE-PIM-01) essential oil enriched with catechin
P1EPP:	Pimento Berry (HE-PIM-01) essential oil enriched with epicatechin
P1FAPP:	Pimento Berry (HE-PIM-01) essential oil enriched with ferulic acid
P1GT1CE:	Pimento Berry (HE-PIM-01) essential oil enriched with crude green tea (EX-THE-01) extract
P1GT1PM:	Pimento Berry (HE-PIM-01) essential oil enriched with green tea polyphenol mix
P1P4CE:	Pimento Berry (HE-PIM-01) essential oil enriched with crude apple (EX-POM-04) extract
P1P4PM:	Pimento Berry (HE-PIM-01) essential oil enriched with apple polyphenol mix
P1PCAPP:	Pimento Berry (HE-PIM-01) essential oil enriched with *p*-coumaric acid

P1QPP:	Pimento Berry (HE-PIM-01) essential oil enriched with quercetin
P1R4CE:	Pimento Berry (HE-PIM-01) essential oil enriched with crude rosemary (EX-ROM-04) extract
P1R4PM:	Pimento Berry (HE-PIM-01) essential oil enriched with rosemary polyphenol mix
P1RA1CE:	Pimento Berry (HE-PIM-01) essential oil enriched with crude grape seed (EX-RAI-01) extract
P1RA1PM:	Pimento Berry (HE-PIM-01) essential oil enriched with grape seed polyphenol mix
P1RAPP:	Pimento Berry (HE-PIM-01) essential oil enriched with rosmarinic acid
P1RHPP:	Pimento Berry (HE-PIM-01) essential oil enriched with rutin hydrate
P1X:	Pimento Berry (HE-PIM-01) essential oil, no enrichment
PCA:	Principal Component Analysis
PMA:	Phorbol 12-myristate 13-acetate
PP:	Polyphenol
rpm:	Revolutions per minute
S12CAPP:	Yellow Sage (HE-SAU-01-02) essential oil enriched with chlorogenic acid
S12CPP:	Yellow Sage (HE-SAU-01-02) essential oil enriched with catechin
S12EPP:	Yellow Sage (HE-SAU-01-02) essential oil enriched with epicatechin
S12FAPP:	Yellow Sage (HE-SAU-01-02) essential oil enriched with ferulic acid
S12GT1CE:	Yellow Sage (HE-SAU-01-02) essential oil enriched with crude green tea (EX-THE-01) extract
S12GT1PM:	Yellow Sage (HE-SAU-01-02) essential oil enriched with green tea polyphenol mix
S12P4CE:	Yellow Sage (HE-SAU-01-02) essential oil enriched with crude apple (EX-POM-04) extract
S12P4PM:	Yellow Sage (HE-SAU-01-02) essential oil enriched with apple polyphenol mix
S12PCAPP:	Yellow Sage (HE-SAU-01-02) essential oil enriched with *p*-coumaric acid
S12QPP:	Yellow Sage (HE-SAU-01-02) essential oil enriched with quercetin
S12R4CE:	Yellow Sage (HE-SAU-01-02) essential oil enriched with crude rosemary (EX-ROM-04) extract
S12R4PM:	Yellow Sage (HE-SAU-01-02) essential oil enriched with rosemary polyphenol mix
S12RA1CE:	Yellow Sage (HE-SAU-01-02) essential oil enriched with crude grape seed (EX-RAI-01) extract

S12RA1PM: Yellow Sage (HE-SAU-01-02) essential oil enriched with grape seed polyphenol mix

S12RAPP: Yellow Sage (HE-SAU-01-02) essential oil enriched with rosmarinic acid

S12RHPP: Yellow Sage (HE-SAU-01-02) essential oil enriched with rutin hydrate

S12X: Yellow Sage (HE-SAU-01-02) essential oil enriched, no enrichment

SET: Single electron transfer

T3CAPP: White thyme (HE-THY-03) essential oil enriched with chlorogenic acid

T3CPP: White thyme (HE-THY-03) essential oil enriched with catechin

T3EPP: White thyme (HE-THY-03) essential oil enriched with epicatechin

T3FAPP: White thyme (HE-THY-03) essential oil enriched with ferulic acid

T3GT1CE: White thyme (HE-THY-03) essential oil enriched with crude green tea (EX-THE-01) extract

T3GT1PM: White thyme (HE-THY-03) essential oil enriched with green tea polyphenol mix

T3P4CE: White thyme (HE-THY-03) essential oil enriched with crude apple (EX-POM-04) extract

T3P4PM: White thyme (HE-THY-03) essential oil enriched with apple polyphenol mix

T3PCAPP: White thyme (HE-THY-03) essential oil enriched with *p*-coumaric acid

T3QPP: White thyme (HE-THY-03) essential oil enriched with quercetin

T3R4CE: White thyme (HE-THY-03) essential oil enriched with crude rosemary (EX-ROM-04) extract

T3R4PM: White thyme (HE-THY-03) essential oil enriched with rosemary polyphenol mix

T3RA1CE: White thyme (HE-THY-03) essential oil enriched with crude grape seed (EX-RAI-01) extract

T3RA1PM: White thyme (HE-THY-03) essential oil enriched with grape seed polyphenol mix

T3RAPP: White thyme (HE-THY-03) essential oil enriched with rosmarinic acid

T3RHPP: White thyme (HE-THY-03) essential oil enriched with rutin hydrate

T3X: White thyme (HE-THY-03) essential oil, no enrichment

TBHQ: tert-Butylhydroquinone

TNF-α: Tumor necrosis factor alpha

VA: Vinyl acetate

VP: Vinyl propionate

XCAPP: Chlorogenic acid, no enrichment

XCPP: Catechin, no enrichment

XEPP: Epicatechin, no enrichment

XFAPP: Ferulic acid, no enrichment

XGT1CE: Crude green tea (EX-THE-01) extract, no enrichment

XGT1PM: Green tea polyphenol mix, no enrichment

XP4CE: Crude apple (EX-POM-04) extract, no enrichment

XP4PM: Apple polyphenol mix, no enrichment

XPCAPP: *p*-coumaric acid, no enrichment

XQPP: Quercetin, no enrichment

XR4CE: Crude rosemary (EX-ROM-04) extract, no enrichment

XR4PM: Rosemary polyphenol mix, no enrichment

XRA1CE: Crude grape seed (EX-RAI-01) extract, no enrichment

XRA1PM: Grape seed polyphenol mix, no enrichment

XRAPP: Rosmarinic acid, no enrichment

XRHPP: Rutin hydrate, no enrichment

INTRODUCTION

Food companies have previously turned to synthetic antioxidants to prevent the degradation of quality and functional value in products containing lipids due to their vulnerability to oxidation and microbial or enzymatic autolysis. However, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (THBQ) and propyl gallate (PG) are suspected to have carcinogenic, mutagenic, and teratogenic effects after chronic use (Y. Li, Fabiano-Tixier, & Chemat, 2014). With increasing concerns regarding the safety of these preservatives as well as increasing consumer demands for more natural products, the industry is now considering essential oils (EOs) as potential natural replacements.

EOs are aromatic, volatile liquids obtained from plant materials (Ríos, 2016). Their name is derived from the word "essence" since they carry the distinctive scent of the plant material from which they are extracted (Attokaran, 2011). The International Organization for Standardization (ISO) has defined EOs as "product obtained from a natural raw material of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase - if any - by physical processes" (ISO:9235, 2013). Produced as secondary metabolites by aromatic plants, EOs have long been used for their medicinal and antiseptic properties as well as their fragrances (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). In addition to their antimicrobial, antiviral, and antifungal properties, they play an important role in protecting aromatic plants from herbivores and in promoting the dispersion of pollen and seeds (Bakkali, Averbeck, Averbeck, 2008).

EOs are generally found in temperate to warm climates, where distillation is the primary method of extraction for these secondary metabolites (Bakkali, Averbeck, Averbeck, 2008). These oils can be enzymatically synthesized in any plant organ, including bark, roots, twigs, leaves, flowers, buds, seeds, stems, or wood (Nazzaro et al., 2013). The enzymes present in these organs will dictate the composition of the EO, therefore knowing the composition of an oil can give information regarding the genetic makeup of the plant from which it originated (Baser & Buchbauer, 2010). Though genetic factors can influence the makeup of EOs, environmental factors, storage conditions, and extraction methods will inevitably affect the composition of EOs (Reyes-Jurado, Franco-Vega, Ramírez-Corona, Palou, & López-Malo, 2015). *Asteraceae* (or *Compositae)*,

Lamiaceae (or *Labiateae)* and *Apiaceae* (or *Umbelliferae)* are the most common plant families from which EOs are extracted (Christaki, Bonos, Giannenas, & Florou-Paneri, 2012).

Due to the intense aroma of EOs, they impart negative organoleptic effects in food products when used in concentrations exceeding an acceptable consumer threshold (Hyldgaard, Mygind, Meyer, & Knapp, 2012). One strategy to circumvent these effects in food products is to modify the chemical profiles of EOs. This can be done through enzymatic pathways such as lipase-catalyzed transesterification. This area of research is relatively new and few studies have investigated the modification of EOs (Antoniotti, 2014).

The overall objective of the study was to investigate the interactions between EOs and plant extracts, as well as modify their chemical profiles to increase their versatility in food products. This was achieved by the following specific objectives:

(1) Investigate the antioxidant capacity of EOs and their enrichments with individual polyphenols, mixtures of polyphenols, and crude extracts.
(2) Develop an enzymatic process based on lipase-catalyzed transesterification to modify selected EOs and investigate the new functional properties.

CHAPTER I.

LITERATURE REVIEW

1.1. Brief Overview

1.1.1. Structures and Chemical Composition

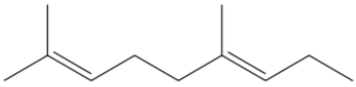
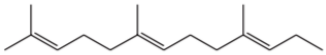
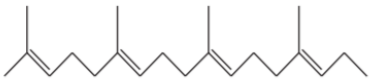
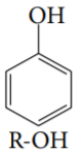
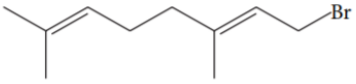
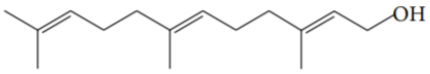
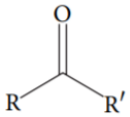
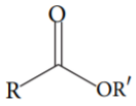
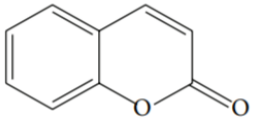
According to Bakkali *et al*. (2008), EOs contain around 20-60 compounds, where two or three of those compounds are present in high concentrations (20-70%). These major constituents are generally terpenes/terpenoids such as monoterpenes (C10) and sesquiterpenes (C15) (Nazzaro et al., 2013). Other major constituents also include aromatic and aliphatic compounds (Bakkali, Averbeck, Averbeck, 2008). As for compounds found in trace amounts, these include acids, aldehydes, alcohols, aliphatic hydrocarbons, acyclic esters or lactones. A general overview of these constituents can be found in **Table 1.1**.

Combinations of isoprene units (C_5H_8) make up hydrocarbons called terpenes (Nazzaro et al., 2013). A combination of two isoprene units will yield a monoterpene, whereas a combination of three isoprene units will yield a sesquiterpene (Bakkali et al., 2008). Diterpenes, formed with four isoprene units, can also be found in EOs, though not as frequently as monoterpenes or sesquiterpenes. Sesquiterpenes are less volatile than monoterpenes and therefore have higher boiling points (Sell, 2007). As such, they are not as potent as their monoterpene counterparts in the aromatic profile of the EO. However, sesquiterpenes that do contribute to the aroma of the EO have low-odor thresholds and are very significant as end notes. The most well-known terpenes present in EOs include sabinene, pinene, limonene, and p-Cymene (Nazzaro et al., 2013). According to Nazzaro *et al*. (2013), most terpenes have very low antimicrobial activity, especially against gram-negative pathogens.

Terpenoids, on the other hand, do show antimicrobial activity. These compounds differ from terpenes in that they have extra oxygen molecules or have methyl groups that have been enzymatically moved or removed (Nazzaro et al., 2013). These oxygenated derivatives exist for both monoterpenes and sesquiterpenes as alcohols, aldehydes, ketones, phenols, esters, oxides, acids, lactones, and coumarins (Attokaran, 2011). The most common terpenoids in EOs include thymol, carvacrol, menthol, linalool, geraniol, linalyl acetate, piperitone, and citronellal (Nazzaro et al., 2013). The most effective terpenoids are carvacrol and thymol, which are major constituents of oregano and thyme oil respectively (Hyldgaard et al., 2012). The effectiveness of the terpenoids' antimicrobial activity is influenced by type and location of functional groups as well as the presence of delocalized electrons.

Occurring in trace amounts, phenylpropenes can be present in some EOs. These compounds contain an aromatic phenol group (6C) and a propene tail (3C) (Nazzaro et al., 2013). Phenylpropenes are principally found in cinnamon, clove, parsley, fennel, nutmeg, tarragon, star anise, and some botanical families such as *Apiaceae*, *Lamiaceae*, *Myrtaceae*, and *Rutaceae* (Bakkali et al., 2008). The most widely studied phenylpropenes are cinnamaldehyde, eugenol, isoeugenol, vanillin and safrole according to Nazzaro and colleagues (2013).

Table 1.1. Chemical classification, molecular formula, structures, and examples of EO constituents. Source: Gautam, Mantha, & Mittal (2014)

Component	General formula	General structure	Example
		Terpene hydrocarbons	
Monoterpene	$C_{10}H_{16}$		Limonene, α-Pinene, β-Myrcene
Sesquiterpenes	$C_{15}H_{24}$		Caryophyllene, Humulene, α-Farnesene
Diterpene	$C_{20}H_{32}$		Cembrene C, Kaurene, Camphorene
		Oxygenated terpenes	
Oxygenated monoterpene	$C_{10}H_{16}O$		Camphor, Carveol, Limonene oxide
Oxygenated Sesquiterpenes	$C_{15}H_{24}O$		Caryophyllene oxide, Humulene epoxide, α-Bisabolene oxide
		Other oxygenated compounds	
Phenols	RC_6H_5OH		Catechol, Eugenol
Simple alcohols	R-OH	R-OH	Isopropyl alcohol, Butyl alcohol
Monoterpene alcohols	$C_{10}H_{17}OH$		Geraniol, Nerol, Eucalyptol
Sesquiterpenes alcohols	$C_{15}H_{25}OH$		Farnesol, Nerolidol
Ketones	$RC(=O)R'$		Acetophenone, Benzophenone
Esters	RCO_2R'		Bornyl acetate, Ethyl acetate
Lactones and coumarins	$C_3H_6O_3$ (Lactones)		Vernolide, Helenin
	$C_9H_6O_2$ (Coumarins)		Fumarin, Benzofuran

In cases where distillation was not the choice method of extraction, some non-volatile compounds can be found in the EOs (Ríos, 2016). These compounds are, in most cases, precursors such as sesquiterpene lactones or glycosides that would have hydrolyzed or transformed into volatile compounds during the distillation process. In the absence of this method, Ríos (2016) explains that the alternative extraction process would leave these volatile compounds in their original form in the extract.

Other external factors, such as plant organ and geographic variation, can affect the chemical makeup and yield of the oils (Figueiredo, Barroso, Pedro, & Scheffer, 2008; Santos-Gomes & Fernandes-Ferreira, 2001). Seasonal changes also affect the composition of EOs due to changes in temperature, humidity, and variations in plant metabolism (Hussain, Anwar, Hussain Sherazi, & Przybylski, 2008). To ensure some uniformity in the composition, the oils must be extracted in practically identical conditions and plant organ (Bakkali et al., 2008).

1.1.2. Sources of EOs

All plants are capable of producing volatile compounds, but some only do so in trace amounts. The following two scenarios represent circumstances where a plant will be used as a source of EOs (Franz & Novak, 2010):

1) The presence of a unique blend of volatiles such as those extracted from rose (*Rosa* spp.), jasmine (*Jasminum sambac*), or tuberose (*Polyanthes tuberosa*). It is important to note that once these volatiles are produced, they are immediately released by the plant through the epidermal layers of their petals. Therefore, the yield, though intense in aroma, is very low.
2) The secretion and accumulation of aromatic volatiles in specialized anatomical storage structures in the plant. These areas of storage can be secretory idioblasts, cavities/ducts, or glandular trichomes (Nazzaro et al., 2013). In this scenario, higher concentrations of EOs are available for extraction.

Plants that fit these criteria are either collected in nature ("wild collection") or are cultivated (Franz & Novak, 2010). **Table 1.2** illustrates important EO-bearing plants that are cultivated.

Table 1.2. Overview of some oil-bearing plants. Adapted from (Franz & Novak, 2010).

Trade Name	Species	Plant Family	Used Plant Part(s)
Basil	*Ocimum basilicum L.*	Lamiaceae	Herb
Cedarwood, Chinese	*Cupressus funebris* Endl.	Cupressaceae	Wood
Cedarwood, Texas	*Juniperus mexicana* Schiede	Cupressaceae	Wood
Cedarwood, Virginia	*Juniperus virginiana* L.	Cupressaceae	Wood
Celery seed	*Apium graveolens* L.	Apiaceae	Seed
Cinnamon bark, Ceylon	*Cinnamomum zeylanicum* Nees	Lauraceae	Bark
Cinnamon bark, Chinese	*Cinnamomum cassia* Blume	Lauraceae	Bark
Cinnamon leaf	*Cinnamomum zeylanicum* Nees	Lauraceae	Leaf
Clove buds	*Szygium aromaticum* (L.)	Myrtaceae	Leaf/bud
Clove leaf	*Szygium aromaticum* (L.)	Myrtaceae	Leaf
Coriander	*Coriandrum sativum* L.	Apiaceae	Fruit
Cumin	*Cuminum cyminum* L.	Apiaceae	Fruit
Cypress	*Cupressus sempervirens* L.	Cupressaceae	Leaf/twig
Dill	*Anethum graveolens* L.	Apiaceae	Herb/fruit
Garlic	*Allium sativum* L.	Alliaceae	Bulb
Ginger	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome
Lemon	*Citrus limon* (L.)	Rutaceae	Fruit peel
Lemongrass, Indian	*Cymbopogon flexuosus*	Poaceae	Leaf
Lemongrass, West Indian	*Cymbopogon citratus* (DC.)	Poaceae	Leaf
Marjoram	*Origanum majorana* L.	Lamiaceae	Herb
Nutmeg	*Myristica fragrans* Houtt.	Myristicaceae	Seed
Onion	*Allium cepa* L.	Alliaceae	Bulb
Oregano	*Origanum* spp.	Lamiaceae	Herb
Palmarosa	*Cymbopogon martinii* (Roxb.)	Poaceae	Leaf
Pepper	*Piper nigrum* L.	Piperaceae	Fruit
Pimento leaf	*Pimenta dioica (L.) Merr.*	Myrtaceae	Fruit
Pine white	*Pinus palustris Mill.*	Pinaceae	Leaf/twig
Rosemary	*Rosmarinus officinalis L.*	Lamiaceae	Leaf
Sage, Dalmatian	*Salvia officinalis* L.	Lamiaceae	Herb
Sage, Spanish	*Salvia lavandulifolia* L.	Lamiaceae	Leaf
Sage, three lobed (Greek. Turkish)	*Salvia fruticosa* Mill., *S. triloba* L.	Lamiaceae	Herb
Tarragon	*Artemisia dracunculus* L.	Asteraceae	Herb
Thyme	*Thymus vulgaris* L., *T. zygis* Loefl.	Lamiaceae	Herb
Ylang Ylang	*Cananga odorata*	Annonaceae	Flower

1.2. Biological Properties of EOs

1.2.1. Antioxidant Properties

The process of degradation is initiated by a radical species that can react with a substrate RH, resulting in the production of an alkyl radical R• (Amorati, Foti, & Valgimigli, 2013). This radical can then go on to react with oxygen to form a peroxyl radical ROO• which will react with another molecule to create another radical along with a hydroperoxide ROOH. This reaction will continue until two radical species react together to quench each other and subsequently terminate the chain reaction. Antioxidants that impair this chain reaction are referred to as *direct antioxidants*, which can be broken down into two groups: *preventive antioxidants* and *chain-breaking antioxidants*. The former, as its name implies, prevents the formation of radical species by interfering with the initiation process. The latter group reacts with peroxyl radicals, forming compounds that do not contribute to the propagation step. In other words, the chain-breaking antioxidants react with the radicals more rapidly than the oxidizable substrate. Comparing the two groups, the chain-breaking antioxidants are significantly more important than the preventive antioxidants since the latter is useless when radical species have already formed in the food product. (Amorati et al., 2013)

The antioxidant capacity of EOs cannot be fully judged using one test method; the evaluation of their antioxidant capacity must consider the results from a variety of methods. These methods include, but are not restricted to, the 2,2'-diphenyl-picrylhydrazyl (DPPH) radical scavenging method, and the oxygen radical absorbance capacity (ORAC) assay (Antolovich, Prenzler, Patsalides, Mcdonald, & Robards, 2001; T Kulisic, Radonic, Katalinic, & Milos, 2004). These assays can be classified as single electron transfer (SET) reaction assays, hydrogen atom transfer (HAT) assays or both (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009).

The DPPH assay is a spectrophotometric technique based on quenching a stable colored radical (DPPH•), thus bleaching of the radical occurs in the presence of an antioxidant at 515 nm (Schaich, Tian, & Xie, 2015). The DPPH assay can be classified as both a SET and HAT assay. However, when using strong hydrogen-bonding solvents such as methanol, intermolecular hydrogen-bonding slows the transfer of hydrogen atoms, thus favoring SET over HAT (Barclay, Edwards, & Vinqvist, 1999). This assay does not measure reaction rates. Results are generally reported as an IC_{50} value, which is the concentration at which 50% of DPPH radical is scavenged by the antioxidant.

The oxygen radical absorbance capacity (ORAC) assay is a HAT-based method that measures the quenching of a fluorescent molecule, commonly fluorescein, in the presence a radical generator, such as 2,2′-azobis-2-amidinopropane (AAPH) (Schaich et al., 2015). More specifically, by heating AAPH, its azide decomposes, eliminating nitrogen gas, and leaves behind two carbon-centered radicals (R•). These radicals are then converted to reactive peroxyl radicals (ROO•) in the presence of oxygen, which then go on to quench fluorescein or react with antioxidants. This assay determines an antioxidant's capacity by its ability to delay the loss of fluorescence by reacting with the peroxyl radicals.

The ability to stop or delay the aerobic oxidation of organic matter lies in the action of certain components of the EOs (Amorati et al., 2013). Specifically, phenols present in the oils are major players in their antioxidant activity due to their high reactivity with peroxyl radicals. Following the reaction illustrated in **equation 1**, the resulting stable phenoxyl radical will react with a second peroxyl radical and quench it (**Equation 2**).

$$PhOH + ROO\bullet \rightarrow PhO\bullet + ROOH \qquad (1)$$

$$PhO\bullet + ROO\bullet \rightarrow \text{nonradical products} \qquad (2)$$

Though phenols contribute greatly to the antioxidant activity of EOs, oils lacking in phenols do not necessarily exhibit zero antioxidant activity at all. These phenol-free oils gain their antioxidant properties from the radical chemistry of some terpenoids and other volatile constituents according to Amorati *et al*. (2013). These components autoxidize like unsaturated lipids and therefore propagate the oxidative chain due to the formation of a peroxyl radical. In other words, when the antioxidant is mixed with the substrate to be protected, both will co-oxidize. This mechanism is characterized by a fast termination process and occurs with terpenoids that have a cyclohexadiene structure such as γ-terpinene and α-phellandrene. The fast termination process is advantageous since this reduces the overall rate of oxidation, therefore these components behave as antioxidants despite propagating the oxidative chain. (Amorati et al., 2013)

The antioxidant activity of oregano is high enough to suggest that its activity is comparable to that of α-tocopherol as well as BHT (T Kulisic et al., 2004). The authors of the study noticed that the CHO fraction of the oil showed the highest antioxidant activity and hypothesized that this was possibly due to minor synergy between the oxygenated compounds. Other EOs with high antioxidant capacity include ylang ylang (*Cananga odorata*), lemongrass (*Cymbopogon citratus*),

rosemary, and thyme (Sacchetti et al., 2005). In another study, researchers found that caraway (*Carum corui*) oil had a higher antioxidant activity than sage (*Salvia officinalis*), which had a higher activity than cumin, followed by rosemary, thyme, and finally, clove (Farag, Badei, Hewedi, & El-Baroty, 1989). To explain the differences in activity, Farag *et al.* (1989) highlighted the importance of an electron repelling group located in the *ortho*-position in the phenolic ring. They also noted that increasing the concentration of the EOs resulted in an increase in their antioxidant activity. In contrast to these findings, another study found that clove oil had the highest antioxidant activity among other EOs tested (Viuda-Martos, Ruiz Navajas, Sanchez Zapata, Fernandez-Lopez, & Perez-Alvarez, 2010). They attributed its high activity to the fact that it contained the highest levels of phenolic compounds among the oils tested. Therefore, positioning on phenolic moieties, as well as concentration of EO influences antioxidant activity.

In butter, thyme and cumin EOs have been able to inhibit lipolytic activity better than BHT (Farag, Ali, & Taha, 1990). The phenolic OH groups in thymol increased the activity of the thyme oil, making it more effective than cumin oil. The researchers suggested that BHT could be replaced with thyme and cumin oil, as these would be capable of prolonging the shelf life of butter. They justified this claim by explaining that the primary cause of butter's spoilage is hydrolytic rancidity rather than oxidative rancidity, therefore these oils' higher anti-hydrolytic activities were suitable for such a task.

1.2.2. Antibacterial Properties

Gram-positive bacteria are less resistant to EOs than gram-negative bacteria due to differences in their cell walls (Nazzaro et al., 2013). Nazzaro *et al*. (2013) explain that those of gram-positive bacteria allow hydrophobic molecules to penetrate more easily than those of gram-negative bacteria since the latter have an outer membrane linked to their peptidoglycan layer by Braun's lipoprotein, differentiating them from their gram-positive counterparts. This outer membrane contains lipopolysaccharides (LPS) which consist of lipid A and an O-side chain. The latter constituent is responsible for the increased resistance to EOs. The LPS layer in gram-negative bacteria will therefore limit the diffusion rate of hydrophobic molecules into the cells (Tongnuanchan & Benjakul, 2014). EOs must, therefore, use alternative methods to enter gram-negative cells if they cannot do so freely.

Once the phenolic compounds of the EOs have entered the cell, they can disrupt the enzymes involved in the production of energy at low concentrations. At high concentrations of phenolic compounds, proteins in the gram-positive bacteria's cells will be denatured.

Oils with the highest antibacterial activity have aldehydes or phenols as major components, followed by oils containing terpene alcohols (Bassolé & Juliani, 2012). It is important to note that antimicrobial activity *in vitro* is not a true reflection of the oils' activity in actual food products due to interactions with the food matrix (Hyldgaard et al., 2012). Antibacterial activity can be increased by manipulating the ingredient composition of the food product—EOs in proteinaceous foods and/or foods with low pH values have shown to exhibit a higher antibacterial activity (Gutierrez, Barry-Ryan, & Bourke, 2008).

1.2.2.1. Mode of Action

The mode of action of EOs is dependent on their chemical composition and the structure of these constituents (Nazzaro et al., 2013). For example, compounds such as carvacrol, eugenol, and thymol have antibacterial properties thanks to their phenolic ring. Dorman and Deans (2000) confirmed that the phenolic ring is responsible for their antibacterial activity by comparing thymol and carvacrol to *p*-cymene, a cyclic monoterpene hydrocarbon which was found to have lacked activity. Furthermore, when comparing the activities of carvacrol with its methyl ether form, it was apparent that the former's hydroxyl group in its phenolic structure was responsible for its greater antimicrobial activity (Saad, Muller, & Lobstein, 2013). Additionally, after comparing the activity of the isomers thymol and carvacrol, it was also confirmed that the relative position of the hydroxyl group influences the terpenes' activity against gram-positive and gram-negative bacteria (Dorman & Deans, 2000). Dorman and Deans (2000) also note that alkyl substitution in the phenol nucleus tends to enhance the antimicrobial activity of EOs. They explain that this alters the distribution ratio of aqueous and non-aqueous phases, thus reducing the surface tension or altering the species selectivity.

The constituents of the EOs may have several targets or a single target when working against bacteria. Their activity can affect both the cell membrane (usually the first target) and the cytoplasm (Nazzaro et al., 2013). When the former is disrupted by the hydrophobicity of the EOs, there is an increase in permeability since the membrane is incapable of separating from the oils. The permeability is also affected by the disruption of the proton motive force—both the pH

gradient and the electrical potential are dissipated by the EOs (Sikkema, De Bont, Poolman, & Wageningen, 1995). The outer membrane can also be disintegrated, causing it to release material from the cells to the external environment, which would then lead to cell death (S. Burt, 2004; Helander et al., 1998). Helander and colleagues (1998) have found that carvacrol and thymol were capable of such action, hypothesizing that the phenolic nature of the compounds was responsible for this activity. In their study, they explained that this was possible due to the fact that phenolics make it easier for the oils to insert themselves into the phospholipid bilayer. Another study also confirmed that carvacrol and thymol increased the permeability of the bacterial cell membrane (Burt & Reinders, 2003). Interestingly, though carvacrol can disintegrate the outer membrane of gram negative bacteria (which then releases LPS), its main target is thought to be the cytoplasmic membrane (Hyldgaard et al., 2012). In affecting it, carvacrol is enabling the passive transport of ions across the membrane.

Phenolic compounds are also capable of disrupting active transport as well as coagulating the material inside the cell (Burt, 2004). Helander *et al*. (1998) found that *trans*-cinnamaldehyde significantly inhibited enterobacterial growth without disintegrating the outer membrane. With these findings, they concluded that *trans*-cinnamaldehyde was able to access the periplasm as well as deeper parts of the cell thanks to the outer membrane-traversing porin proteins.

Another accepted view is that the concentration of active constituents in the EOs influences the mode of action, where low concentrations would affect enzymes associated with energy production and high concentrations would precipitate bacterial proteins (Tassou, Chorianopoulos, Skandamis, & Nychas, 2012). By acting on the proteins in bacteria, the EO components are able to affect cell division (Nazzaro et al., 2013). For example, cinnamaldehyde can inhibit cell separation in *B. cereus* by decreasing the assembly reaction and bundling of FtsZ, a prokaryotic homolog of tubulin. Thymol, on the other hand, can cause stress to the bacterial envelope by the up-regulation of certain proteins. Additionally, according to Nazzaro *et al.* (2013), thymol will also cause the accumulation of misfolded outer membrane proteins, contributing to the aforementioned stress. By interacting with the membrane proteins and intracellular material, thymol also hinders cell recovery after temporary exposure to it (Hyldgaard et al., 2012).

Tea tree (*Melaleuca alternifolia*) oil's mode of action has been studied against both gram-negative and gram-positive bacteria (Cox et al., 2001). It was found that at minimum inhibitory

concentrations, tea tree oil was able to disrupt bacterial membranes, thereby increasing membrane permeability and causing potassium ion leakage. Additionally, cell respiration was also inhibited at minimum inhibitory concentrations. The researchers concluded that these activities are likely the cause of cell death.

1.2.2.2. Synergy

Blends of EOs can have an additive, synergistic, antagonistic, or indifferent effect. In an additive effect, the sum of the individual components will be equal to the combined activity of the blend of oils (Hyldgaard et al., 2012). When the combined effect of the blend of oils is greater than the sum of their individual components, the effects are synergistic. When the activity of the sum of the components is equal to the combined effect of the blend, the effect is considered to be indifferent (Gunnison, Kunishige, Coleman, & Jawetz, 1955). When antagonism occurs, the combined effect is less than the sum of their individual components.

According to Hyldgaard *et al*. (2012), to assess the type of combined effect, the fractional inhibition concentration index (FIC_{Index}) is calculated using measurements of the minimum inhibition concentration (MIC). The equations are as follows:

$$FIC_A = MIC_{A+B}/MIC_A \quad (3)$$

$$FIC_B = MIC_{B+A}/MIC_B \quad (4)$$

$$FIC_{Index} = FIC_A + FIC_B \quad (5)$$

Where MIC_{A+B} is the MIC of A in the presence of B and vice versa for MIC_{B+A}. When $FIC_{Index} < 0.5$, the effect is synergistic. When $0.5 < FIC_{Index} < 1$, the effect is additive and when $1 < FIC_{Index} < 4$, the effect is indifferent. Lastly, when $FIC_{Index} > 4$, the effect is antagonistic (Odds, 2003).

In one study, no synergistic effects were observed when blends of lemon balm (*M. officinalis*), marjoram (*O. majorana*), oregano (*O. vulgare*), and thyme (*T. vulgaris*) were used against *Enterobacter* spp., *Listeria* spp., *Lactobacillus* spp., and *Pseudomonas* spp. (Gutierrez, Barry-Ryan, & Bourke, 2009). They did find, however, that combinations of oregano with thyme or lemon balm showed an additive effect against *L. monocytogenes*. A blend of thyme and lemon balm also had an additive effect against *L. innocua*. The only combination that showed an additive effect against both spoilage bacteria was that of oregano and thyme. Another study assessing combinations of basil (*O. basilicum*), lemon balm, marjoram, oregano, rosemary (*R. officinalis*),

sage (*S. triloba*), and thyme also did not find any synergistic effects, but rather, additive effects (Gutierrez et al., 2008).

In the studies summarized in **Table 1.3**, combinations of phenolic monoterpenes (thymol, carvacrol) and phenylpropanoids (eugenol) with other components increased the bioactivities of these mixtures (Bassolé & Juliani, 2012). Synergistic effects were observed against microorganisms when combinations of phenolics and monoterpene alcohols were used. More specifically, combinations of thymol, carvacrol, and eugenol were synergistically active against *E. coli* strains. Cinnamaldehyde with carvacrol or thymol also showed synergistic effects against *E. coli* and *S. typhinurium*.

Table 1.3. Activities of Combinations of EOs and their components. Adapted from (Bassolé & Juliani, 2012).

Pair combinations	Organism	Methods	Interaction	References
Thymol/Carvacrol	*Staphylococcus aureus, Pseudomonas aeruginosa*	Half dilution	Additive	(Lambert et al., 2001)
	Escherichia coli	Checkerboard	Synergism	(Pei et al., 2009)
	S. aureus, Bacillus cereus, E. coli	Checkerboard	Antagonism	(Gallucci et al., 2009)
	S. aureus, P. aeruginosa	Mixture	Additive	(Lambert et al., 2001)
	E. coli	Checkerboard	Additive	(Rivas et al., 2010)
	Salmonella typhinurium	Mixture	Synergism	(Zhou et al., 2007)
Thymol/Eugenol	*E. coli*	Checkerboard	Synergism	(Pei et al., 2009)
Carvacrol/Eugenol	*E. coli*	Checkerboard	Synergism	(Pei et al., 2009)
Cinnamaldehyde/ Carvacrol	*S. aureus, B. cereus, E. coli*	Checkerboard	Antagonism	(Gallucci et al., 2009)
	E. coli	Checkerboard	Additive	(Pei et al., 2009)
Cinnamaldehyde/ Thymol	*S. typhinurium*	Mixture	Synergism	(Zhou et al., 2007)
	E. coli	Checkerboard	Synergism	(Pei et al., 2009)
Cinnamaldehyde/ Eugenol	*S. typhinurium*	Mixture	Synergism	(Zhou et al., 2007)
	Staphylococcus sp., Micrococcus sp., Bacillus sp., Enterobacter sp.	Mixture	Additive	(Moleyar & Narasimham, 1992)
Origanum vulgare/ Rosmarinus officinalis	*L. monocytogenes, Yersinia enterocolitica, Aeromonas hydrophilla, P. fluorescens*	Mixture	Synergism	(de Azeredo et al., 2011)
O. vulgare/ Thymus vulgaris	*P. fluorescens*	Mixture	Additive	
O. vulgare/ O. basilicum	*B. cereus, E. coli, P. aeruginosa*	Checkerboard	Additive	(Gutierrez et al., 2008)
O. vulgare/ Melissa officinalis	*B. cereus*			
O. vulgare/ O. majorana	*B. cereus, E. coli*			
O. vulgare/ R. officinalis	*B. cereus*			
O. vulgare/ T. vulgaris	*Enterobacter cloacae, P. fluorescencs, Listeria Innocua*	Checkerboard	Additive	(Gutierrez et al., 2009)

1.2.3 Immunomodulatory Properties

In addition to their antioxidant and antibacterial properties, EOs can also stimulate the immune system and provide beneficial results to those who utilize these oils. As such, many people employ various EOs as an alternative, holistic medicine to ease their ailments.

1.2.3.1. Anti-inflammatory Properties

Inflammation can be triggered in human hosts by the LPS-layer in gram-negative bacteria (Chao et al., 2005). In this process, the production of TNF-α, IL-1β, and IL-6 is stimulated by the activation of macrophages due to the presence of the LPS. Though the stimulation of these inflammatory cytokines is helpful, their overstimulation could result in septic shock. Therefore, in cases of overstimulation, suppression of these cytokines is necessary. Chao *et al.* (2005) have investigated the inflammatory bioactivity of Taiwanese cinnamon (*Cinnamomum osmophloeum* Kaneh.) leaf EO and found that it was able to inhibit the expression of proIL-1β, IL-1β, and IL-6. They remarked that the EO was unable to inhibit the expression of TNF-α and hypothesized that it was due to the fact that the oil's anti-inflammatory bioactivity occurred *in vitro*.

According to a study on episiotomy recovery, it was found that the use of lavender oil had significantly reduced redness and inflammation without side effects (Vakilian, Atarha, Bekhradi, & Chaman, 2011). In this study, the participants took a sitz bath with 5-7 drops of lavender oil twice a day for 10 days. After comparison with the control group, it was clear that the oil had positive effects on the patients. The researchers concluded that lavender oil is a viable candidate for postpartum episiotomy wound care. They mentioned that the oil owed its anti-inflammatory properties to caryophyllene oxide and noted that in other studies, 1,8-cineole and terpenoid oxide have shown inhibitory effects on inflammation in rats. This is confirmed in a study where rosemary oil (which contains 1,8-cineole) did indeed inhibit inflammation in rats and the researchers expressed their support for the use of rosemary oil as folk medicine for ailments and inflammation (Takaki et al., 2008).

Interestingly, carvacrol, a component of oregano, has shown to be able to tackle two aspects of inflammation, namely, edema and leukocyte infiltration (Silva et al., 2012). In Silva *et al.*'s (2012) study, the use of carvacrol also sped up the healing process in rats suffering from chronic

gastric ulcers induced by acetic acid. The researchers note that this activity could be due to the inhibition of the cyclooxygenase enzymes COX-1 and COX-2.

1.2.3.2. Anticancer Properties

No available drug on the market is able to treat most or all cancers. Thus, there is a call for new sources of effective drugs against cancer that have low toxicity and low environmental impacts. To affect carcinogenesis, the drugs should interfere with the modulation steps (initiation, promotion, and progression) (Fresco, Borges, Diniz, & Marques, 2006). In addition to the numerous carcinogens available, some cancers are due to oxygen-centred free radicals and other reactive oxygen species that cause oxidative damage to biomolecules (Bhalla, Gupta, & Jaitak, 2013).

The monoterpenes present in EOs have chemopreventive and chemotherapeutic activities in mammary tumor models and may, therefore, be viable therapeutic agents (Edris, 2007). Chemoprevention involves the prevention of the interaction of chemical carcinogens with DNA during the initiation phase. This is done by induction of phase I and phase II drug metabolizing enzymes, which could help with organ protection from carcinogenesis. In chemotherapy, tumor cells are prevented from proliferating during the promotion phase. It also involves the acceleration of tumor cell death and/or induction of tumor cell differentiation (Edris, 2007). EOs are able to (a) induce apoptosis by damaging cancer cell DNA (Cardile et al., 2009), (b) change expression levels of genes (Cha, Kim, & Kim, 2010), (c) inhibit angiogenesis (Chen et al., 2011), (d) prevent metastasis (Manjamalai & Grace, 2013), and (e) inhibit tumour growth (Ferraz et al., 2013).

In mice, the EO of *Lippia gracilis* Schauer (Verbenaceae) displayed cytotoxicity to different tumor cell lines by causing apoptosis and inhibiting tumor cell growth (Ferraz et al., 2013). The major component of this EO was thymol (55.50%), which probably contributed to its potent cytotoxic activity. Additionally, it was found that the oil was able to affect the cell cycle and arrest it at the G1-phase, which would allow the cells to either repair themselves or enter an apoptotic pathway. In the latter scenario, mutated neoplastic and hyperproliferating neoplastic cells can be eliminated.

Myristicin, the main component of nutmeg oil, has shown to have hepatoprotective activity against liver damage (Morita et al., 2003). This activity could be due to the inhibition of TNF-α release from macrophages and suppression of liver cell apoptosis. Murine hepatocarcinogenesis was inhibited by the action of curcumin oil in another study, confirming its chemopreventive properties

(Chuang et al., 2000). At high concentrations of *Origanum onites* (Turkish oregano), cancer cell viability was observed to have decreased (Özkan & Erdoğan, 2011). In the same study, carvacrol and thymol protected cells pre-treated with *O. onites* oil against H_2O_2-induced cytotoxicity.

The consumption of *Allium* vegetables has been associated with a reduced risk of stomach cancer due to the action of organosulfur compounds found in the vegetables (Bhalla et al., 2013; Dorant, van den Brandt, Goldbohm, & Sturmans, 1996). The strong cancer-inhibiting abilities of garlic were also observed in another study that noted differences in the number of cases of gastric cancer in different regions of Italy—regions with an increased number of gastric cancer cases were those where garlic (among other foods) was not consumed as frequently (Buiatti et al., 1989). Sulfur-containing foods inhibit carcinogenesis by increasing the levels of phase II enzymes, thereby increasing detoxification (Bhalla et al., 2013). These sulfur compounds can also decrease the levels of cytochrome P450, a phase I enzyme. This is favorable since cytochrome P450s catalyze the bioactivation of carcinogens into electrophilic species, allowing them to exert their genotoxic and cytotoxic effects (Jana & Mandlekar, 2009).

Though these results seem promising, there is no evidence pointing to EOs as being the next cure for cancer (Harris, 2010).

1.2.3.3. Aromatherapy

Aromatherapy involves the use of EOs as an alternative medicine to improve well-being and immune function (Bhalla et al., 2013; Dunning, 2006). Ailments such as depression, muscular pain, swollen joints, headaches, and respiratory problems have been alleviated by various forms of aromatherapy (Ali et al., 2015). This practice relieves stress by controlling hormones secreted by adrenal glands, which will then stimulate an immune response and the production of immune-boosting cells (Bhalla et al., 2013). During this process, harmful microorganisms are also destroyed. Dunning (2006) outlines the three main methods of aromatherapy as follows:

1. Medical aromatherapy: Popular in France, this form of aromatherapy is practiced by and restricted to medical practitioners as it involves the internal use of EOs in high doses. Carrier substances used include gel and wax capsules and suppositories. Medical aromatherapy is also known as aromatology or aromatic medicine.
2. Subtle aromatherapy: This form of aromatherapy is popular in Germany and is also called aromachology. In this practice, EO aromas are used to promote well-being.

3. Topical application and inhalation: This practice is popular in the UK and Australia.

Sesquiterpenes (e.g. caryophyllene, viridiflorene, elemene) have a calming effect and alcohols (e.g. geraniol, linalool, citronellol) are considered the most therapeutically beneficial (Dunning, 2006). The latter has a soft, sweet, herbaceous or woody odour and can be found in ho leaf, rosewood, tea tree, palmarosa, rosa damascene, and sweet marjoram. Dunning (2006) also remarks that aldehydes (e.g. citral, neral, cinnamaldehyde) have an odor that is generally floral or sharp and lemony, whereas ketones (e.g. camphor, piperitone, carvone) have a range of aromas. This range includes green and oily, fruity, fresh mint, and dry. Some ketones cause convulsions when ingested in large doses and are therefore not used in aromatherapy, with a few exceptions (e.g. ho leaf, sage). Esters that form during distillation generally have fruity aromas.

Once in the body, the oils remodulate themselves and work on the affected area or site of malfunction (Ali et al., 2015). The use of aromatherapy has shown to increase positive feelings and is used in hospitals in the USA to boost morale (Lis-Balchin, 1997). Additionally, Japanese offices and factories have also used EOs to enhance productivity in the workplace. One study found that rosemary EO and lavender EO were able to elevate contentedness and maintain good mood respectively (Moss, Cook, Wesnes, & Duckett, 2003). Another study came to the same conclusions—lavender and rosemary oils decreased stress and anxiety levels in graduate nursing students (McCaffrey, Thomas, & Kinzelman, 2009). However, Moss *et al*. (2003) found that following aromatherapy with lavender oil, working memory performance levels decreased, as did reaction times for memory and attention-based tasks. McCaffrey *et al.* (2009) reported that lavender oil decreased focus in students, whereas rosemary oil made things clearer and sharper. In patients suffering from severe dementia, it was found that aromatherapy with essential balm oil (*M. officinalis*) was a viable treatment for clinically significant agitation and that it improved the overall quality of life of these patients (Ballard, O'Brien, Reichelt, Perry, & Ballard, 2002).

On the other hand, there are cases where aromatherapy has not shown to contribute any beneficial effects. For example, in a randomized trial with 66 patients awaiting surgical abortion, aromatherapy was used to try to reduce their levels of anxiety (Wiebe, 2000). However, in this study, the EOs used did not reduce the women's anxiety any more than the placebo. In another study with 313 patients undergoing radiotherapy, anxiety levels were also measured following

aromatherapy (Graham, Browne, Cox, & Graham, 2003). Disappointingly, the results from this study showed that this method did not help with the patients' anxiety.

1.3. Techno-functional Properties

1.3.1. Flavor enhancing properties

The main component of licorice oil, glycyrrhizin, is known to be able to impart a sweet taste to foods and has flavour-enhancing properties (Quirós-Sauceda & Ovando-Martínez, 2015). In fact, it is reputed to be 50 times sweeter than regular refined sugar (Isbrucker & Burdock, 2006). In addition, glycyrrhizin has salt-softening properties and is used in Japan to attenuate the saltiness in soy sauce (Quirós-Sauceda & Ovando-Martínez, 2015). As a flavor enhancer, glycyrrhizin can replace 25% of the cocoa in food products and can be used in combination with various sweeteners to enhance their sweetness. Ice cream, sweet snacks, toffee bars, instant noodles, and sauces are examples of products that have utilized the properties of licorice oil to enhance their flavors. The beverage industry has also taken advantage of the beneficial properties of licorice oil, using it to satisfy the thirst sensation. Beers and ales have used glycyrrhizin for its foaming properties and ability to attenuate bitterness in foods (Isbrucker & Burdock, 2006). However, since its accompanying licorice flavor is incompatible with other flavors, this has limited its use in various products (Quirós-Sauceda & Ovando-Martínez, 2015). Additionally, glycyrrhizin also imparts an undesirable brownish color and loses its sweetness in acidic foods, further limiting its use on the market (Isbrucker & Burdock, 2006).

1.3.2. Effect on Gut Microbiota and Digestibility

The addition of EOs to animal feed has been investigated by numerous researchers in response to stricter regulations regarding antibiotic growth promoters (AGP) in animal feed (S. Y. Li et al., 2012). These regulations came about due to increasing concerns regarding antibiotic resistance in poultry and swine. Since it has been previously reported that EOs can improve immune functions and support gut health of farm animals, these oils are of great interest as possible replacements for AGPs. In a study with 240 piglets, encapsulated EOs containing thymol and cinnamaldehyde as their primary components were used (S. Y. Li et al., 2012). In the piglets, daily weight gain was improved with the addition of the EOs to their diets, as did beneficial *Lactobacillus* counts. Additionally, fecal samples revealed a decrease in *E. coli* count, suggesting that the added EOs were able to target pathogenic bacteria while also promoting the growth of beneficial microbiota.

Similar results were obtained in a study with broiler chickens treated with EOs also containing thymol and cinnamaldehyde (Tiihonen et al., 2010). The broilers increased their body weight by 4.5% in 42 days and an increase in beneficial caecal microbiota was observed. The researchers remarked that the treated broiler group had lower levels of *E. coli*, which probably contributed to the increase in beneficial bacteria such as propionibacteria.

On the other hand, in an *in-vitro* study involving oregano and thyme oils, it was found that these oils were active against both pathogenic and beneficial bacteria (Roldan, Diaz, & Duringer, 1978). Interestingly, the researchers also found that basil (*O. basilicum*) oil was more preferentially active against pathogenic bacteria than beneficial bacteria since the former's MBC (minimum bactericidal concentration) was much lower than that for the latter (10mg/ml or lower versus 80mg/ml). They note that if the right dose of basil oil were used to control pathogenic bacteria, it would not affect the beneficial bacteria present in the gut.

Lower levels of *E. coli* were also detected in another broiler chicken study, where the modulation of digestive enzymes was also explored (Jang, Ko, Kang, & Lee, 2007). Broilers that were fed with diets high in a commercial EO blend (50mg EO/kg diet) had greater pancreatic trypsin activity than those fed with antibiotics. Furthermore, pancreatic α-amylase activity was also higher. With an increased secretion of pancreatic enzymes, the researchers suggested that digestion of nutrients in the intestine was being enhanced.

1.4. Applications of EOs in foods

In candies and confectionaries that enhance the sweetness of their formulas with licorice oil, about 2-10% of their recipe consists of EO (Quirós-Sauceda & Ovando-Martínez, 2015).

To investigate its potential as a natural preservative, a study added tarragon (*Artemisia dracunculus*) oil to beef burger patties at varying concentrations; the formulation containing 0.25% tarragon oil decreased the growth rate of *S. aureus* in the patties the greatest (Sharafati-Chaleshtori et al., 2014). Patties containing 0.125% tarragon oil were found to be the most pleasant tasting, whereas those containing 0.25% tarragon oil had a lower overall acceptance. The authors suggested using the oil in conjunction with other preservation systems since high concentrations of the oil impart unfavorable sensorial effects.

Due to the intense aroma of EOs, they impart negative organoleptic effects in food products when used in concentrations exceeding an acceptable consumer threshold (Hyldgaard et al., 2012). This is problematic because high concentrations of EOs are needed for food products to benefit from their antimicrobial activity. Cilantro (*Coriandrum sativium*) oil was tested for its activity against *Listeria monocytogenes* on vacuum-packed ham and *in vitro*, which revealed the discrepancy in its activity on the inoculated media (Gill, Delaquis, Russo, & Holley, 2002). In the *in vitro* tests, the minimum inhibitory concentration (MIC) was 0.018%, whereas in vacuum-packaged ham, treatments containing 6% of the oil were required to inhibit *L. monocytogenes*. Since interactions with food matrices hinder antimicrobial activity, the only foods that can employ high concentrations of EOs are spicy foods (Hyldgaard et al., 2012).

To circumvent this obstacle, nanoencapsulation of EOs can be done to reduce the effect of their potent aromas as well as increase their antimicrobial activity (Donsì, Annunziata, Sessa, & Ferrari, 2011). EOs can be incorporated in polymeric nanoparticles (e.g. nanocapsules, nanospheres), lipid nanoparticles (e.g. liposomes, niosomes, solid lipid nanoparticles, nanostructured lipid carries) and nanoemulsions (São Pedro, Santo, Silva, Detoni, & Albuquerque, 2013). When carvacrol was loaded into chitosan nanoparticles, researchers found that the nanoparticles showed antimicrobial activity against *Staphylococcus aureus, Bacillus cereus,* and *E. coli* (Keawchaoon & Yoksan, 2011). The MIC of the carvacrol-loaded chitosan nanoparticles was close to the MIC of free carvacrol. The encapsulation of oils can also increase solubility (Ajazuddin & Saraf, 2010) and protect against volatilization (Flores et al., 2011). The latter is highly favorable since this creates a controlled release system which reduces the oils' organoleptic effects in food products (São Pedro et al., 2013). When tea tree oil was incorporated into nanocapsules, its stability was improved and its intense aroma was decreased (Flores et al., 2011). Additionally, the oil was protected from evaporation when encapsulated.

In the carvacrol-loaded chitosan nanoparticle study mentioned above, the researchers found that the release of carvacrol from the nanoparticles followed a Fickian behavior; during release, a small burst was initially released, then the rate of release decreased significantly in the second stage of release, and then again during the third stage (Keawchaoon & Yoksan, 2011). Similar results were found with oregano-loaded chitosan nanoparticles—an initial burst was first observed, where 82% of the oregano oil was released in the first 3 hours, followed by a slow accumulated release of 12% (Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013).

Nanoencapsulation of EOs is a very promising strategy to facilitate the incorporation of these intensely aromatic antimicrobials. In addition to increasing their solubility in food matrices and controlling their release, nanoencapsulation can maintain (or increase) the oils' antimicrobial activity against pathogenic bacteria, making it more attractive in the application of EOs in foods.

Alternatively, another strategy to circumvent the negative organoleptic effects of EOs in food products is to modify their chemical profiles. This can be done through enzymatic pathways such as lipase-catalyzed transesterification. This area of research is relatively new and few studies have investigated the modification of EOs (Antoniotti, 2014).

1.5. Modification of EOs

1.5.1. Modification of Extraction Methods

When EOs are extracted from plant materials, the general yield is typically below 5% (w/w) (Groussin & Antoniotti, 2012). As such, investigations have been carried out to find enzymatic methods that can increase the yield of these extractions. According to Groussin and Antoniotti (2012), this can be done by weakening cell walls with cellulases to facilitate the release of metabolites. Glucosidases can also be used to increase yields by cleaving metabolites existing as conjugates with sugars.

Enzymatic pre-treatment of celery (*Apium gravelolens* L.) oil with cellulase, pectinase, protease, and viscozyme was able to increase its yield from 1.8% to 2.2-2.3% (Sowbhagya, Srinivas, & Krishnamurthy, 2010). Furthermore, limonene, a major constituent of the oil which has shown to be able to reduce breast tumor cyclin D1 expression (Miller et al., 2013), also increased from 63% to 82%. Aside from this increase, the flavour profile and physicochemical properties of the celery oil were not drastically modified (Sowbhagya et al., 2010). Similar results were found when garlic was treated with pectinase, cellulase, protease, and viscoenzyme prior to extraction (Sowbhagya, Purnima, Florence, Appu Rao, & Srinivas, 2009). The yield increased from 0.28% to 0.39-0.51% and 0.31% to 0.45-0.57% by steam distillation and hydrodistillation respectively. Minor changes were observed in the flavour profile and physicochemical properties of the garlic oil.

Pre-treatment of ground mustard seeds with cellulase increased their yield by 50% (Szakács-Dobozi, Halász, Kozma-Kovács, & Szakács, 1988). In the oil, it was found that this treatment also

increased the yield of allyl isothiocyanate, an isothiocyanate found to be able to inhibit tumour-specific angiogenesis (Thejass & Kuttan, 2007).

1.5.2 Modification of Aromatic Profiles

1.5.2.1 Lipases

Found in animals, plants, yeasts, molds, and bacteria, lipases (EC 3.1.1.3) are hydrolases that act on carboxylic ester bonds in triacylglycerols and other substrates (Houde, Kademi, & Leblanc, 2004). In comparison to chemical catalysts, lipases are very advantageous thanks to their high substrate specificity and mild reaction conditions, such as lower temperature and lower pressure, resulting in greener practices. In the food industry, lipases are extensively used in bakery products, confectionery products, dairy products, and more. These enzymes can extend bread shelf life (Moayedallaie, Mirzaei, & Paterson, 2010), accelerate cheese ripening (Rani & Jagtap, 2019), and improve flavoring in alcoholic beverages (Sharma, Chisti, & Banerjee, 2001) to name a few uses.

To catalyze reactions, these enzymes must be in their active (open) form, which is activated by exposure to a lipophilic substrate. The open form is the only form that can interact with hydrophobic compounds (Palomo et al., 2002). Indeed, in the presence of a lipophilic substrate, conformational changes occur so that the helical oligopeptide chain known as the "lid" displaces, exposing the active site. These active sites vary from lipase to lipase and can serve as a basis to categorize these enzymes in the following way: (1) lipases with a hydrophobic, crevice-like binding site located near the protein surface; (2) lipases with a funnel-like binding site; (3) lipases with a tunnel-like binding site (Pleiss, Fischer, & Schmid, 1998). In certain lipases, such as *Candida antarctica* lipase B (funnel-like active site), there is limited space in the pocket of the active site, therefore increasing the selectivity of the enzymes.

The reactions they catalyze include hydrolysis, esterification, and transesterification. Under aqueous conditions, they catalyze hydrolysis, whereas in non-aqueous conditions, the reverse reactions are catalyzed, which include esterification and transesterification. In aqueous media, the hydrophobicity of the substrates can also influence the catalytic behavior of lipases (Sun & Liu, 2015).

1.5.2.1.1 Hydrolysis

Lipases naturally catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, fatty acids and glycerol. Aqueous conditions promote hydrolysis since water molecules are responsible for the splitting of fats/esters into their constituent acid and glycerol/alcohol (Gandhi, 1997). This can be illustrated as follows:

$$RCOOR' + H2O \Leftrightarrow RCOOH + R'OH \qquad (5)$$

Partial hydrolysis of esters may occur in aqueous media, leading to the production of di- and mono-glycerides, which can serve as biocompatible emulsifiers in the food industry (Kaur, Minhas, & Jooyandeh, 2009). In the dairy industry, hydrolysis can be employed to impart desirable flavors in dairy products through the production of short-chain fatty acids such as butanoic and hexanoic acids, which contribute "buttery" flavors to a product (Omar, Gounga, Liu, Mlyuka, & Wang, 2016). Furthermore, hydrolysis can also be used to create concentrated cheese flavors, as in the case of enzyme modified cheese (Kaur et al., 2009). Following incubation of a dairy substrate in the presence of lipases from sources such as *Aspergillus oryzae* and *Aspergillus niger*, the substrate's fat and protein simultaneously hydrolyze, producing an intense cheese flavour (Moosavi-Nasab, Radi, & Jouybari, 2010). These flavors can then be used to enhance Blue, Mozzarella, Swiss, Gouda, and Parmesan cheese.

1.5.2.1.2 Esterification

Without the need for large quantities of water, esterification can be carried out in an anhydrous media or in solvent-free media (Gandhi et al., 2000). During these reactions, water must be removed either in a multi-step approach by removing it at every step or instantaneously *in situ* (Jeromin & Zoor, 2008; Stergiou et al., 2013). Esterification mainly entails the reaction of an acid with an alcohol, though lipases are capable of also catalyzing the synthesis of sugar esters, thiol esters, peptides, fatty amides, and so on (Gandhi, 1997). Esterification can be illustrated as follows:

$$RCOOH + R'OH \Leftrightarrow RCOOR' + H2O \qquad (6)$$

This reaction can be used to irreversibly esterify carboxylic acids into flavor esters such as isobutyl acetate (fruity), methyl butyrate (apple), ethyl butyrate (pineapple) and benzyl butyrate (flowers/jasmine) (Coleman, 2009; Jeromin & Zoor, 2008). Additionally, esterification can be employed in synergy with novel imidazolium surfactants and microwave irradiation for starch modification (Adak & Banerjee, 2016). Indeed, Adak and Banerjee (2016) reported that the

modification of corn starch catalyzed by *Rhizopus oryzae* lipase improved its hydrophobicity, oil holding capacity and thermoplasticity.

1.5.2.1.3 Transesterification

Transesterification reactions differ from esterification in that no water is produced as a by-product in the former. This, therefore, makes the former more lucrative, as this eliminates a water removal step in the process. During transesterification, three possible reactions can take place: (a) acidolysis, (b) alcoholysis, and (c) interesterification. As such, this reaction results in the production of an acid, alcohol, or ester, as illustrated below:

(a) Acidolysis

$$RCOOR' + R''COOH \Rightarrow R''COOR' + RCOOH \qquad (7)$$

(b) Alcoholysis

$$RCOOR' + R''OH \Leftrightarrow RCOOR'' + R'OH \qquad (8)$$

(c) Interesterification

$$RCOOR' + R''COOR^* \Leftrightarrow RCOOR^* + R''COOR' \qquad (9)$$

Transesterification reactions can be used to synthesize flavor esters such as hexyl butyrate, which impart a "green note" to products in foods, beverages and pharmaceuticals (Chang, Shaw, & Shieh, 2003). Lipases can also be used to catalyze the transesterification of sugar alcohols into aromatic esters to increase their solubility in oil-based formulations and emulsions (Croitoru et al., 2011), which is a relatively new area of research. Another interesting area of research is the production of cocoa butter substitutes—these enzymatically-catalyzed reactions have gained much attention due to the discovery of the advantages of regio-selecteive lipases in comparison to chemical catalysts (Ghazani & Marangoni, 2018). Interesterification reactions can be used to produce these cocoa butter substitutes from other oils such as palm and hydrogenated soybean oil (Abigor et al., 2003). The production of a cocoa butter equivalent has been widely studied, as cocoa butter is one of the most expensive fats on the market and with growing demands for chocolate, shortages could render it even more expensive. In addition to producing these equivalents through interesterification reactions, studies have shown that acidolysis is also a plausible method to transform cheaper oils and fats into cocoa butter equivalents (Mohamed, 2013, 2014)

1.5.2.2 Lipases and EOs

EOs are can be modified through transesterification reactions, where vinyl esters are generally used as the acyl donor, and a component of the oil is the acceptor. Few studies have explored the modification of aromatic profiles of EOs. To date, lipases have been used to catalyze the modification of palmarosa oil (Daniel, Malik, & Albert, 2011; Ramilijaona et al., 2013) and citrus oils (K. Zhang et al., 2017), including Brazilian mandarin oil and American sweet orange. These modifications have resulted in fruitier aromatic profiles and have permitted minor components to gain more spotlight. When acetic acid is used as an acyl donor, geraniol is converted to geranyl acetate, a floral and fruity compound with a higher antimicrobial activity than geraniol (Dorman & Deans, 2000). When vinyl (ethenyl) esters are used as acyl donors, the fruity aromas of rose oil increase, as do aromas of minor components such as linalool and farnesene (Ramilijaona et al., 2013). Enzymatic modification of EOs is a promising and interesting area of research, which could eventually lead to their ubiquitous use as natural antioxidants in the food industry.

CONNECTING STATEMENT I

A literature review on the biological, immunomodulatory, and techno-functional properties of EOs, as well as their applications in food and their modifications through enzymatic pathways were presented in Chapter I.

Chapter II investigates the antioxidant capacity of 38 EOs as well as their enrichments with individual polyphenols, multiple polyphenols, as well as crude extracts. Two *in vitro* methods were used to assess their antioxidant capacities and combinatorial interactions. Predictive models were also presented in this chapter to understand the relationship between the chemical profiles and the antioxidant capacity of EOs as well as the prediction of the effects of EO enrichment.

CHAPTER II

INVESTIGATION OF THE COMBINATORIAL EFFECTS BETWEEN SELECTED EOS AND PLANT EXTRACTS

2.1. Abstract

The antioxidant capacity of 38 essential oils (EOs) and their enrichments were evaluated using two *in-vitro* methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Oxygen Radical Absorption Capacity (ORAC) assays. Individually, pimento berry (HE-PIM-01) showed the greatest overall antioxidant capacity (IC_{50} = 0.0816 mg/mg DPPH; ORAC Value = 8174.75 µmol TE/g), whereas white pine needles (HE-PIN-01) showed the lowest overall antioxidant capacity (IC_{50} = 652.4518 mg/mg DPPH; ORAC Value = 7.64 µmol TE/g). Phenols were found to significantly contribute ($p < 0.05$) to the synergistic effects within EOs in both the DPPH and ORAC assays. Combining EOs with polyphenols (PPs) can lead to synergistic effects, reducing the quantity needed to extend shelf life. These effects were investigated using a multiple-method approach where 6 selected EOs were enriched with 8 individual PPs, 4 mixtures of major plant extract PPs, and 4 crude plant extracts. As the number of advantageous functional groups increased in individual PPs, the antioxidant capacity of their enrichments with EOs increased proportionally. As such, the phenolic acid that improved the overall antioxidant capacity of EOs the most was rosmarinic acid, whereas *p*-coumaric acid improved it the least. Furthermore, the flavonoid that improved the overall antioxidant capacity of EOs the most was quercetin, whereas rutin hydrate showed the least improvement. Enrichments with mixes of major PPs of plant extracts, as well as crude plant extracts, showed synergistic and additive effects in the DPPH assay. In the ORAC assay, the latter showed an increase in additive effects and a decrease in antagonistic effects. EOs enriched with the major PPs of green tea extract showed the lowest IC_{50} values among the 4 enrichments (0.0090-0.0681 mg/mg DPPH) and exhibited moderate to high values (6,207.50-13,681 µmol TE/g sample) in the ORAC assay. Crude green tea (EX-THE-01) and apple (EX-POM-04) extracts did not exhibit any antagonistic effects in both assays, suggesting that both their major and minor components interacted synergistically or additively with the selected EOs. Crude grape seed (EX-RAI-01) and rosemary (EX-ROM-04) extracts equally exhibited antagonistic effects. The former exhibited more additive effects, whereas the latter exhibited more synergistic effects when enriching EOs. These combinatorial effects could partly be explained by the possible formation of stable intermolecular complexes that subsequently enables the regeneration of certain antioxidants.

2.2. Introduction

Lipids are highly susceptible to oxidative degradation during storage and distribution, which negatively impacts the sensory quality of food products, as well as their nutritional or functional quality. To limit this, the food industry has incorporated synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ) in food products as an economical and effective solution. However, these synthetic ingredients have fallen under great scrutiny in the public eye, which has consequently driven consumer interest towards more wholesome products. The advent of increasing consumer health consciousness has pushed the food industry to explore natural alternatives such as essential oils (EOs) (Fasseas, Mountzouris, Tarantilis, Polissiou, & Zervas, 2008; Patrignani, Siroli, Serrazanetti, Gardini, & Lanciotti, 2015) and plant extracts (Ahn, Grun, & Fernando, 2002; Schwarz et al., 2001).

EOs are composed of volatile compounds produced by the secondary metabolism of aromatic plants. Their composition is influenced by several factors, including seasonality, genetic constitution of plants, agronomic conditions and extraction methods (Dehghan, Torbati, Mohammadian, Movafeghi, & Talebpour, 2018; Duarte, Santos, Seraphin, & Ferri, 2010; Moniodis, Renton, Jones, Barbour, & Byrne, 2018; Sriti Eljazi et al., 2018). Though phenols are mostly responsible for the antioxidant nature of EOs, EOs with an absence of these compounds also exhibit some antioxidant activity due to the presence of other compounds such as allylic alcohols, including nerol and geraniol, and selected monoterpenes such as α- and γ-terpinene (Cutillas, Carrasco, Martinez-Gutierrez, Tomas, & Tudela, 2018; Ruberto & Baratta, 2000). The antioxidant activity of some EOs cannot fully be explained by the presence of certain major components. Some activity can also be attributed to underlying synergistic effects between these components and the EO's minor components (Araújo Couto et al., 2019). EOs are known as direct antioxidants, as they directly target the radical chain reaction by competing with propagation reactions (Amorati et al., 2013). The phenols (PhOH) they contain will react with a peroxyl radical ROO• to form a stable phenoxyl radical (ROOH), which will then quickly react with another peroxyl radical to quench it, forming a non-radical product.

Plant extracts from herbs, fruits, and vegetables have also evoked considerable interest as natural (Bitalebi, Nikoo, Rahmanifarah, Noori, & Ahmadi Gavlighi, 2019; Z. Li et al., 2018; Shah, Bosco, & Mir, 2014). These sources contain a wide variety of health-promoting phytochemicals, including

flavonoids, polyphenols, carotenoids, and catechins (Liu, 2003; Rodriguez-Casado, 2016). These phytochemicals are capable of scavenging free radicals and studies have investigated their use in food products, such as meats and vegetable oils, for oxidative stability (Shah et al., 2014; Yang et al., 2016). Indeed, apple peel extract has been shown to slow protein and lipid oxidation in fish mince (Bitalebi et al., 2019), grape seed extract was effective in inhibiting lipid oxidation in cooked turkey meat during cold storage (Mielnik, Olsen, Vogt, Adeline, & Skrede, 2006), rosemary extract has been shown to be able to improve oxidative stability in vegetable oils (Yang et al., 2016), and green tea was an effective antioxidant in uncured pork sausages (Jayawardana, Warnasooriya, Thotawattage, Dharmasena, & Liyanage, 2019). Mixtures of green tea and grape seed extracts have also been reported to interact synergistically together (El-Beltagi, El-Desouky, & Yousef, 2016). The mechanisms of phytochemicals depend on their molecular structure—it has been shown that additional galloyl, catechoyl, or hydroxyl groups in phenolic compounds have increased antioxidant activity in these phytochemicals (Capitani, Carvalho, Rivelli, Barros, & Castro, 2009). Due to the presence of catechol groups, flavonoids are capable of creating stable complexes with phenols through π- π stacking of their B-rings to the aromatic rings of phenols (Peyrat-Maillard, Cuvelier, & Berset, 2003). This phenomenon allows for the regeneration of the more efficient antioxidant (primary antioxidant) or less efficient antioxidant (synergist/co-antioxidant) in a multi-antioxidant system, resulting in a synergistic or antagonistic effect, respectively.

In addition to the antioxidants' abilities to scavenge free radicals (Edge & Truscott, 2018), it is necessary to also understand the extent of their effectiveness in different lipid systems, all of which depends on their physicochemical properties. According to the ''polar paradox theory'', non-polar antioxidants are more effective in oil-in-water emulsions than in bulk oils, and the opposite applies for polar antioxidants (Jayasinghe, Gotoh, & Wada, 2013; Porter, 1993). Additionally, Jayasinghe *et al.* (2013) have shown that polar antioxidants in a crude extract behaved differently in a lipid medium than when they are used alone. It is important to note that although the polar paradox theory holds true in some studies, it does not take into account other factors that may influence antioxidant activity such as concentration, mobility and micellization of the antioxidant, as well as presence and type of emulsifiers in the system (Shahidi & Zhong, 2011).

Few studies have investigated the antioxidant properties of multi-component mixtures (Capitani, Carvalho, Botelho, Carrapeiro, & Castro, 2009; El-Beltagi et al., 2016; Peyrat-Maillard et al.,

2003). Combinations of some antioxidants have shown to exhibit synergistic effects (Araújo Couto et al., 2019; Bag & Chattopadhyay, 2015; Peyrat-Maillard et al., 2003; Romano, Abadi, Repetto, Vojnov, & Moreno, 2009), where the antioxidant activity of the final mixture is greater than the sum of their individual activities. Other combinations have resulted in additive or antagonistic effects (Araújo Couto et al., 2019; Peyrat-Maillard et al., 2003), where the activity of the combinations is equal to or lesser than that observed for each compound. To the best of our knowledge, no study so far has explored the interactive effects between the major compounds of EOs and plant extracts. The understanding of such interactions can enhance the use of antioxidants from different sources in a synergistic way.

To measure the extent of the chain-breaking abilities of potential antioxidants, methods have been developed based on two radical-quenching mechanisms: single electron transfer (SET), where antioxidants transfer electrons to reduce radicals, and hydrogen atom transfer (HAT), where antioxidants donate hydrogen atoms to quench free radicals (Schaich et al., 2015). Mixture effects such as synergy, addition, and antagonism arise in these methods depending on the antioxidants' abilities to jointly transfer electrons or hydrogen atoms more efficiently, comparatively, or less efficiently, respectively, than their individual components. In this study, two methods utilizing one of each mechanism were used—the DPPH (2,2′-diphenylpicryl hydrazyl free radical) assay (SET) and ORAC (Oxygen Radical Absorbance Capacity) assay (HAT). The present work has two main objectives. First, a comparative study of the antioxidant activity of EOs from different sources was carried out. Using selected EOs and plant extracts, combinations of EOs with individual major PPs, a mix of multiple major PPs or whole plant extracts (containing major and minor PPs) were assessed for their antioxidant activities. Selected EOs included pimento berry (HE-PIM-01), clove (HE-CLO-01), oregano (HE-ORI-03), white thyme (HE-THY-03), yellow sage (HE-SAU-01-02), and Ceylon cinnamon (HE-CAN-04), while selected plant extracts included grape seed (EX-RAI-01), green tea (HE-THE-01), apple (EX-POM-04), and rosemary (EX-ROM-04). Such combinations were conducted to understand the component interactions that contribute to the synergistic effects between EOs and plant extracts. Doing so will enable optimization of antioxidant activity of combinations of EOs and plant extracts.

2.3. Materials and Methods

2.3.1. Materials

EOs were obtained from commercial suppliers (**Table 2.1**). 2,2-diphenyl-1-picrylhydrazyl (DPPH) (>90%), α-tocopherol (96%), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), fluorescein sodium salt, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from MilliporeSigma (Burlington, Massachusetts, United States). Acetone (≥99.5%) and methanol (≥99.9%) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, United States). Anhydrous ethanol (≥99.9%) was purchased from Commercial Alcohols Inc. (Brampton, Ontario, Canada).

2.3.2. Preparation of Antioxidant Components and their Mixes

EO dilutions were made in ethanol. The major PP mixes were prepared in ethanol in such a way that reflects the proportions present in their respective crude plant extracts (grape seed, green tea, apple, rosemary) (**Table 2.2**). All EOs were enriched with individual major PPs, PP mixes, and crude extracts at a 1:1 ratio.

2.3.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging Assay

The DPPH assay was carried out according to the method reported by Lewis (2012) with modifications. 50µL aliquots of varying concentrations of EOs in ethanol were mixed with 450µL of Tris-HCl buffer (pH 7.4). 1mL of 0.1mM DPPH was added to all samples, which were then incubated in the dark for 30min at room temperature. In preliminary trials, 30 min was identified as the appropriate time at which the steady state of scavenging was achieved. Following incubation, the absorbance of each sample was read at 517nm using a Beckman spectrophotometer. Water was used as the blank. 10% methanol in Tris-HCl buffer was used as the negative control. The inhibition ratio (%) was calculated as follows:

$$\text{Inhibition ratio (\%)} = [(As - As)/Ac] \times 100 \quad \text{(Eq. 1)}$$

Where: Ac is the absorbance of the control; As is the absorbance of the sample

The different concentration of EOs were plotted against the estimated inhibition ratio. The IC_{50} was defined as the concentration required to reduce DPPH by 50%. The IC_{50} was expressed as mg sample/mg DPPH.

Table 2.1. List of EOs and their origins

Type	ID	EO	Latin name	Distilled part	Origin	Supplier
Spice	HE-PIM-01	Pimento Berry	*Pimenta officinalis Lindley*	Seed	-	BSA
	HE-PIM-02	Pimento Leaf	*Pimenta officinalis Lindley*	Leaf	-	BSA
	HE-CAN-04	Ceylon Cinnamon	*Cinnamomum verum (zeylanicum)*	Leaf	-	BSA
	HE-CLO-01	Clove	*Eugenia caryophyllus*	Flower bud	Madagascar	Aliksir
	HE-CLO-05	Clove	*Eugenia caryophyllata*	Flower bud	Madagascar	Novotaste
	HE-CLO-03	Clove	*Eugenia caryophyllata*	Leaf	-	BSA
	HE-CLO-02	Clove	*Eugenia caryophyllata*	Bud	Indonesia	New Directions
	HE-CLO-04	Clove	*Eugenia caryophyllata*	Flower bud	-	BSA
	HE-CAN-02	Cinnamon	*Cinnamomum verum (Zeylanicum)*	Bark	Madagascar	Aliksir
	HE-CHI-01	Chili	*Capsicum annum L.*	Flower bud	India	New Directions
	HE-CAN-03	Chinese Cinnamon	*Cinnamomum cassia*	Bark	-	BSA
	HE-CUM-01	Cumin	*Cuminum cyminum*	Seed	Iran	Aliksir
	HE-CAN-01	Chinese Cinnamon	*Cinnamomum cassia*	Bark	China	Aliksir
Herb	HE-ORI-02	Oregano	*Origanum vulgare v. kaliteria*	Leaf	-	BSA
	HE-THY-02	Thyme	*Thymus vulgaris*	Aerial part	France	Aldrich
	HE-SAU-01-02	Sage	*Salvia officinalis L.*	Aerial part	-	BSA
	HE-THY-03	White thyme	*Thymus zygis*	Leaves	France	Novotaste
	HE-ORI-03	Oregano	*Origanum vulgare v. kaliteria*	Aerial part	Hungary	Novotaste
	HE-ORI-01	Oregano	*Origanum vulgare v. kaliteria*	Aerial part	Bolivia	Aliksir
	HE-THY-01	Thyme	*Thymus vulgaris*	Flowering tops	France	Aliksir
	HE-MEL-01	Melissa Leaf	*Melissa officinalis*	Leaves	Slovenia	New Directions
	HE-VER-01	Lemon Verbena	*Lippia citriodora*	Aerial parts	Spain	Aliksir
	HE-SAU-02	Dalmatian Sage	*Salvia officinalis L.*	Leaf	Albania	Novotaste
	HE-ROM-02	Rosemary	*Rosmarinus officinalis*	Aerial part	Tunisia	Novotaste
	HE-ROM-01	Rosemary	*Rosmarinus officinalis*	Aerial part	Morocco	Aliksir

Table 2.1. Continued

Type	ID	EO	Latin name	Distilled part	Origin	Supplier
Botanical (Fruits, vegetables, conifers)	HE-CNB-01	Cranberry	*Vaccinium oxycoccos*	Seed	Canada	Atoka
	HE-AIL-01	Garlic	*Allium sativum L.*	Crushed bulb	-	BSA
	HE-OIG-01	Onion	*Allium cepa L.*	Bulb	-	BSA
	HE-AIL-02	Garlic	*Allium sativum L.*	Crushed bulb	China	Novotaste
	HE-AIL-03	Mexican Garlic Blend	*Allium sativum L.*	Crushed bulb	Mexico	Novotaste
	HE-CIT-02	Lemon	*Citrus limonum*	Peel	Canada	Novotaste
	HE-CIT-01	Lemon	*Citrus limonum*	Peel	-	BSA
	HE-PIN-02	Pine	*Pinus strobus L.*	Needles and Twigs	Bulgaria/Hungary	Novotaste
	HE-CED-01	Cedar	*Thuja occidentalis*	Leaf	-	Nascent
	HE-CYP-01	Cypress	*Cupressus sempervirens*	Branches	Spain	Aliksir
	HE-CED-02	Cedar	*Thuja occidentalis*	Leaf	Canada	Novotaste
	HE-CYP-02	Cypress	*Cupressus sempervirens L.*	Needles and cones	France	Novotaste
	HE-PIN-01	White Pine	*Pinus strobus*	Needles and Twigs	Canada	Aliksir

Table 2.2. Preparation of multi-component polyphenol mixes as analogues of major compounds present in selected plant extracts.

Extract	Country of Origin	Supplier	ID	Polyphenol	% (w/w) Total Polyphenols
Grape Seed	China	New Directions	EX-RAI-01	Catechin	48
				Epicatechin	52
Green Tea	China	New Directions	EX-THE-01	Chlorogenic Acid	1.1
				Catechin	21
				Epicatechin	76
				p-Coumaric Acid	0.4
				Rutin Hydrate	1.2
Apple	-	Diana Foods	EX-POM-04	Chlorogenic Acid	81
				Epicatechin	2.1
				Rutin Hydrate	4.3
				Quercetin	12
Rosemary	-	BSA	EX-ROM-04	p-Coumaric Acid	80
				Rosmarinic Acid	20

2.2.4. Oxygen Radical Absorption Capacity (ORAC) Assay

The ORAC assay was carried out according to the method outlined by Cao, Alessio, and Cutler (1993), with modifications. AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) was used as a free-radical generator and varying concentrations of selected EOs was used to prevent the decay of Fluorescein Sodium Salt. Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (100, 50, 25, 12.5, 6.25, and 0mM) was used as a standard and phosphate buffer (75mM, pH 7.4) was used as a blank. Aliquots of 25µL of EOs at different concentrations (acetone/phosphate buffer pH 7.4) and 150µL of 16 mM fluorescein (prepared in PBS 10mM pH 7.4) was pipetted into a 96-well black-walled plate. Following incubation at 37^{o}C for 30min in a Synergy HTX Multi-Mode Reader, all wells were injected with 25µL of freshly prepared AAPH (79.65mmol/L). Fluorescence readings were taken for 1 hour at 485nm (excitation wavelength) and 520nm (emission wavelength) every 60 seconds. The AUC (area under the curve) and Net AUC of the standards and samples were determined using Gen5 Data Analysis Software using the following equations respectively:

$$\mathrm{AUC} = \left(\frac{R_1}{R_1}\right) + \left(\frac{R_2}{R_1}\right) + \left(\frac{R_3}{R_1}\right) + \cdots + \left(\frac{R_n}{R_1}\right) \qquad \text{(Eq. 2)}$$

Where: $\mathbf{R_1}$ is the fluorescence reading at the initiation of the reaction; $\mathbf{R_n}$ is the last measurement

$$\text{Net AUC} = \mathrm{AUC_{sample}} - \mathrm{AUC_{blank}} \quad \text{(Eq. 3)}$$

The standard curve consisted of the Net AUC of different Trolox concentrations plotted against their concentration. Finally, to calculate the Trolox Equivalents (TE) of each sample range, the following equation was used:

$$\text{TE (range of concentrations)} = \mathrm{m_{compound}}/\mathrm{m_{Trolox}} \quad \text{(Eq. 4)}$$

Where: $\mathbf{m_{compound}}$ is the slope of the linear regression analysis of the compound; $\mathbf{m_{Trolox}}$ **i**s the slope of the linear regression analysis of Trolox

Results are expressed as µmol Trolox Equivalents (TE)/g sample.

2.3.5. Statistical analysis

All measurements were taken as triplicates and reported as mean ± standard error. The statistical analyses were carried out using the Microsoft Excel 2019 software package (Microsoft Corp., Redmond, WA). The Kruskal Wallis and Dunn's multiple comparisons tests were performed to detect significant differences ($p < 0.05$) using GraphPad Prism Version 8.4.2 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

The antioxidant activity of the combinations was compared to their respective simulations. Synergistic effects were defined to have taken place if the measured antioxidant capacity was greater than the sum of the antioxidant capacity of the individual compounds in equimolar concentrations. Antagonistic effects were defined to have place if the measured antioxidant capacity was lower than the sum of the antioxidant capacity of the individual compounds in equimolar concentrations.

Antioxidant samples were clustered according to their IC_{50} and ORAC values and to their types using Ward's method and heat map representations of variables were generated. Multivariate statistical analysis, including principal component analysis (PCA), was completed using R software (version 3.4). R software was also used to establish correlations between variables and responses. Design Expert® Software version 8.0.7 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to identify the best models that fit the relationships between the composition and the antioxidant properties of EOs and for analysis of variance (ANOVA).

2.4. Results and Discussion

2.4.1. Antioxidant Capacity of Non-Enriched EOs

The antioxidant capacities of EOs vary according to their chemical composition and methods of testing. Since antioxidant capacity can proceed through different mechanisms, it should be measured using more than one method to obtain a complete and precise evaluation of this property. Two methods that can respectively act primarily by single electron transfer (SET) or hydrogen atom transfer (HAT) were used in the present study. The antioxidant capacities of 38 EOs were assessed using the DPPH and ORAC assays (**Table 2.3**).

EOs were categorized into three groups, including spices, herbs and botanicals (fruits, vegetables and conifers). These classifications were based on chemical families present in the chemical profiles of the EOs. The main families of the spice EOs fell under phenols, aldehydes, and esters, with phenols being the major compounds in many of them (HE-CLO-01 to -05; HE-PIM-01/02; HE-CAN-04). Herb EOs had a more diverse variety of main families, including alcohols, phenols, monoterpenes, and esters. Botanical EOs, which encompassed fruits, vegetables, and conifers, had profiles mainly consisting of monoterpenes, ketones, and/or sulfur-containing compounds. More specifically, EOs belonging to the *Allium* genus (HE-AIL-01 to -03; HE-OIG-01) were mainly composed of sulfur-containing compounds, as well as esters. EOs derived from fruits (HE-CNB-01, HE-CIT-01 to -02) had aldehydes in common with each other, differing in the presence of alcohol/sulfur-containing (HE-CNB-01) and monoterpenes/sesquiterpenes (HE-CIT-01 to -02). Lastly, EOs derived from conifers (HE-PIN-01 to -02, HE-CED-01 to -02, HE-CYP-01 to -02) were all composed of monoterpenes and differed in the presence of sesquiterpenes, esters, ketones, and/or alcohols.

The IC_{50} values of spices, herbs and botanicals ranged between 0.0816-237.5030, 0.9602-580.0000, and 43.6427-652.4518 mg/mg DPPH, respectively; while the ORAC values ranged between 4.38-8765.50, 11.94-8061.63, and 0.45-43.02 µmol TE/g sample, respectively. There was a significant difference between these three groups (Kruskal Wallis test, $p < 0.05$). Furthermore, there was a significant difference (Dunn's test, $p < 0.05$) between all categories in the DPPH assay and in the ORAC assay, except between the ORAC values of the herbs and spices.

Table 2.3. Antioxidant properties of 38 EOs.

Type	ID	EO	Major Families of compounds[a]	IC_{50} (mg/mg DPPH)	ORAC Value (μmol TE/g sample)
Spice	HE-PIM-01	Pimento Berry	Phenol, Sesquiterpene	0.0816 ± 0.01	8173.75 ± 1.31
	HE-PIM-02	Pimento Leaf	Phenol, Sesquiterpene, Esters	0.1025 ± 0.01	7103.63 ± 1.24
	HE-CAN-04	Ceylon Cinnamon	Phenol, Esters, Alcohol	0.1259 ± 0.01	7063.13 ± 1.18
	HE-CLO-01	Clove bud	Phenol, Sesquiterpene, Monoterpene	0.1355 ± 0.03	8765.50 ± 0.89
	HE-CLO-03	Clove leaf	Phenol, Sesquiterpene	0.1359 ± 0.04	6468.00 ± 1.01
	HE-CLO-05	Clove bud	Phenol, Sesquiterpene	0.1430 ± 0.03	7175.50 ± 1.20
	HE-CLO-02	Clove bud	Phenol, Esters, Sesquiterpene	0.1464 ± 0.02	6048.63 ± 1.27
	HE-CLO-04	Clove bud	Phenol, Esters, Sesquiterpene	0.1497 ± 0.03	6877.00 ± 1.15
	HE-CAN-02	Cinnamon	Aldehyde, Monoterpene, Esters	2.6133 ± 0.17	5.41 ± 0.94
	HE-CHI-01	Chili seed	Esters, Phenol	79.4583 ± 10.60	4.38 ± 0.81
	HE-CAN-03	Chinese Cinnamon	Aldehyde, Esters, Other	138.2133 ± 4.22	48.11 ± 0.78
	HE-CUM-01	Cumin	Aldehyde/Monoterpene, Alcohol	202.4908 ± 3.13	24.16 ± 0.37
	HE-CAN-01	Chinese Cinnamon	Aldehyde, Esters	237.5030 ± 0.73	52.15 ± 1.84
Herb	HE-ORI-02	Oregano	Phenol/Monoterpene, Alcohol	0.9602 ± 2.21	2002.50 ± 1.02
	HE-THY-02	Thyme	Phenol/Monoterpene, Alcohol	1.1738 ± 0.94	11.94 ± 1.15
	HE-SAU-01-02	Yellow Sage	Monoterpene/ketone	1.2422 ± 0.04	6701.00 ± 0.81
	HE-THY-03	White Thyme	Phenol, Monoterpene, Alcohol	1.4533 ± 0.70	6129.00 ± 0.80
	HE-ORI-03	Oregano	Monoterpene, Alcohol	3.2667 ± 0.95	6707.88 ± 1.19
	HE-ORI-01	Oregano	Alcohol/Monoterpene, Phenol	18.6357 ± 5.54	2389.00 ± 0.94
	HE-THY-01	Thyme	Alcohol, Esters, Monoterpene	28.2198 ± 4.76	8061.63 ± 1.15
	HE-MEL-01	Melissa Leaf	Sesquiterpene/Aldehyde, Monoterpene	35.2102 ± 11.51	158.08 ± 0.27
	HE-VER-01	Lemon Verbena	Aldehyde/Monoterpene	87.0619 ± 9.29	87.14 ± 1.36
	HE-SAU-02	Dalmatian Sage	Ketone, Monoterpene	181.1078 ± 22.34	66.28 ± 0.72
	HE-ROM-02	Rosemary	Monoterpene/ketone	417.4035 ± 56.97	13.49 ± 0.31
	HE-ROM-01	Rosemary	Monoterpene/ketone	580.0000 ± 64.81	12.03 ± 0.86

Table 2.3. Antioxidant properties of 38 EOs (Continued).

Type	ID	EO	Major Families of Compounds[a]	IC_{50} (mg/mg DPPH)	ORAC Value (μmol TE/g sample)
Botanical (Fruits, Vegetables, Conifers)	HE-CNB-01	Cranberry Seed	Alcohol/Sulfur containing, Aldehyde	43.6427 ± 4.01	1.01 ± 0.92
	HE-AIL-01	Garlic	Sulfur containing, Esters	58.2348 ± 2.81	21.77 ± 0.62
	HE-OIG-01	Onion	Sulfur containing, Esters	65.8506 ± 14.00	0.45 ± 0.07
	HE-AIL-02	Garlic	Sulfur containing, Esters	87.9036 ± 1.28	20.95 ± 0.73
	HE-AIL-03	Mexican Garlic Blend	Sulfur containing, Esters	174.8453 ± 2.78	39.86 ± 2.65
	HE-CIT-02	Lemon	Monoterpene, Aldehyde, Sesquiterpene	274.0933 ± 12.55	21.03 ± 1.30
	HE-PIN-02	White Pine	Monoterpene, Sesquiterpene, Esters	276.0524 ± 23.45	43.02 ± 0.16
	HE-CIT-01	Lemon	Monoterpene, Aldehyde, Sesquiterpene	298.0260 ± 50.21	20.62 ± 0.94
	HE-CED-01	Cedar	Ketone, Monoterpene, Alcohol	300.9034 ± 81.30	35.56 ± 0.84
	HE-CYP-01	Cypress	Monoterpene, Alcohol	335.6896 ± 58.80	37.14 ± 1.68
	HE-CED-02	Cedar	Ketone, Monoterpene, Esters	420.6031 ± 73.89	10.94 ± 0.15
	HE-CYP-02	Cypress	Monoterpene, Alcohol, Esters	451.1640 ± 54.11	20.93 ± 0.18
	HE-PIN-01	White Pine Needles	Monoterpene, Sesquiterpene, Esters	652.4518 ± 99.35	7.64 ± 0.72

[a]The chemical profiles of EOs were analyzed by GC-MS in our labs (Khodaei, Nguyen, Mdimagh, Bayen, & Karboune, 2020).

The EO with the best overall antioxidant capacity in both assays was pimento berry (HE-PIM-01, IC_{50} = 0.0816 mg/mg DPPH; ORAC Value = 8174.75 µmol TE/g), followed by pimento leaf (HE-PIM-02, IC_{50} = 0.1025 mg/mg DPPH; ORAC Value = 7,103.63 µmol TE/g) and clove bud oil (HE-CLO-01, IC_{50} = 0.1355 mg/mg DPPH; ORAC Value = 8765.50 µmol TE/g). According to a previous study (Khodaei et al., 2020), these oils are mainly composed of eugenol (phenol family)—it comprises up to 86% of both pimento chemical profiles and up to 94% of the clove bud profile. This could suggest that eugenol was the main driver behind the excellent performance of the oils. Misharina and colleagues (2015) found that pimento and clove oils showed comparable, high antioxidant capacity regardless of having different levels of eugenol. Eugenol was also found to be the main driver for the antioxidant capacity of clove, as its other components did not exhibit any antioxidant activity in the DPPH assay (Dawidowicz & Olszowy, 2014). Additionally, it has been shown that eugenol has efficient antiradical activity as one molecule of eugenol is capable of reducing almost two molecules of DPPH despite only having one available hydrogen on a hydroxyl group, making its activity comparable to butylated hydroxytoluene (BHT) (Brand-Williams, Cuvelier, & Berset, 1995; Mastelić et al., 2008).

The high radical scavenging activity of pimento EOs (HE-PIM-01 to -02) found in this study is consistent with the literature. Padmakumari, Sasidharan, and Sreekumar (2011) found that the two pimento EOs they tested had IC_{50} values of 4.82 ± 0.08 and 5.14 ± 0.11 µg/mL. Misharina and colleagues (2015) found that pimento EO displayed synergistic effects *in situ* between eugenol and its minor components when its activity was measured in the DPPH assay (IC_{50} = 20mg/L).

Clove, thyme, oregano, and cinnamon EOs have been shown to exhibit high antioxidant capacity (ORAC value = 2.43, 0.79-1.24, 2.24-2.62, 2.11 g Trolox/g EO, respectively) (Bentayeb, Vera, Rubio, & Nerín, 2014a), which agrees with this present study. The authors also investigated the combinatorial effects within the oils themselves and concluded that about 72-115% of antioxidant capacity of the EOs could be explained by the activity of their major compounds, noting that the fluorescein decay curves of some EOs matched the shape of the curves of their major compounds. They also remarked that these oils showed near-additive effects in the ORAC assay.

The oils with the lowest overall antioxidant capacity in both assays were white pine needles (HE-PIN-01, IC_{50} = 652.4518 mg/mg DPPH; ORAC Value = 7.64 µmol TE/g), followed by cypress (HE-CYP-02, IC_{50} = 451.1640 mg/mg DPPH; ORAC Value = 20.93 µmol TE/g) and cedar oil

(HE-CED-02, IC_{50} = 420.6031 mg/mg DPPH; ORAC Value = 10.94 µmol TE/g). These botanical oils from conifers are mainly composed of monoterpenes (**Table 2.3**), apart from cedar oil (HE-CED-02)—ketones make up its most abundant chemical family and monoterpenes make up its second-most abundant family. In fact, most oils with monoterpenes as the most abundant chemical family performed poorly in both the DPPH and ORAC assays. One of the most abundant monoterpenes present in these profiles is α-pinene, which is known to autoxidize alongside oxidizable material and propagate the oxidative radical chain (Amorati et al., 2013). These EOs are also composed of non-isoprenoid components (e.g. ketones, esters, etc.) and sesquiterpenes, which have been shown to have very low antioxidant activity (Ruberto & Baratta, 2000). This, coupled with the lack of phenols in the oils, could explain the lowered antioxidant capacity in the oils.

The weak antioxidant activity of pine EOs (HE-PIN-01, to -02) has not been reported in the literature. On the contrary, six EOs from the *Pinus* taxa sourced from China has been found to exhibit acceptable antioxidant capacity, with IC_{50} values ranging between 892.45-1851.65 µmol TE/g DW (Xie, Liu, & Li, 2015). Another study found that pine needle EO sourced from Korea to have an antioxidant activity (IC_{50} = 95.12 µg/ml) comparable to α-tocopherol (IC_{50} = 95.12 µg/ml) (Kwak, Moon, & Lee, 2006). The researchers hypothesized that the strong antioxidant capacity of the EOs could be from the presence of 4-hydroxy-5-methyl-3[2H]-furan, which was not found in the EOs used in this study (Khodaei et al., 2020).

Rosemary EOs (HE-ROM-01 to -02) displayed low antioxidant activity in both assays, which is consistent with a study that investigated the antioxidant properties of six rosemary EOs (Cutillas et al., 2018). They mention that the main components of rosemary did not show ORAC activity, concluding that the antioxidant activity measured (88.3 ± 10.9 to 226.1 ± 13.3 mg TE/g EO) was due to the action of its minor components. Furthermore, the researchers found that the only main components displaying reactivity towards the DPPH radical were α- and γ-terpinene. On the other hand, another study stated that rosemary EO had a strong radical scavenging activity (IC_{50} = 77.6 µl/ml). After comparing its activity to that of α-tocopherol (IC_{50} = 25.3 µg/ml), they found that 1 µg of the latter was equivalent to 3.1 µL of the former.

Both lemon oils (HE-CIT-01; HE-CIT-02), mainly composed of D-limonene, a monoterpene, also performed very poorly (IC_{50} = 298.0260 mg oil/mg DPPH; ORAC Value = 20.62 µmol TE/g; IC_{50}

= 274.0933 mg oil/mg DPH; ORAC value = 21.03 µmol TE/g, respectively). It has been shown that citrus oils mainly composed of limonene showed very low DPPH radical scavenging activity levels (Choi, Sun Song, Ukeda, & Sawamura, 2000).

Two clustering diagrams based on homology and chemical composition (**Fig. 2.1**) grouped EOs together based on the inverse IC_{50} and ORAC values, where EOs dispersed from each other bore little resemblance in activity with each other. Though some EOs may originate from the same botanical source of origin, this figure showed that there is some variance in their antioxidant capacity. That is, EOs of the same botanical source, but different plant material, were dispersed from each other, indicating that anatomy plays a role in chemical composition. This is especially true with cypress EOs (HE-CYP-01, branches; HE-CYP-02 needles and cones), which were composed of branches and needles/cones, respectively. Furthermore, "identical" EOs from different commercial suppliers had dissimilar antioxidant capacities, as observed in the dispersion of some outliers between the oregano (HE-ORI-03), thyme (HE-THY-02), sage (HE-SAU-01-02 and HE-SAU-02), and cypress oils (HE-CYP-01 and HE-CYP-02). These differences arise from the many factors that can influence the chemical profile of an EO, including geographical origin, climate, method of extraction, as well as storage conditions (Reyes-Jurado et al., 2015). On the other hand, some clove EOs displayed similar antioxidant capacities (HE-CLO-02 to -05), even though they were purchased from different suppliers and originated from different countries. The same can be said about selected cinnamon EOs (HE-CAN-01, HE-CAN-03) and garlic EOs (HE-AIL-01, HE-AIL-02).

Figure 2.1A shows the distribution of EO clusters based on their type (botanical, herb, spice), whereas **Figure 2.1B** illustrates the EO clusters based on the chemical families of their major compounds (>80%). It is important to note that each EO contains a plethora of major and minor compounds that fit under at least three chemical families. EOs with high antioxidant capacities in both assays (yellow/red squares) were all spices, with phenols as their main chemical family. This comes as no surprise since phenols are known to have excellent antioxidant activity (Tea Kulisic, Radonic, & Milos, 2005). Oils where the main chemical families were split equally between two families were dispersed towards the lower antioxidant capacity area of the diagram (blue squares). More specifically, families that included monoterpenes had weaker activity, notably alcohol/monoterpene and phenol/monoterpene. As previously mentioned, this could be due to the presence of pro-oxidants such as α-pinene.

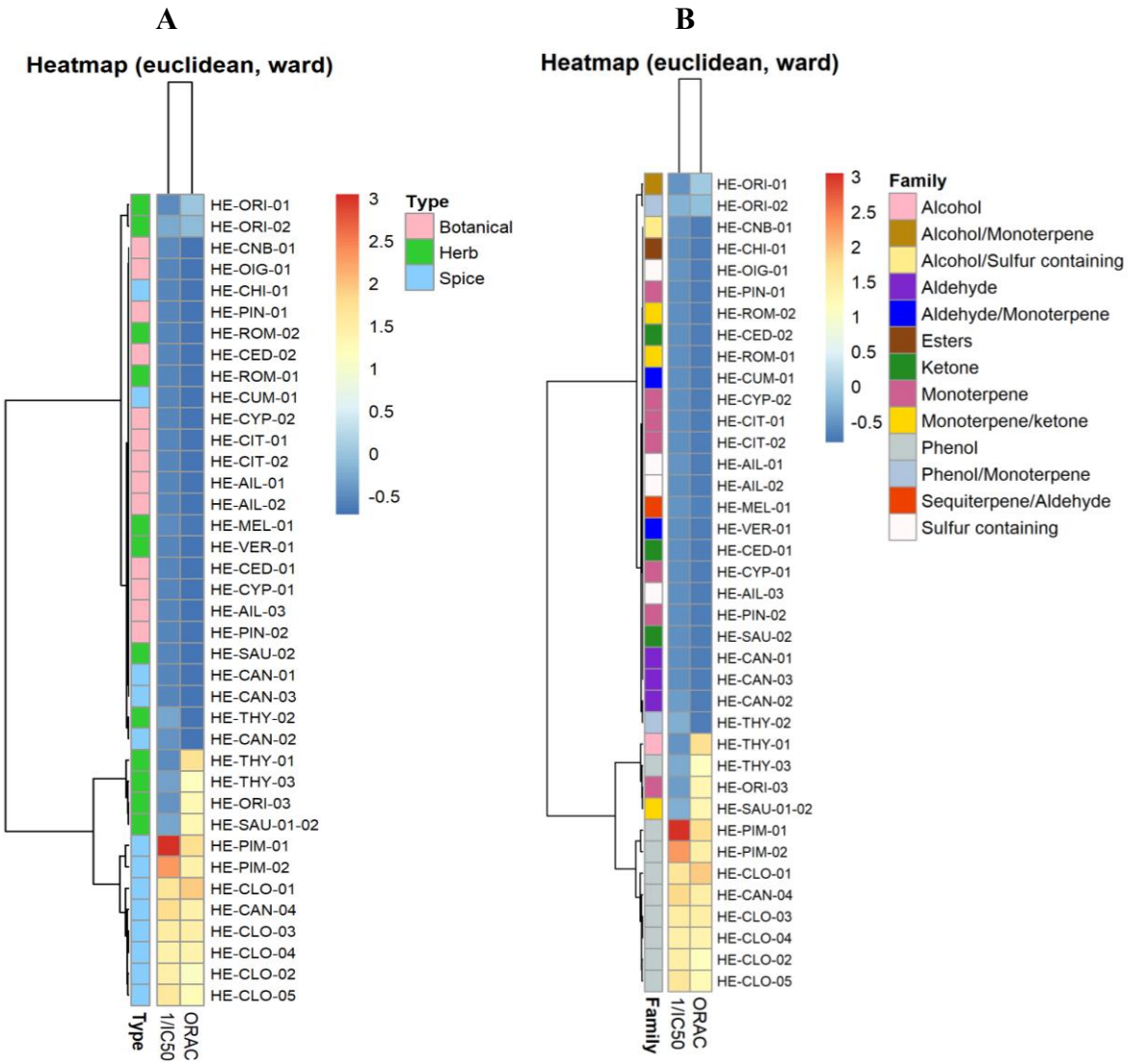


Figure 2.1. Heat map and clustering diagram of IC_{50} and ORAC values of EOs based on homology.

There was no significant difference between oils with monoterpenes and monoterpenes/ketones.

The heat maps revealed a misalignment in antioxidant capacity of some herb EOs (HE-THY-01, HE-THY-03, HE-ORI-03, HE-SAU-01-02) when it was measured with the DPPH and ORAC assays (**Fig. 2.1**), as shown by the presence of both blue and yellow squares in the $1/IC_{50}$ and ORAC columns, respectively. The DPPH and ORAC assays represent typical SET and HAT-based methods, respectively. The results show that the $1/IC_{50}$ and ORAC values of botanical EOs were well aligned. In comparison to the higher ORAC values of these selected herb EOs, the lower $1/IC_{50}$ values may be attributed to the presence of monoterpenes that demonstrate a lower antioxidant capacity in the DPPH assay, but display higher antioxidant activity when paired with active oxygen- or nitrogen-containing radicals (Misharina et al., 2015; Sacchetti et al., 2005). The majority of monoterpene hydrocarbons are known to have poor antioxidant activity, with the exception of α-terpinene, γ-terpinene, and terpinolene (Graßmann, 2005). These compounds stand out from their hydrocarbon counterparts due to the presence of methylene groups in their chemical structure (Ruberto & Baratta, 2000). Therefore, the presence of these compounds could contribute to the lower $1/IC_{50}$ value of the EOs, while also contributing to their higher ORAC values. The difference between the ORAC and DPPH assays has also been reported for different major tea catechins in which the ORAC assay was found to poorly reflect the antiradical activity based on their phenolic hydroxyl groups compared to the DPPH assay (Roy et al., 2010a). The same authors have hypothesized that a compound having a lower antioxidant capacity in the ORAC assay, compared with that of the DPPH assay, would exhibit a pro-oxidant behavior by generating reactive oxygen species that further react with the fluorescent compound (fluorescein), thereby enhancing the apparent loss of fluorescence. Such can be seen in pimento EOs (HE-PIM-01 to -02), where their $1/IC_{50}$ values showed greater antioxidant activity than their ORAC value.

To delineate the relationship between the $1/IC_{50}$ and ORAC values, a principal component analysis (PCA) was carried out (**Fig. 2.2**). The PCA revealed that the first principal component accounts for 88.5% of the total variance. The ORAC Value and $1/IC_{50}$ vectors were not parallel, indicating that there was no correlation between the two values. Therefore, a certain increase in one value will not automatically correspond to an identical increase in the other value. This could be due to the different mechanisms (SET and HAT) involved in both tests.

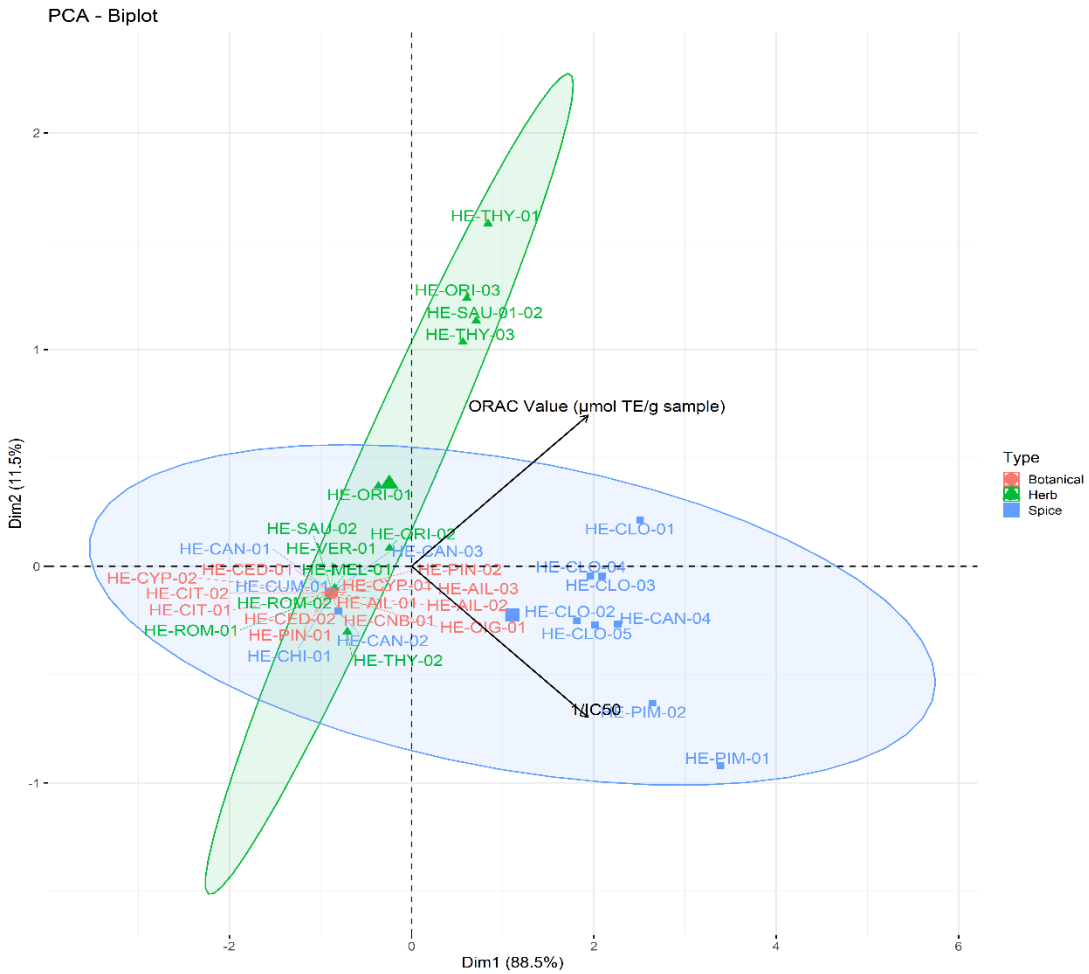


Figure 2.2. Principal component analysis of 38 botanical (■), herb (■), and spice (■) EOs based on their IC_{50} and ORAC values.

As such, the antioxidant activities of EOs will vary according to the transfer mechanism governing the chosen assay. Additionally, there could be some solvent effects that influence the outcome of these assays by modifying the bond dissociation energies of the active components (H. Y. Zhang & Wang, 2005). In polar solvents, the bond dissociation energy of -OH groups increase due to intermolecular hydrogen bonding. This results in a slower transfer of hydrogen atoms, subsequently decreasing radical-scavenging activity. In other words, compounds that favor hydrogen atom transfer will show a decrease in activity in such solvents.

EOs classified as spices very loosely followed the $1/IC_{50}$ vector, whereas EOs classified as herbs followed more or less the ORAC vector. The dispersion of the herbs in the same direction as the ORAC vector indicates that the ORAC assay could be a suitable method to use when comparing the antioxidant capacity of herbs, as it differentiates their activity quite well. Perhaps this category of EOs is best differentiated by the ORAC assay due to its solvent effects in the DPPH assay. The use of polar solvents such as ethanol and methanol in the assay could result in a decrease in activity and differentiation due to the intermolecular hydrogen bonding of the solvents with the hydroxyl moieties of compounds in these families.

To further understand the relationship between the chemical profile and the antioxidant capacity of EOs, these parameters were analyzed by response surface methodology, where the compositional data of the EOs were the independent variables, and the IC_{50} and the ORAC values were the dependent ones. The chemical profiles were previously analyzed by GC-MS in our labs (Khodaei et al., 2020). The results of ANOVA analyses are shown in **Table 2.4**. The IC_{50} and the ORAC value responses were both fitted into linear models, which were found to be statistically significant with an F-value of 25.78 and 15.9, respectively, and a P value of <0.0001. The coefficient of determination (R^2) was 0.865 and 0.79, the lack of fit was not statistically significant. In the predictive model of the ORAC value, the most significant independent variables were phenols (*F-value* of 99.88, *p-value* of < 0.0001), alcohols (*F-value* of 11.91, *p-value* of 0.0018), and esters (*F-value* of 4.11, *p-value* of 0.0523). While in the predictive model of the IC_{50} value, the most significant independent variables were phenols (*F-value* of 6.87, *p-value* of 0.0140), monoterpenes (*F-value* of 46.61, *p-value* of < 0.0001), alcohols (*F-value* of 7.08, *p-value* of 0.0127), and ketones (*F-value* of 11.51, *p-value* of 0.0021).

Table 2.4. ANOVA Analysis of the relationship between the chemical composition[a] and the antioxidant capacity (IC_{50} and ORAC values).

	IC_{50}		ORAC Values	
Source	F-value	*p*-value	F-value	*p*-value
Model	15.90	< 0.0001	25.78	< 0.0001
A-Aldehydes	0.0076	0.9313	0.1138	0.7384
B-Monoterpenes	46.61	< 0.0001	0.0631	0.8035
C-Alcohols	7.08	0.0127	11.91	0.0018
D-Sesquiterpenes	0.6547	0.4252	0.1838	0.6714
E-Ketones	11.51	0.0021	0.0045	0.9470
F-Phenols	6.87	0.0140	99.88	< 0.0001
G-Esters	1.14	0.2946	4.11	0.0523

[a]The chemical profiles of EOs were analyzed by GC-MS in our labs (Khodaei et al., 2020).

The equations of the predictive models generated by regression can be illustrated as follows:

ORAC Values = -410.21*-0.374 * Aldehyde -0.214 * Monoterpene +7.519*Alcohol +1.34* Sesquiterpene -0.084 * Ketone +7.915* Phenol +12.51* Esters

IC50 = 74.15 - 0.0056*Aldehyde + 0.3368*Monoterpene - 0.335*Alcohol - 0.1470*Sesquiterpene + 0.246*Ketone -0.120*Phenol + 0.381*Esters

Selected interactive effects of chemical components are shown in contour plots (**Fig. 2.3**). Contributions to antioxidant capacity are illustrated using a color-scale transitioning from blue (low/negative) to red (high/positive). **Figure 2.3A/2.3B** shows the positive synergistic interactive effect of phenols/esters and phenols/alcohols on the ORAC value since the color-scale moves further away from blue as each component increases in concentration. In this case, the phenols lead to a more significant increase in the antioxidant capacity. Interestingly, monoterpenes and ketones in the presence of phenols do not affect the ORAC value, as an increase in their concentrations reveal a constant antioxidant capacity (**Fig. 3C/3D**), such as in the case of clove bud (HE-CLO-01, 8,766 μmol TE/g sample). In contrast, monoterpenes or ketones paired with phenols exhibited an antagonistic interactive effect on the anti-scavenging activity, increasing the IC_{50} values as their contents increased (**Fig. 2.3G/2.3H**). This can be seen in oregano EO (HE-ORI-02, IC_{50} = 0.96 mg/mg DPPH; ORAC value = 2,003 μmol TE/g sample).

Phenols paired with alcohols showed a slightly synergistic effect in the IC_{50} value, whereas esters did not affect the IC_{50} and increasing the phenolic content resulted in an increase in antioxidant activity (**Fig 2.3E/2.3F**). It is expected that the oxygenated constituents (e.g. alcohols, aldehydes, esters, ketones, phenols, and oxides) display a larger contribution to the antioxidant activities of EOs, in comparison to their non-oxygenated counterparts. However, it is important to note that some non-oxygenated terpenes, especially cyclic monoterpenes with 1,4-cyclohexadiene moieties, enhance free radical scavenging activity (Wojtunik, Ciesla, & Waksmundzka-Hajnos, 2014). Furthermore, Wojtunik *et al.* (2014) remark that the presence of conjugated double bonds is especially helpful since π-bonds are responsible for chain-breaking antioxidant activity in monoterpenes.

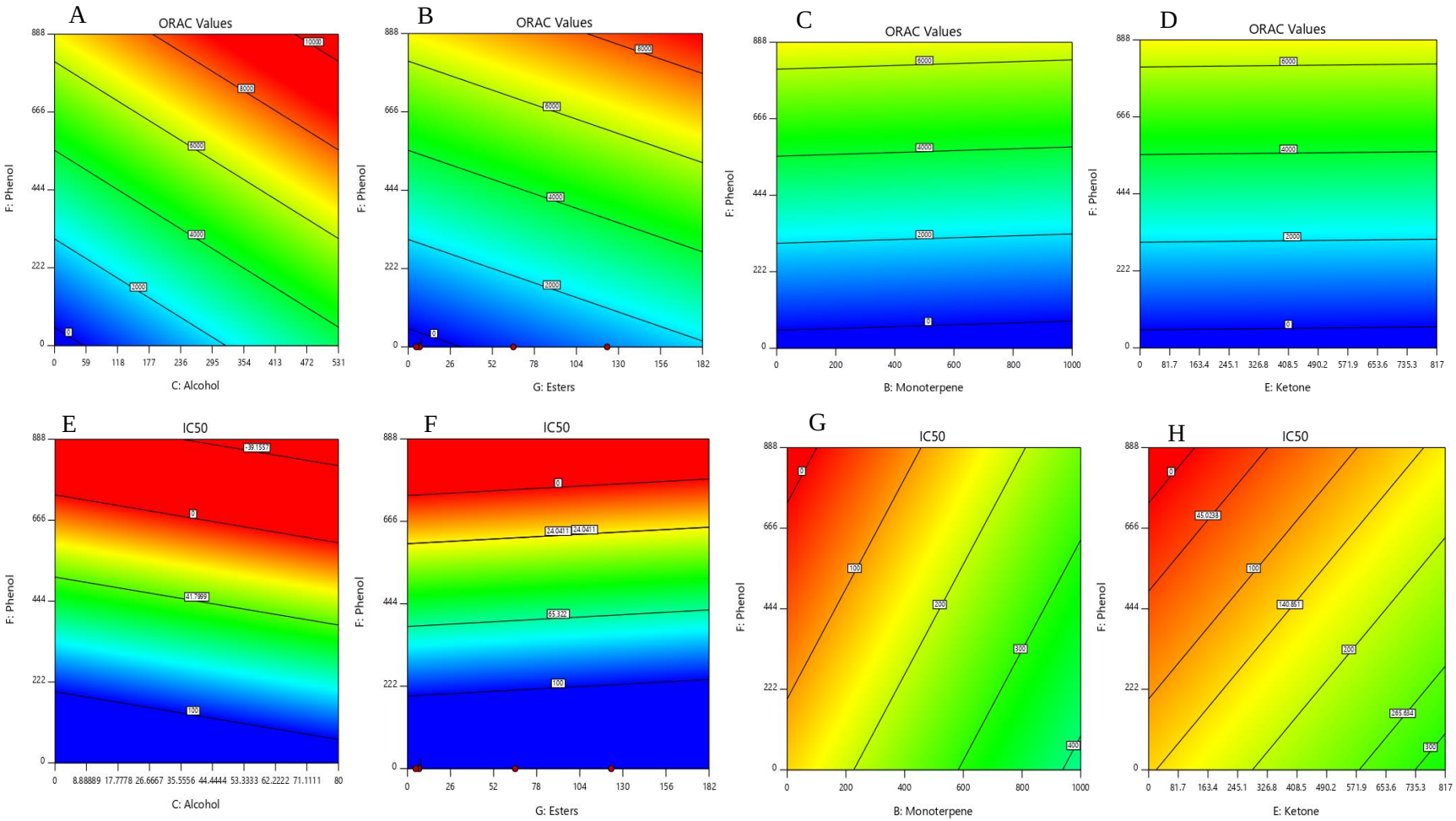


Figure 2.3. Counter Plots of ORAC (0 8800) and IC50 values (123 0) from the predictive models.

2.4.2. Antioxidant Capacity of EOs Enriched with Individual PPs

The addition of singular major PPs of plant extracts to six selected EOs showed some improvement in the antioxidant capacity of the EOs but did not greatly improve the capacities of some major PPs (**Table 2.5**). These major compounds included four flavonoids (rutin hydrate, quercetin, epicatechin, catechin) and four phenolic acids (rosmarinic acid, *p*-coumaric acid, chlorogenic acid, ferulic acid). Regardless of interactions with EOs, the IC_{50} and ORAC values of the enrichments followed the "strength" of their respective PPs enrichment. For the phenolic acids, the average antioxidant activities of the EOs were as follows according to enrichment: rosmarinic acid > chlorogenic acid > ferulic acid > *p*-coumaric acid. This pattern can be attributed to the number of -OH groups available for hydrogen atom transfer, notably those in the phenyl moieties (rosmarinic acid: 2x2, chlorogenic acid: 2, ferulic acid: 1, *p*-coumaric acid: 1) (Peyrat-Maillard et al., 2003). The difference in activity between ferulic acid and *p*-coumaric acid lies in the methoxy substitution in the ortho position to its hydroxyl group (Rice-Evans, Miller, & Paganga, 1996). The same can be said for the flavonoids - the average antioxidant activities of the EOs depended on the structural advantages of the compounds. In descending order, these advantages resulted in the following antioxidant efficiencies based on enrichment: quercetin > epicatechin > catechin > rutin hydrate. Quercetin held the best advantage thanks to its double bond and 4-oxo function in its C-ring, as well as its catechol group in its B-ring. The saturated heterocyclic ring in epicatechin's and catechin's structure leads to their lower antioxidant capacity. The difference in activity between epicatechin and catechin lies in the stereo-position of catechin's 3-OH group in its C-ring (Peyrat-Maillard et al., 2003). Flavonoids and phenols are known as hydrogen-donating radical scavengers and their efficiencies are increased with increasing hydroxyl groups. More specifically, the presence and availability of -OH groups in catechol groups in flavonoids are highly desirable, as are the -OH groups attached to the aromatic moieties of phenolic acids (Jovanovic, Steenken, Hara, & Simic, 1996; Rice-Evans et al., 1996). Rice-Evans and colleagues (1996) note that unsaturation in the C-ring of flavonoids is important, as it allows electron delocalization. Therefore, the 2,3-double bond and 4-oxo function in the C-ring offers structural advantage to quercetin (Jovanovic et al., 1996) when compared to epicatechin, which has a saturated heterocyclic ring. Additionally, glycosylation of flavonoids at the 3-OH group tends to decrease their activity, such as in the case with the glycosylation of quercetin, producing rutin (Shahidi, Janitha, & Wanasundara, 1992).

Table 2.5. Antioxidant Capacity of EOs Enriched with Individual Polyphenols.

Enrichment	Oil ID[a]	IC_{50} (mg oil + polyphenol/mg DPPH)	Effect[b]	ORAC Value (µmol TE/g oil +polyphenol)	Effect[b]
Rutin Hydrate	N/A	0.1779 ± 0.0029	N/A	7,075.90 ± 2.11	N/A
	HE-CLO-01	0.1614 ± 0.0038	+	3,716.95 ± 4.32	−
	HE-PIM-01	0.1140 ± 0.0048	+	6,227.00 ± 5.98	−
	HE-ORI-03	0.3112 ± 0.0003	+ + +	5,427.50 ± 6.23	−
	HE-THY-03	0.3058 ± 0.0005	+ + +	4,986.50 ± 2.72	−
	HE-SAU-01-02	0.2837 ± 0.0008	+ + +	2,298.50 ± 1.25	−
	HE-CAN-04	0.1326 ± 0.0070	+ + +	6,329.50 ± 3.23	+
Rosmarinic Acid	N/A	0.0635 ± 0.0010	N/A	8,296.20 ± 1.94	N/A
	HE-CLO-01	0.1210 ± 0.0041	−	17,879.50 ± 4.08	+ + +
	HE-PIM-01	0.0975 ± 0.0008	−	13,511.00 ± 3.83	+ + +
	HE-ORI-03	0.1355 ± 0.0023	+ + +	10,067.00 ± 1.19	+ + +
	HE-THY-03	0.1340 ± 0.0024	+ + +	8,680.00 ± 1.36	+ + +
	HE-SAU-01-02	0.1448 ± 0.0010	+ + +	5,772.00 ± 1.80	−
	HE-CAN-04	0.0891 ± 0.0033	+ + +	12,916.00 ± 3.86	+ + +
Quercetin	N/A	0.0513 ± 0.0020	N/A	13,143.00 ± 0.48	N/A
	HE-CLO-01	0.1060 ± 0.0039	+	14,567.00 ± 1.89	+ + +
	HE-PIM-01	0.0698 ± 0.0017	+	13,502.50 ± 1.87	+ + +
	HE-ORI-03	0.0891 ± 0.0024	+ + +	12,714.00 ± 0.86	+ + +
	HE-THY-03	0.0921 ± 0.0022	+ + +	12,228.50 ± 1.77	+ + +
	HE-SAU-01-02	0.0906 ± 0.0021	+ + +	9,776.00 ± 1.99	+
	HE-CAN-04	0.0682 ± 0.0012	+ + +	12,921.50 ± 1.67	+ + +
p-Coumaric acid	N/A	0.7590 ± 0.0100	N/A	10,762.00 ± 3.17	N/A
	HE-CLO-01	0.2155 ± 0.0028	+ + +	3,400.00 ± 4.77	−
	HE-PIM-01	0.1822 ± 0.0021	+ + +	11,588.00 ± 4.50	+ + +
	HE-ORI-03	0.7749 ± 0.0071	+ + +	10,242.50 ± 0.98	+
	HE-THY-03	0.5832 ± 0.0056	+ + +	10,349.00 ± 2.07	+ + +
	HE-SAU-01-02	1.1424 ± 0.0050	+	7,451.00 ± 2.56	−
	HE-CAN-04	0.1865 ± 0.0065	+ + +	10,892.50 ± 5.33	+ + +
Epicatechin	N/A	0.0494 ± 0.0028	N/A	6,593.00 ± 1.05	N/A
	HE-CLO-01	0.1581 ± 0.0058	−	2,252.45 ± 2.61	−
	HE-PIM-01	0.0750 ± 0.0021	+	9,170.50 ± 2.10	+ + +
	HE-ORI-03	0.0940 ± 0.0057	+ + +	11,907.50 ± 1.63	+ + +
	HE-THY-03	0.0817 ± 0.0015	+ + +	11,489.00 ± 0.71	+ + +
	HE-SAU-01-02	0.0853 ± 0.0058	+ + +	9,058.50 ± 2.77	+ + +
	HE-CAN-04	0.0699 ± 0.0030	+ + +	12,459.00 ± 2.38	+ + +

Table 2.5. Antioxidant Capacity of EOs Enriched with Individual Polyphenols (Continued)

Enrichment	Oil ID[a]	IC_{50} (mg oil + polyphenol/mg DPPH)	Effect[b]	ORAC Value (µmol TE/g oil +polyphenol)	Effect[b]
Chlorogenic Acid	N/A	0.0932 ± 0.0012	N/A	6,438.20 ± 2.72	N/A
	HE-CLO-01	0.1032 ± 0.0006	+	13,014.50 ± 3.25	+ + +
	HE-PIM-01	0.1342 ± 0.0037	−	9,497.50 ± 3.11	+ + +
	HE-ORI-03	0.2160 ± 0.0009	+ + +	10,272.00 ± 2.67	+ + +
	HE-THY-03	0.2068 ± 0.0026	+ + +	9,599.50 ± 2.81	+ + +
	HE-SAU-01-02	0.2207 ± 0.0010	+ + +	4,709.40 ± 2.34	−
	HE-CAN-04	0.1259 ± 0.0023	+	9,870.00 ± 3.25	+ + +
Catechin	N/A	0.0491 ± 0.0022	N/A	11,950.00 ± 6.92	N/A
	HE-CLO-01	0.0916 ± 0.0036	+ + +	9,887.00 ± 3.01	+
	HE-PIM-01	0.0878 ± 0.0023	−	12,937.00 ± 3.04	+ + +
	HE-ORI-03	0.0791 ± 0.0013	+ + +	12,603.00 ± 1.52	+ + +
	HE-THY-03	0.1289 ± 0.0046	+ + +	12,669.50 ± 3.41	+ + +
	HE-SAU-01-02	0.1310 ± 0.0043	+ + +	8,947.00 ± 2.27	+
	HE-CAN-04	0.0806 ± 0.0032	+ + +	13,511.00 ± 1.98	+ + +
Ferulic Acid	N/A	0.2070 ± 0.0012	N/A	18,915.00 ± 0.14	N/A
	HE-CLO-01	0.1337 ± 0.0006	+ + +	18,915.00 ± 1.68	+ + +
	HE-PIM-01	0.3675 ± 0.0037	−	16,703.50 ± 2.30	+ + +
	HE-ORI-03	0.3896 ± 0.0009	+ + +	7,787.50 ± 0.55	−
	HE-THY-03	0.2564 ± 0.0026	+ + +	19,273.50 ± 0.37	+ + +
	HE-SAU-01-02	0.2413 ± 0.0010	+ + +	16,581.00 ± 0.44	+ + +
	HE-CAN-04	0.3739 ± 0.0023	−	16,004.00 ± 0.26	+ + +

[a]*HE-CLO-01*: Clove EO; *HE-PIM-01*: Pimento Berry EO; *HE-ORI-03*: Oregano EO; *HE-THY-03*: Thyme EO; *HE-SAU-01-02*: Yellow Sage EO; *HE-CAN-04*: Cinnamon EO

[b]Interaction effects were identified to be *antagonistic* (−), *additive* (+), *or synergistic* (+ + +) when the antioxidant capacity of the mixture was respectively less than, equal to, or greater than the antioxidant capacity of the sum of its individual compounds (expected value).

This decrease can even be seen in the antioxidant activity of EOs enriched with rutin hydrate. Therefore, the effects of these nuances can be seen even in complex-antioxidant systems. In other words, the differences in their antioxidant capacities due to their structural differences can be seen even when enriching and interacting with EOs, as can be seen in **Table 2.5**. In addition to structural advantages, it is also important to take interactions into account to determine synergism, addition, and antagonism between mixtures. The EO/PP pair with the lowest IC_{50} (highest antioxidant activity) was Ceylon cinnamon (HE-CAN-04)/quercetin (IC_{50} = 0.0681 mg/mg DPPH) and the pair with the highest IC_{50} was yellow sage (HE-SAU-01-02)/*p*-coumaric acid (IC_{50} = 1.1424 mg/mg DPPH). The former pair's high antioxidant capacity is supported by the fact that quercetin can be found *in situ* in *Cinnamomum* species (K. N. Prasad et al., 2009), therefore the addition of supplementary quercetin reinforces the already-present synergistic effects within the oil itself. The latter pair exhibited the lowest antioxidant capacity, which, based on a previous analysis of yellow sage oil's chemical profile (Khodaei et al., 2020), could be due to the lack of phenols present in the EO, in addition to *p*-coumaric acid's general poor performance in the DPPH assay (Nenadis & Tsimidou, 2002; Ohnishi et al., 1994) due to the lack of a catechol group.

In general, mixtures enriched with rutin hydrate, quercetin, and *p*-coumaric acid did not show antagonistic effects in the DPPH assay. Instead, they exhibited additive and synergistic effects. Rutin hydrate has been shown to exhibit synergistic effects with terpenoids such as γ-terpinene (Graßmann, 2005), which could explain the interaction effects between it and the EOs. Interestingly, many pimento berry EO (HE-PIM-01) enrichments exhibited antagonistic effects, including those with rosmarinic acid (IC_{50} = 0.1025 mg/mg DPPH), chlorogenic acid (IC_{50} = 0.1342 mg/mg DPPH), catechin (IC_{50} = 0.0878 mg/mg DPPH), and ferulic acid (IC_{50} = 0.3675 mg/mg DPPH).

As for the ORAC assay, the EO/PP pair with the highest ORAC value was white thyme (HE-THY-03)/ferulic acid (ORAC value = 19,273.50 µmol TE/g oil + polyphenol). Whereas the EO/PP pair with the lowest antioxidant capacity was clove (HE-CLO-01)/epicatechin (ORAC value = 2,252.45 µmol TE/g oil + polyphenol). Quercetin and catechin were the only enrichments that did not show antagonistic effects. Rutin hydrate exhibited the greatest number of antagonistic effects when enriching EOs in the ORAC assay. Of the EOs, yellow sage (HE-SAU-01-02) exhibited the most antagonistic effects when enriched with PPs, notably rutin hydrate (ORAC value = 2,298.50 µmol TE/g oil + polyphenol), rosmarinic acid (ORAC value = 5,772.00 µmol TE/g oil + polyphenol), *p*-coumaric acid

(ORAC value = 7,451.00 µmol TE/g oil + polyphenol), and chlorogenic acid (ORAC value = 4,709.40 µmol TE/g oil + polyphenol).

The synergistic interactive effects could be due to the ability of the antioxidants to regenerate other antioxidants (Peyrat-Maillard et al., 2003). Peyrat-Maillard and colleagues (2003) remarked in their study that some antioxidant mixtures tended to exhibit synergistic effects when weaker antioxidants regenerated more efficient antioxidants. Indeed, this synergistic effect arise when a primary antioxidant (more effective free radical scavenger) has a higher reduction potential than the co-antioxidant/synergist (less effective free radical) (Choe & Min, 2009; Pedrielli & Skibsted, 2002). It has been shown that quercetin, epicatechin and catechin are capable of regenerating α-tocopherol in tert-butyl alcohol and chlorobenzene (Pedrielli & Skibsted, 2002), thereby resulting in a co-antioxidant effect. In the case of antagonism, the opposite is true—the more efficient antioxidant will regenerate the less efficient antioxidant, thus resulting in a lower overall antioxidant capacity. Peyrat-Maillard and colleagues (2003) confirmed that the antagonistic effects in their study between rosmarinic acid and α-tocopherol, as well as caffeic acid and α-tocopherol resulted from the regeneration of the least efficient antioxidant (α-tocopherol) by the most efficient antioxidant in both pairs (rosmarinic acid, caffeic acid, respectively). However, it is important to note that this explanation is not the phenomenon behind the synergy and antagonism in multi-component systems. Other factors include the polarity of the molecules (Cuvelier, Bondet, & Berset, 2000) and the influences of the microenvironment (Koga & Terao, 1995).

2.4.3. Antioxidant Capacity of EOs Enriched with PP mixes

Aside from rosemary's PP mix, the IC_{50} of all PP mixes (without enrichment) were lower than their expected IC_{50}, thus displaying antagonistic effects (**Table 2.6**). This could be due to flavonoid-flavonoid interactions, where hydrogen bonding between flavonoids results in a decrease in the availability of -OH groups (Hidalgo, Sánchez-Moreno, & de Pascual-Teresa, 2010). This, in turn, decreases the possibility of interaction with the DPPH radical, which then lowers the resulting antioxidant capacity in this assay. As for the ORAC assay, all PP mixes, except grape seed's PP mix, showed synergistic effects. It has been found that the ORAC value of a pair of flavonoids can significantly increase when a third flavonoid with a low reduction potential energy is added to them (Freeman, Eggett, & Parker, 2010).

Table 2.6. Combinatorial effects of polyphenol mixtures.

Polyphenol Mix[a]	Expected IC_{50} (mg/mg DPPH)	Measured IC_{50} (mg/mg DPPH)	Effect[b]	Expected ORAC (μmol TE/g mix)	Measured ORAC (μmol TE/g mix)	Effect[b]
EX-RAI-01	0.0492	0.0622	−	9,170.91	1,772.50	−
EX-THE-01	0.0543	0.0714	−	7,732.46	19,167.00	+ + +
EX-POM-04	0.0908	0.1010	−	7,288.54	12,318.00	+ + +
EX-ROM-04	0.6184	0.3480	+ + +	10,263.39	16,611.00	+ + +

[a]*EX-RAI-01*: Grape seed extract; *EX-THE-01*: Green tea extract; *EX-POM-04:* Apple extract; *EX-ROM-04:* Rosemary extract
Polyphenol mixes consist of the major compounds present in their respective plant extracts. Expected values calculated based on the sum of the individual compounds. See Table 2.2 for proportions.

[b]Interaction effects were identified to be *antagonistic* (−), *additive* (+), *or synergistic* (+ + +) when the antioxidant capacity of the mix was respectively less than, equal to, or greater than the antioxidant capacity of the sum of its individual compounds (expected value).

The authors found that in the case of a ternary mixture, the concentration of flavonoids was more important, with a lower concentration being more favorable, than the presence of favorable functional groups or the efficiency of electron donation.

Another study found that in mixtures of flavonoids from strawberries, mixtures with compounds that had similar reduction potential energies showed lower antioxidant activities (Reber, Eggett, & Parker, 2011), which could account for the antagonism within the grape seed PP mix. They hypothesized that the compounds were drawing electrons away but were not donating them as readily to the AAPH radical, thus lowering the overall antioxidant capacity. The authors note that although the ORAC assay is a HAT-based method, an electron transfer does take place in the mechanism, justifying the importance of analyzing reduction potentials of compounds.

When enriched with grape seed PP mix **(Table 2.7)**, all EOs showed synergistic effects in the DPPH assay, except the enrichment of pimento berry (HE-PIM-01, IC_{50} = 0.0744 mg/mg DPPH), which showed additive effects. Similarly, all enrichments showed synergistic effects in the ORAC assay, except the enrichment of oregano (HE-ORI-03, ORAC value = 1,219.40 μmol TE/g sample), which exhibited antagonistic effects. All EOs containing eugenol (clove, HE-CLO-01; pimento berry, HE-PIM-01; Ceylon cinnamon, HE-CAN-01) showed similar IC_{50} and ORAC values. This suggests that the mixture of epicatechin and catechin mostly interacted with the eugenol present in the EOs. EOs whose major compounds did not include phenols (oregano, HE-ORI-03; yellow sage, HE-SAU-01-02) exhibited lower ORAC values. As previously mentioned, oregano (HE-ORI-03)/grape seed mix showed antagonistic effects in the ORAC assay, whereas all other enrichments exhibited synergistic effects. This suggests that despite the antagonistic effects within the grape seed PP mix *in situ* (**Table 2.6**), enriching these mixes with EOs, except that of oregano (HE-ORI-03), overcomes the antagonism in the ORAC assay. Perhaps there is an antagonism in the oregano (HE-ORI-03)/grape seed PP mix enrichment due to the large quantity of α-pinene (55%, Khodaei et al., 2020) present in the oil. Therefore, in addition to the antagonism in the grape seed PP mix, the co-oxidizing activity of α-pinene further decreases the overall antioxidant activity of the enrichment.

EOs enriched with the major PPs of green tea extract (EX-THE-01) showed the lowest IC_{50} values among the 4 enrichments, thus exhibiting the highest antioxidant capacity.

Table 2.7. Antioxidant capacity of EOs enriched with polyphenol mixtures and pure plant extracts.

		Polyphenol Mix								Crude Plant Extract							
Enrichment[a]	Essential Oil ID	IC_{50} (mg oil/mg DPPH)			Effect[b]	ORAC Value (μmol TE/g sample)			Effect	IC_{50} (mg oil/mg DPPH)			Effect	ORAC Value (μmol TE/g sample)			Effect
EX-RAI-01	N/A	0.0622	±	0.0046	N/A	1,772.50	±	0.92	N/A	0.0888	±	0.0044	N/A	49,119.00	±	0.64	N/A
	HE-CLO-01	0.0713	±	0.0017	+ + +	12,764.50	±	1.85	+ + +	0.0139	±	0.0004	+ + +	25,758.50	±	3.42	+
	HE-PIM-01	0.0744	±	0.0027	+	13,058.00	±	1.64	+ + +	0.0146	±	0.0004	+ + +	12,468.50	±	3.42	−
	HE-ORI-03	0.1348	±	0.0061	+ + +	1,219.40	±	0.39	−	0.0135	±	0.0004	+ + +	36,607.50	±	1.55	+ + +
	HE-THY-03	0.1022	±	0.0015	+ + +	11,870.00	±	0.74	+ + +	0.0143	±	0.0004	+ + +	25,178.00	±	2.40	+
	HE-SAU-01-02	0.1006	±	0.0019	+ + +	7,483.00	±	1.39	+ + +	0.0138	±	0.0004	+ + +	26,152.50	±	1.36	+
	HE-CAN-04	0.0849	±	0.0024	+ + +	12,796.50	±	3.74	+ + +	0.0143	±	0.0003	+ + +	12,790.50	±	2.40	−
EX-THE-01	N/A	0.0714	±	0.0047	N/A	19,167.00	±	1.48	N/A	0.0718	±	0.0031	N/A	38,095.00	±	1.72	N/A
	HE-CLO-01	0.0642	±	0.0004	+ + +	12,473.50	±	2.42	+	0.0208	±	0.0005	+ + +	24,684.00	±	1.62	+
	HE-PIM-01	0.0090	±	0.0003	+ + +	13,681.00	±	1.85	+	0.026	±	0.0005	+ + +	39,921.50	±	2.86	+ + +
	HE-ORI-03	0.0144	±	0.0001	+ + +	6,207.50	±	0.83	−	0.0208	±	0.0209	+ + +	21,298.50	±	6.59	+
	HE-THY-03	0.0137	±	0.0001	+ + +	11,692.50	±	1.68	+	0.0192	±	0.0002	+ + +	23,003.00	±	2.96	+
	HE-SAU-01-02	0.0132	±	0.0001	+ + +	9,685.50	±	0.5	−	0.0201	±	0.0006	+ + +	20,030.00	±	1.94	+
	HE-CAN-04	0.0681	±	0.0005	+ + +	12,413.00	±	4.82	+ +	0.0204	±	0.0004	+ + +	24,199.00	±	2.36	+
EX-POM-04	N/A	0.101	±	0.0012	N/A	12,318.00	±	2.02	N/A	0.2148	±	0.0134	N/A	24,721.00	±	3.52	N/A
	HE-CLO-01	0.0131	±	0.0043	+ + +	12,720.50	±	3.04	+ + +	0.0406	±	0.0009	+ + +	20,954.00	±	1.68	+ + +
	HE-PIM-01	0.1043	±	0.0035	+	11,740.00	±	3.41	+	0.0442	±	0.0010	+ + +	23,100.00	±	1.79	+ + +
	HE-ORI-03	0.1863	±	0.0014	+ + +	1,151.65	±	1.15	−	0.0376	±	0.0002	+ + +	14,475.00	±	0.87	+
	HE-THY-03	0.1836	±	0.0017	+ + +	1,156.40	±	1.58	−	0.0459	±	0.0008	+ + +	21,128.00	±	1.15	+ + +
	HE-SAU-01-02	0.1779	±	0.0013	+ + +	601.65	±	0.71	−	0.0456	±	0.0005	+ + +	15,410.50	±	1.18	+
	HE-CAN-04	0.1160	±	0.0046	+ + +	11,338.50	±	2.38	+	0.0482	±	0.0008	+ + +	21,813.50	±	1.90	+ + +

Table 2.7. Antioxidant Capacity of EOs enriched with polyphenol mixtures and pure plant extracts (Continued)

		Polyphenol Mix							Crude Plant Extract						
Enrichment[a]	Essential Oil ID[b]	IC_{50} (mg oil/mg DPPH)			Effect[c]	ORAC Value (μmol TE/g sample)			Effect	IC_{50} (mg oil/mg DPPH)			Effect	ORAC Value (μmol TE/g sample)			Effect
EX-ROM-04	N/A	0.3480	±	0.0033	N/A	16,611.00	±	1.62	N/A	0.1742	±	0.008	N/A	11,729.00	±	1.28	N/A
	HE-CLO-01	0.1574	±	0.0043	+ + +	12,481.50	±	4.26	+	0.1153	±	0.0044	+ + +	9,529.50	±	2.25	+
	HE-PIM-01	0.1116	±	0.0063	+ + +	16,148.00	±	0.97	+ + +	0.1257	±	0.0035	+	15,089.00	±	2.41	+ + +
	HE-ORI-03	0.6605	±	0.0154	+ + +	11,193.50	±	2.62	+	0.3949	±	0.0038	+ + +	4,487.35	±	0.33	−
	HE-THY-03	0.6674	±	0.0124	+ + +	10,590.00	±	3.79	+	0.4230	±	0.007	+ + +	10,893.50	±	1.84	+ + +
	HE-SAU-01-02	0.6731	±	0.0094	+ + +	8,620.00	±	1.22	−	0.3983	±	0.0048	+ + +	5,605.50	±	1.49	−
	HE-CAN-04	0.1645	±	0.0015	+ + +	18,477.00	±	0.92	+ + +	0.1244	±	0.003	+ + +	13,828.50	±	2.09	+ + +

[a]*EX-RAI-01*: Grape seed extract; *EX-THE-01*: Green tea extract; *EX-POM-04:* Apple extract; *EX-ROM-04:* Rosemary extract

[b]Interaction effects were identified to be *antagonistic* (−), *additive* (+), *or synergistic* (+ + +) when the antioxidant capacity of the enrichment was respectively less than, equal to, or greater than the antioxidant capacity of the sum of its individual compounds (expected value).

As for the ORAC assay, oils paired with green tea mix (EX-THE-01) exhibited moderate to high values (6,207.50-13,681.00 μmol TE/g sample). Pimento berry (HE-PIM-01)/green tea showed the highest overall antioxidant capacity (IC_{50} = 0.0090 mg/mg DPPH; ORAC Value = 13,681.00 μmol TE/g sample). The IC_{50} value of Pimento berry (HE-PIM-01)/green tea is significantly lower than all other enrichments and the interaction between this mix and the eugenol in pimento berry (HE-PIM-01, 86.4% eugenol) alone cannot explain these synergistic effects (**Table 2.7**). Indeed, if the synergistic effects were solely attributed to the interactions between the mix and eugenol, then clove oil (HE-CLO-01) would logically have a higher antioxidant capacity than pimento berry (HE-PIM-01), considering it has a higher amount of eugenol (93.5%). These effects could be due to the contribution of the minor compounds of pimento berry, such as methyleugenol. As with the enrichments with the grape seed (EX-RAI-01) mix, this PP mix showed low ORAC values when enriching oregano (HE-ORI-03) and yellow sage (HE-SAU-01-02) EOs, with both enrichments exhibiting antagonistic effects, which could be due to the lack of phenols in their chemical profiles and/or the presence of pro-oxidants, which can go on to generate reactive species (hydrogen peroxide) that react with the fluorescent probe (fluorescein), thus decreasing the fluorescence and overall antioxidant capacity (Roy et al., 2010b).

The enrichment of EOs with apple PP mix did not show antagonistic effects in the DPPH assay, however, there were antagonistic effects in the ORAC assay when enriching oregano (HE-ORI-03, ORAC value = 1,151.65 μmol TE/g sample), white thyme (HE-THY-03, ORAC value = 1,156.40 μmol TE/g sample), and yellow sage (HE-SAU-01-02, ORAC Value = 601.65 μmol TE/g sample), with the last enrichment exhibiting the poorest activity. Looking back at **Table 2.2**, yellow sage (HE-SAU-01-02) exhibited some of the lowest antagonistic ORAC values when paired with chlorogenic acid and rutin. This antagonism could arise from the oxidation of the more effective antioxidant(s) by the less effective antioxidant(s) or the regeneration of the latter by the former (Peyrat-Maillard et al., 2003). In the case of yellow sage (HE-SAU-01-02), perhaps this oxidation and/or regeneration mechanism occurred in both individual PP enrichments and by combining these mechanisms together, any trace of effective antioxidant is practically lost. Additionally, the presence of possible pro-oxidant monoterpenes in yellow sage could contribute to the antagonistic effects, as previously mentioned. The enrichment of clove (HE-CLO-01) with apple PP mix showed synergistic effects in both assays (IC_{50} = 0.0131 mg/mg DPPH; ORAC Value = 12,720.50 μmol TE/g sample), whereas pimento berry (HE-PIM-01)/apple mix (EX-POM-04)

(IC_{50} = 0.1043 mg/mg DPPH; ORAC value = 11,740.00 μmol TE/g sample) and Ceylon cinnamon (HE-CAN-04)/apple PP mix (IC_{50} = 0.1160 mg/mg DPPH; ORAC Value = 11,338.50 μmol TE/g sample) showed additive/synergistic and synergistic/additive effects in the DPPH/ORAC assays, respectively. The enrichment of these three oils with the apple PP mix exhibited ORAC values more than ten times greater than enrichments with the other oils. As previously mentioned, such synergistic effects could be due to the interaction of the mix with the eugenol in all three oils. Indeed, the ORAC values seem to increase with increasing eugenol content in the three oils (clove, HE-CLO-01, 93.5%, 12,720.50 μmol TE/g sample > pimento berry, HE-PIM-01, 86.4%, 11,740.00 μmol TE/g sample > Ceylon cinnamon, HE-CAN-04, 84.6%, 11,338.50 μmol TE/g sample). Clove (HE-CLO-01)/apple PP mix has the greatest activity in the DPPH assay, which could be due to the number of compounds present in the mixture. Since clove oil (HE-CLO-01) is composed of less compounds than the other two oils, its synergistic effects with the apple mix could arise from a lack of hindrance to the DPPH radical from its components.

All EOs paired with the rosemary PP mix did not exhibit any antagonism in the DPPH assay, however, this enrichment yielded some of the highest IC_{50} values (lowest antioxidant capacity), though all were synergistic. The high absolute values of the IC_{50} of these EO/PP mix enrichments could be attributed to the presence of *p*-coumaric acid (80% of the PP mix), which was shown to have poor antioxidant activity on its own (**Table 2.5**). This activity could be due to the absence of a catechol group in its structure. In the ORAC assay, enrichments of all EOs exhibited synergistic and additive effects, except in the case of yellow sage (HE-SAU-01-02, ORAC value = 8,620.00 μmol TE/g sample). Pimento berry EO (HE-PIM-01) and Ceylon cinnamon EO (HE-CAN-04) exhibited high ORAC values (16,148.00 μmol TE/g sample, 18,477.00 μmol TE/g sample, respectively). Though these eugenol rich EOs (86.4%, 84.6%, respectively) demonstrated ORAC values greater than EOs lacking eugenol, interactions with this compound were not the sole contributors to the synergistic effects. This is apparent when these effects and values are compared to the enrichment of clove EO (ORAC value = 12,481.50 μmol TE/g sample), which exhibited additive effects, even with a greater amount of eugenol present in the EO (93.5%, Khodaei et al., 2020). This indicates that eugenol played a part in the synergy, but it did not provide the full effect. Perhaps interactions with minor oxygenated compounds, such as alcohols, contributed to the synergistic effects found in the pimento berry (HE-PIM-01) and cinnamon (HE-CAN-04) EO

enrichments. This phenomenon has been reported in basil—where synergism was observed between minor components in addition to eugenol's contributions (Araújo Couto et al., 2019).

2.4.4. Antioxidant Capacity of EOs Enriched with Crude Extracts

Enrichment of all EOs with crude extracts (**Table 2.7**) yielded synergistic effects in the DPPH assay, with the exception of pimento berry (HE-PIM-01)/rosemary extract, which exhibited additive effects. No antagonistic effects were observed in the DPPH assay. As for the ORAC assay, less antagonistic effects were observed in comparison to enrichments with PP mixes (**Table 2.7**) (4 antagonistic effects versus 7, respectively). Additionally, there was an increase in additive effects in the ORAC assay and there was the same amount of synergistic effects in comparison to the PP mix.

Enrichment of all oils, except pimento berry (HE-PIM-01) and Ceylon cinnamon (HE-CAN-04), with crude grape seed extract (EX-RAI-01) greatly improved the antioxidant capacities of the EOs in both assays, in comparison to their corresponding values when enriched with the PP mix. When clove EO (HE-ORI-03) was enriched with the grape seed PP mix (**Table 2.7**), it exhibited antagonistic effects; however, when enriched with the crude extract, this pair showed great synergistic effects (**Table 2.7**). This suggests that interactions with the minor components of the extract contributed a great deal to the antioxidant capacity of the enrichment. Yilmaz and Toledo (2004) found that a mix of the major compounds in grape seed skins, including epicatechin, catechin, and gallic acid, contributed to less than 26% of the antioxidant properties of the extract, confirming that other minor components have a role in the combinatorial effects. As for pimento berry (HE-PIM-01) and Ceylon cinnamon (HE-CAN-04), their ORAC values remained relatively similar to those enriched with the PP mix and exhibited antagonistic effects, indicating that the major PPs were the main compounds interacting with the oils.

Enrichment of all oils with crude green tea extract (EX-THE-01) increased their antioxidant capacity, especially pimento berry's (HE-PIM-01) ORAC value (39,921.50 μmol TE/g sample), which showed synergistic effects. No antagonistic effects were observed in both assays—all enrichments in the DPPH assay were synergistic, whereas all interactions in the ORAC assay were additive, except with pimento berry (HE-PIM-01). The ORAC values of the oils nearly doubled, which suggests that the major compounds contribute to half of the effects and that there are interactions between the major and minor PPs of the green tea crude extract (EX-THE-01) with

the oils. Green tea is known to have the highest PP content in comparison to other teas and a very high antioxidant activity (Forester & Lambert, 2011). The presence of all the flavonoids, catechins, gallic acids, and the like all contribute to the excellent antioxidant capacity of green tea. With an abundance of these compounds, combining EOs with green tea could result in the regeneration of many powerful antioxidants and stable phenoxyl radicals.

Enrichments with crude apple extract (EX-POM-04) improved both the IC_{50} and ORAC values of all EOs, except the IC_{50} of clove (HE-CLO-03)/crude apple extract (IC_{50} = 0.0406 mg/mg DPPH), compared to enrichments with the PP mixes. No antagonistic effects were observed in any enrichments in both assays. Enrichments that once were antagonistic between EOs and the apple PP mix were then additive (oregano, HE-ORI-03; yellow sage, HE-SAU-01-02) or synergistic (white thyme, HE-THY-03) when enriched with the crude extract. This suggests that the presence of the minor components of the crude extract and the interaction between them and the EOs contribute positively to the overall antioxidant activity of the enrichments. Furthermore, all synergistic combinations included EOs with phenols, whereas those that exhibited additive effects included EOs that lacked phenols. This suggests that when crude apple extract is used to enrich EOs, this extract interacts synergistically with phenols in the ORAC assay, thereby increasing the overall antioxidant capacity significantly. As previously mentioned, all enrichments in the DPPH assay were synergistic, suggesting that both major and minor components of crude extract interact synergistically with all EOs, regardless of the presence of phenols.

Crude rosemary extract (EX-ROM-04) showed the lowest overall improvement in antioxidant capacity, in terms of absolute values. In comparison to the PP mix enrichment, the addition of crude extract showed improvements in the IC_{50}, which showed synergistic effects across all enrichments, except that with pimento berry (HE-PIM-01, IC_{50} = 0.1257 mg/mg DPPH), which exhibited additive effects. As for the ORAC assay, enrichment with the crude rosemary extract exhibited one more antagonistic effect in comparison to enrichments with the PP mix. In addition to the already-existing antagonistic effects between yellow sage (HE-SAU-01-02)/crude rosemary extract (EX-ROM-04), the combination of oregano (HE-ORI-03)/crude rosemary extract (EX-ROM-04) also yielded antagonistic effects. Therefore, the pre-existing antagonistic effects between yellow sage (HE-SAU-01-02) and the major PP mix of rosemary carried over to its enrichment with the crude extract (EX-ROM-04), suggesting that interactions with the minor components did not contribute to the antioxidant capacity, nor did they help overcome the

antagonism. Additionally, the synergy that was previously present between oregano (HE-ORI-03) and the major PPs of crude rosemary extract was then lost when this oil was enriched with the crude extract (EX-ROM-04). This reveals that the minor components of rosemary extract interact poorly with EOs that lack phenols in the ORAC assay. The enrichment of clove EO (HE-CLO-01) remained synergistic, albeit with a lower ORAC value than that of the enrichment with the PP mix, whereas, enrichment of pimento berry (HE-PIM-01) and Ceylon cinnamon (HE-CAN-04) remained synergistic, though enrichments with the crude extract resulted in lower ORAC values. This suggests that the major compounds of rosemary extract were mostly responsible for the synergistic effects in the aforementioned EOs.

2.4.5. Prediction of the Effects of EO Enrichment

Overall, each enrichment step behaved differently in both assays, as shown in the final PCA (**Fig. 2.4**). Unenriched oils, as well as oils enriched with the PP mixes followed the $1/IC_{50}$ vector. The ORAC values of the EOs enriched with the PP mixes did not vary very much between each enrichment. For example, the ORAC values for the enrichment of clove oil (HE-CLO-01) with PP mixes stayed between 12,473.50 and 12,764.50 μmol TE/g sample, meaning these enrichments behaved similarly in the ORAC assay and their differences cannot be differentiated. Therefore, to assess the contribution of each PP mix and the different interactions between the mixes and the oils, the DPPH assay could be used. Oils enriched with individual PPs, on the other hand, followed the ORAC vector, as their IC_{50} values were not dispersed enough to be differentiated between each other. Finally, though all oil/crude extract enrichments were loosely dispersed from each other, oils enriched with the same crude extract uniformly clustered together along the ORAC vector, with a few outliers (oil/green tea crude extract enrichments). This, therefore, suggests that these enrichments are loosely differentiated by the ORAC assay.

Figure 2.5 shows the correlations between the observed and the expected IC_{50} and ORAC values. There is a weak to moderate correlation between the expected and observed IC_{50} ($R^2 = 0.37$). The difference between the two variables is more pronounced in the high IC_{50} value range. In addition, the correlation is not a proportional relationship, in which the ratio between the observed and expected IC_{50} decreased with the decrease of the antioxidant capacity. The following model can explain 48% of the variability of observed IC_{50} ($R^2 = 0.48$):

$$\text{Predicted } IC_{50} = -0.0007 + 0.09*(\text{Oil } IC_{50}) + 1.4*(\text{Enrichment } IC_{50})$$

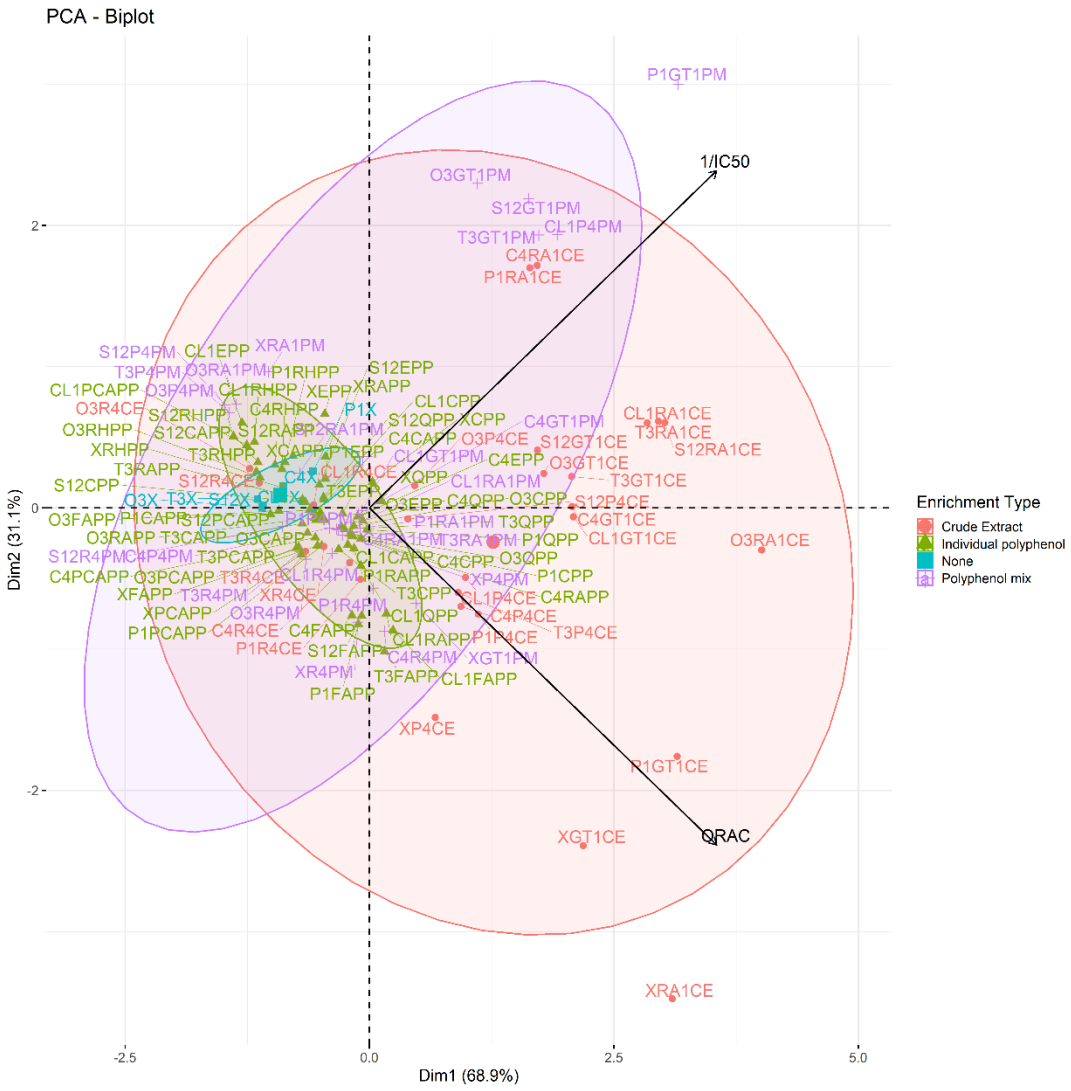


Figure 2.4. Principal Component Analysis of non-enriched (■) and enriched EOs with individual polyphenols (▲), polyphenol mixes (▲), and crude extracts (■).

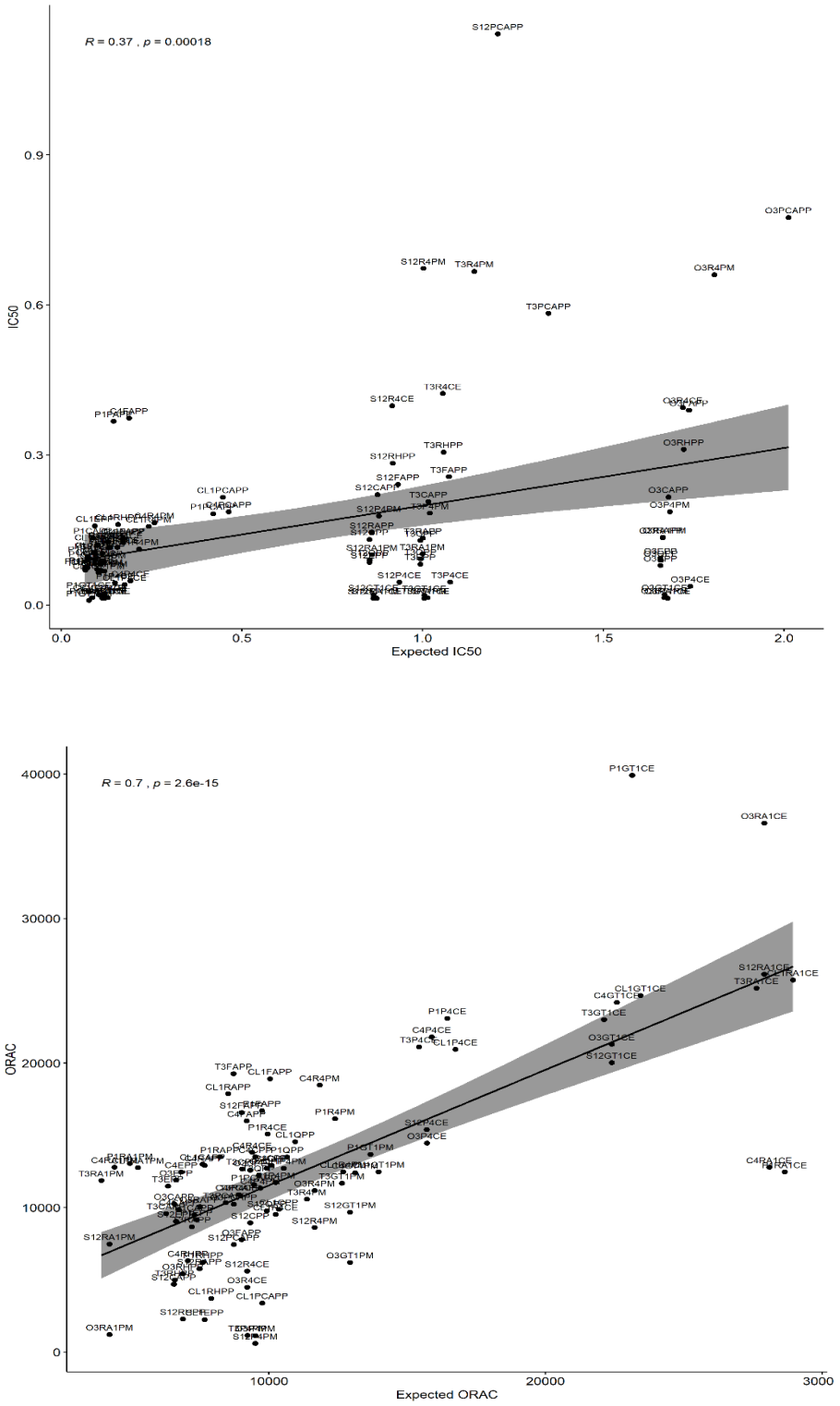


Figure 2.5. Correlations between the observed and expected IC50 and ORAC.

In contrast, a moderate to strong correlation between the expected and observed ORAC values was observed ($R^2 = 0.7$).

The observed variability was mainly due to the synergistic and antagonistic effects observed upon EO enrichment with crude extracts than with individual polyphenols and PP mixes. The following model can explain 50% of the variability of the observed IC_{50} ($R^2 = 0.48$).

Predicted ORAC = -1014+ 2.073*(Oil ORAC) + 0.79*(Enrichment ORAC).

2.5. Conclusion

The antioxidant capacities of 38 EOs and their enrichments with individual PPs, PP mixes, and crude extracts were measured using the DPPH and ORAC assays. Overall, enrichments of selected EOs with PP mixtures and crude extracts showed no antagonistic interactions in the DPPH assay. On the other hand, the ORAC assay showed some antagonistic effects with PP mixes, however, enrichments with crude extracts exhibited a decrease in antagonistic effects, suggesting that both major and minor compounds of plant extracts such as green tea (EX-THE-01) and apple (EX-POM-04) extracts interact positively with EOs.

No direct correlation was found between the IC_{50} and ORAC values, indicating that EOs behaved differently in both assays. The differences in their values could be due to solvent interactions affecting the bond dissociating energies, as well as the presence of pro-oxidants.

Predictive models were developed to explain 48% and 50% of the variability in the IC_{50} and ORAC values, respectively.

CONNECTING STATEMENT II

The antioxidant capacities of 38 EOs and their enrichments were studied. Furthermore, their interactions and combinatorial effects were also investigated in Chapter II.

In Chapter III, the modification of terpenes catalyzed by two lipases was explored in toluene with vinyl esters at several reaction times. In this chapter, a solvent-free method was also developed, utilizing lemon oil as the reaction medium. The cytotoxicity of modified and unmodified lemon oils was also investigated, as well as their respective anti-inflammatory properties.

CHAPTER III

DEVELOPMENT OF AN ENZYMATIC PROCESS BASED ON LIPASE-CATALYZED TRANSESTERIFICATION TO MODIFY SELECTED EOS AND INVESTIGATE THE NEW FUNCTIONAL PROPERTIES

3.1. Abstract

The reaction capacities of 3 terpenes (eugenol, thymol, geraniol) with vinyl esters (vinyl acetate (VA), vinyl propionate (VP)) and commercial lipases from *Candida rugosa* and *Candida antarctica* (B fraction) (Novozym® 435) were studied. Phenolic terpenes were not detected to have participated in the transesterification reaction and were therefore not modified, which could be attributed to the position of the hydroxyl group on the phenyl moiety. Geraniol was successfully modified in toluene and in a solvent-free medium utilizing lemon oil as the reaction medium, with bioconversion yields as high as 100% and 88%, respectively. Modified and unmodified lemon oil emulsions exhibited the same cytotoxic behavior. Production of interleukin-8 (IL-8) was significantly ($p < 0.05$) increased in lipopolysaccharide (LPS)-stimulated U-937 cells but showed a significant ($p < 0.05$) decrease in the presence of unmodified and modified lemon oil emulsions, as well as geraniol emulsions. The anti-inflammatory activity of the oils was not dose-dependent and could potentially be attributed to a plateau in activity in either the oils. The modification of oil did not affect the response to the anti-inflammatory activity test. These findings suggest that solvent-free modification of terpene alcohols in EOs is possible. This method could, therefore, be considered to modify EOs to be used in food products.

3.2. Introduction

Increasing health consciousness in consumers has pushed the food industry to turn to natural alternatives. Essential oils (EOs) are excellent, natural solutions for the food industry thanks to their high antioxidant (Bentayeb, Vera, Rubio, & Nerín, 2014b; Misharina et al., 2015; Padmakumari et al., 2011) and antimicrobial (Bassolé & Juliani, 2012; Lee, Kim, Beuchat, Kim, & Ryu, 2020; Semeniuc, Pop, & Rotar, 2017) activities. However, their applications are limited to selected products due to their strong aromatic and flavor profiles (Hyldgaard et al., 2012). To overcome this limitation, this study proposes the modification of monoterpenes present in the EOs through lipase-catalyzed transesterification. A structure-odor relationship study on geraniol and nerol has shown that structural changes on the basic skeleton of these monoterpenes led to derivatives that can be less potent or more pleasant than their corresponding parent monoterpenes (Elsharif & Buettner, 2018). For instance, these authors have reported that geraniol was the most potent compound with a very low odor threshold and was characterized by panelists as having fresh, citrus-like and fatty odor attributes, while geranyl acetate was less potent and exhibited sweet and floral attributes.

The use of enzymatic processes is a plausible route to research new natural ingredients, as opposed to chemical synthesis. Biocatalysts offer greener, environmentally friendly alternatives to traditional chemical methods, as they transform materials under mild reaction conditions and require very low energy to do so (Ferreira-Dias, Sandoval, Plou, & Valero, 2013). The key step in the enzymatic acylation of monoterpene alcohols is the selection of the appropriate reaction medium that modulates the properties of lipases towards the synthesis. Both the polarity of the solvent and the solvent-enzyme interactions can alter the synthesis activity of the lipase through modification of its three-dimensional active structure (Kamal, Yedavalli, Deshmukh, & Rao, 2013; Palomo et al., 2002). There has been growing interest in reducing the amount of organic solvent in the reaction media, as such solvent-free reaction media are becoming more attractive solutions. Solvent-free media have been shown to enhance the productivity of an enzymatic reaction, shorten reaction time, prevent or minimize hazardous products, require simpler downstream separation, and make the process greener by being more economical and environmentally friendly (Himaja, Poppy, & Of, 2011). Furthermore, the use of vinyl esters in such reactions results in the formation of a vinyl alcohol, which will then tautomerize to acetaldehyde (Nakagawa, Watanabe, Shimura, Kirimura, & Usami, 1997). This carbonyl can then no longer serve as a substrate for the reverse

reaction (Franken, Eggert, Jaeger, & Pohl, 2011), resulting in an irreversible reaction, which is highly favorable. The volatility of acetaldehyde also, therefore, favors downstream processing.

The use of enzymes to improve the extraction of EOs has been reported in the literature (Hosni et al., 2013; Sowbhagya et al., 2009; Zhiping, Weirong, & He, 2006). Examples regarding the application of lipase-catalyzed synthesis of esters in EOs to enhance and modify their aromatic profiles are limited. It has been shown that lipase-catalyzed transesterification has increased fruity notes in the aromatic profile of palmarosa oil (Ramilijaona et al., 2013) and brought minor components forward in the modification of citrus EOs (K. Zhang et al., 2017).

This study seeks to investigate the possibility of modifying the chemical profile of lemon oil through lipase-catalyzed transesterification and subsequently studying the potential cytotoxic effects of the newly modified oils. To the best of our knowledge, no study has investigated the modification of lemon oil through lipase-catalyzed transesterification.

3.3. Materials and Methods

3.3.1. Materials

Major compounds (eugenol, thymol, geraniol), lipases (from *Candida rugosa* and *Candida antarctica,* B fraction (Novozym® 435)), and vinyl esters (vinyl acetate (VA), vinyl propionate (VP)) were purchased from Sigma Chemical Co. (St-Louis, MO, USA). Lemon oil was obtained from a commercial supplier (Novotaste, Qc, Ca). A Cell Counting Kit-8 (CCK-8) was purchased from Tebu-bio (Le Perray en Yvelines, France, http://www.tebu-bio.com). A Human IL-8/CXCL8 Quantikine ELISA Kit (Catalog Number D8000C) was purchased from R&D Systems (Minneapolis, Minnesota, United States). Human monocyte cell line U-937 (ATCC®CRL-1593.2) was purchased from the American Type Culture Collection (ATCC).

3.3.2. Lipase-Catalyzed Transesterification Reaction in Solvent media

The transesterification reaction was carried out according to the method reported by Ramilijaona et al. (2013) with modifications. Acyl acceptors (geraniol or EOs, 1% w/w) were mixed with varying molar equivalents (0.5, 1, 1.5) of donors (VA, VP) in toluene. Unless otherwise specified, the total reaction volume was a maximum of 5 mL. Then, 0.1mg lipase/mL reaction was added to the mixture. Both Novozym® 435 from *C. antarctica* and lipase from *C. rugosa* were used as biocatalysts. The flasks were then vacuum-sealed and incubated (40h and 175h) at 40^{o}C and shaken at 150rpm. When gram scaled-up, 1 molar equivalent of VP was reacted with geraniol (1%

v/v, 10% v/v, 30% v/v) in toluene (up to 5 mL reaction volume). This reaction was catalyzed by Novozym® 435 (2.5, 25, 75 mg/mL, respectively). The end-products and their structures were analyzed and confirmed by Gas Chromatography-Mass Spectrometry (GC-MS). The internal standard used was hexadecane. The blank reaction consisted of substrate, acyl donor, and reaction medium. Reactions were performed in duplicate. Results are reported as the mean bioconversion yield, which was estimated as the peak area of the substrate and/or product, divided by that of the substrate, multiplied by 100%.

3.3.3. Lipase-Catalyzed Transesterification Reaction in Solvent-Free Media

The transesterification reaction was carried out according to the method reported by Ramilijaona et al. (2013) with modifications. Lemon EO (*Citrus limon* L.) was enriched with geraniol (0%, 2%, 5%) and reacted with vinyl esters (VA, VP) at a 1:1 molar ratio with respect to geraniol. Novozym® 435 from *C. antarctica* was added at a concentration of 0.0125g/mL with respect to the final reaction volume. The flasks were then vacuum-sealed and incubated (15-175h) at 40^{o}C and shaken at 150 rpm. The end-products and their structures were analyzed and confirmed by GC-MS. The internal standard used was hexadecane. The blank reaction consisted of substrate, acyl donor, and reaction medium. Reactions were performed in duplicate. Results are reported as the mean bioconversion % yield ± standard deviation %.

3.3.4. Gas Chromatography - Mass Spectrometry (GC-MS) Analysis

GC was performed using a fused fused-silica capillary column, coated with 5% diphenyl –95% dimethylsiloxane (HP-5ms GC column, 30 m, 0.25 mm, 0.25 µm, Agilent Technologies, Santa Clara, CA, USA). The oven temperature was increased from 50 to 155°C at a rate of 5.0°C/min, then from 155 to 250°C at 10.0°C/min, with a final 5 min post run at 300°C. The injection volume was 0.4 µL of diluted sample in toluene (1:100) (spitless mode). Helium was used as the carrier gas at 12 psi with an average velocity of 43 cm/sec and the inlet was at 225°C. Both detector and interface temperature were set at 250°C. Electron ionization (EI; 70 eV) was used and the ion source temperature was 230°C. Acquisition mass-to-charge ratio (m/z) range was 40–500 Da with the scan rate of 5.92 scans/sec. Agilent MassHunter Workstation Software Version B.09.00 was used to analyze peak changes and qualitatively identify compounds.

3.3.5. Assessment of Immunomodulatory Activity

3.3.5.1. Sample Preparation

Stock solutions consisted of 16% (v/v) lemon oil, 0.8% (v/v) geraniol, and 1% (v/v) Tween®80 in sterile water. These were prepared under sterile conditions and homogenized at 12,000 rpm for 1 minute using a homogenizer (IKA T 25 ULTRA-TURRAX High-Speed Homogenizer 115VAC). Dilutions were performed using sterile water.

3.3.5.2. Cytotoxicity (Viability Test)

Human monocyte cell line U-937 (ATCC®CRL-1593.2) was grown and maintained in a humidified incubator at 37^{o}C, in 5% CO_2 atmosphere. Complete Roswell Park Memorial Institute medium (RPMI 1640; Dutscher) supplemented with 10% foetal calf serum (FCS; Gibco), 2 mM L-glutamine (PanBiotech GmbH), 100 U/mL penicillin (PanBiotech GmbH) and 100 µg/mL streptomycin (PanBiotech GmbH) was used for cell culture in T75 flasks. All experimental tests were conducted in 96-well microtiter plates. Following cell culture, the U-937 cells were centrifuged and re-suspended in fresh RPMI-1640 containing 2 mM L-glutamine, free of antibiotics or serum. The cell concentration was adjusted to 1 x 10^6 cells/mL and were then dispensed into a 96-well plate. After adding EO/geraniol samples at selected concentrations, the plates were pre-incubated for 24h in a humidified incubator (37^{o}C, 5% CO_2). Following incubation, 5% CCK-8 solution was added to all wells. The plates were then incubated for 2h and their absorbance was read at 450nm using a microplate reader (SpectraMax® iD3 (Molecular Devices)). Wells containing only RPMI 1640 and U-937 cells served as the control. Wells containing RPMI 1640 and EO samples served as the blank. Each sample was done in n=6.

3.3.5.3. Enzyme-Linked Immunosorbent Assay (ELISA)

The U-937 cells were seeded on 96-well microtiter plates at a concentration of 0.5 x 10^6 cells/mL and differentiated with PMA (60 ng/mL). The cells were stimulated with LPS (50 µg/mL) and the control was treated with dexamethasone (10 µM). Another control without any inducers (no LPS or PMA) was also performed in parallel. The plates were then incubated for 24 hours. Following incubation, the supernatants were collected and frozen.

A Human IL-8/CXCL8 Quantikine ELISA Kit (R&D Systems Catalog Number D8000C) was then used to investigate the anti-inflammatory properties of the EO/geraniol samples. The experiments were carried out according to the procedure outlined by the kits. Each sample was done in n=4.

3.3.5.4. Statistical Analyses

All measurements were taken as n=6 (cytotoxicity test) or n=4 (ELISA). The statistical analyses were carried out using GraphPad Prism version 8.00 for Windows 10™ (GraphPad Software, San Diego, CA, USA). The Kruskal Wallis and Dunn's multiple comparisons tests were performed (cytotoxicity test), as well as one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test (ELISA). Data was considered statistically significant if $p < 0.05$.

3.4. Results and Discussion

3.4.1. Transesterification of Selected Terpene Alcohols with Vinyl Esters

Table 3.1 summarizes the bioconversion yield of the transesterification reaction of selected terpene alcohols (e.g. geraniol, eugenol, thymol) with vinyl acetate (VA) and vinyl propionate (VP). Geraniol is a major constituent of several EOs, such as lemon (*Citrus* genus), rose (*Rosa* genus), and citronella (*Cymbopogon* genus), and exhibits antioxidant (S. N. Prasad & Muralidhara, 2017) and anti-inflammatory (Su, Chao, Lee, Ou, & Tsai, 2010) properties. Eugenol and thymol can both be found in thyme (*Thymus* genus) and individually found in clove (*Syzygium* genus) and ajwain (*Trachyspermum* genus), respectively. These compounds are known to exhibit excellent antioxidant (Gülçin, 2011; Hossain, Brunton, Barry-Ryan, Martin-Diana, & Wilkinson, 2008) and antimicrobial (Höferl et al., 2009) properties. The results show that no reaction was observed when eugenol and thymol were used as acyl acceptors (**Table 3.1**). To the best of our knowledge, no published studies have reported the transesterification of either of these phenols. However, one study was able to successfully acetylate *p*-cresol (4-methyl phenol) with Novozym® 435 under different conditions (Torres et al., 2008). The researchers noted that reactions were faster with *p*-cresol due to its small size; however, even though eugenol and thymol bear close phenolic structures to this compound, there was no indication that any reaction had occurred in the present study. The alkyl substituents as well as their positioning on the phenol moiety may have sterically hindered the binding of eugenol (2-methoxy-4-allyl phenol) and thymol (5-methyl-2-iso-propylphenol) to the active site of the lipases. Indeed, with a funnel-like binding site in Novozym® 435 and a tunnel-like binding site in lipase from *C. rugosa* (Pleiss et al., 1998), it is possible that these phenols do not properly enter or fit these active sites when in toluene.

Table 3.1. Acylation of terpenes with Novozym® 435 and lipase from *Candida rugosa* using vinyl acetate (VA) and vinyl propionate (VP) as substrates.

Lipase Type	Substrate	Acyl Donor	Molar Equivalent (Donor)	Reaction Time (h)	Bioconversion yield %		
Novozym® 435	Eugenol	VA	0.5, 1, 1.5	40, 175		ND	
		VP	0.5, 1, 1.5	40, 175		ND	
	Thymol	VA	0.5, 1, 1.5	40, 175		ND	
		VP	0.5, 1, 1.5	40, 175		ND	
	Geraniol	VA	0.5	40	57	±	-
				175	41	±	1
			1	40	66	±	-
				175	82	±	0.2
			1.5	40	79	±	10
				175	100	±	11
		VP	0.5	40	48	±	-
				175	48	±	6
			1	40	94	±	13
				175	98	±	1
			1.5	40	96	±	21
				175	96	±	16
Lipase from *Candida rugosa*	Eugenol	VA	0.5, 1, 1.5	40, 175		ND	
		VP	0.5, 1, 1.5	40, 175		ND	
	Thymol	VA	0.5, 1, 1.5	40, 175		ND	
		VP	0.5, 1, 1.5	40, 175		ND	
	Geraniol	VA	0.5	40	31.5	±	16.4
				175		<10*	
			1	40		<10*	
				175	33	±	1
			1.5	40		<10*	
				175	13	±	3
		VP	0.5	40		<10*	
				175	15	±	6
			1	40		<10*	
				175	28	±	12
			1.5	40	21.5	±	10.6
				175	41	±	9

* High standard deviation due to limited acetylation
ND, Not Detected
VA, Vinyl Acetate
VP, Vinyl Propionate
Reaction conditions: 0.1mg/mL lipase, incubation at 40^{o}C at 150rpm

In contrast, a study was able to synthesize eugenyl acetate through lipase-catalyzed esterification of eugenol with acetic anhydride using Novozym® 435 in a solvent-free system (Chiaradia et al., 2012). Vinyl acetate and vinyl propionate may have acted as competitive substrates to eugenol in the investigated transesterification reaction. As far as we are aware, no study regarding the synthesis of eugenol propionate, thymol acetate, or thymol propionate could be found.

Other transesterification reactions have efficiently been carried out with phenolic acid substrates containing phenyl moieties (Safari, Safari, Karboune, St-Louis, & Kermasha, 2006; Weitkamp, Vosmann, & Weber, 2006). These reactions involved a nucleophilic hydroxyl group that was not directly attached to the phenyl moiety. In the case of eugenol and thymol, their only hydroxyl group is attached to their phenyl moiety. This could suggest that the access of lipases to their hydroxyl group may be restrained because of the limited rotation of the phenyl moiety and the bulkiness of their alkyl substituents. Additionally, it has been noted that Novozym® 435 has a high enantioselectivity for racemic mixtures of small secondary alcohols and that lipase from *C. rugosa* is generally used for bulky secondary alcohols with ring structures (Kazlauskas & Bornscheuer, 2008).

Reactions between geraniol and both vinyl esters resulted in a bioconversion yield varying from <1% to 100% (**Table 3.1**). It has been reported that geraniol can be successfully acetylated using various acyl donors (vinyl acetate, vinyl propionate, vinyl crotonate) with varying bioconversion yields such as 97.5% (Nakagawa et al., 1997), 20-100% (Ramilijaona et al., 2013), and 22.45-96.30% (Xiong, Huang, Zhang, & Hou, 2014). At 0.5 molar equivalent, the reaction between geraniol and VA catalyzed by Novozym® 435 started with a bioconversion yield of 57% at 40h, then dropped to 41% after 175h. The VP/geraniol reaction system did not show such a decrease, instead it seems to have reached an equilibrium at 40h with a maximum yield of 48%. With lipase from *C. rugosa*, the VA/geraniol reaction system exhibited the same pattern as with Novozym® 435—it started with a higher bioconversion yield (32%) and dropped to <10%. On the other hand, the use of VP as an acyl donor in the reaction system catalyzed by lipase from *C. rugosa* resulted in lower bioconversion yields of <10% and 15% after 40h and 175h, respectively. At 1 molar equivalent, VP was the most effect acyl donor, regardless of lipase source or reaction time. With Novozym® 435, the reaction with VP seems to have already reached a complete bioconversion at 40h (94%) since it does not increase much by 175h (98%). For reactions at 1.5 molar equivalents, VP (21-41%) was slightly more effective than VA (<10%-13%) in reactions catalyzed with lipase

from *C. rugosa*. However, geraniol and VA reactions catalyzed by Novozym® 435 showed high yields (79-100%) alongside reactions with VP (96%), which reached equilibrium by the 40h reaction.

Since yields were lower at lower acyl donor equivalents, this could suggest that there was an excess of geraniol, which could have slowed lipase activity. Indeed, it has been shown that in the presence of excess geraniol, this compound is capable of inhibiting lipase-catalyzed transesterification (Chulalaksananukul, Condoret, & Combes, 1992). Furthermore, reactions utilizing VA could have entailed a side reaction involving its hydrolysis, therefore resulting in free acetic acid in the system. This acetic acid could then go on and compete with the acylation reaction and inhibit lipase activity as well (Torres et al., 2008; Xiong et al., 2014), resulting in lower yields in comparison to reactions with VP.

Water activity was also not controlled throughout the experiments; therefore, the flasks could have been exposed to fluctuating humidity in the air. This discrepancy in conditions could have subsequently affected the conversion yields and resulted in a variability. From a practical point of view, the accidental addition of water could have favored bioconversion, since it has been shown that increasing water activity increases enzyme activity (Zaks & Klibanov, 1988). Indeed, to a certain extent, increasing water content has been shown to slightly increase enzyme activity of both Novozym® 435 (Talukder, Wu, Van Nguyen, Fen, & Melissa, 2009) and lipase from *C. rugosa* (Herbst, Peper, & Niemeyer, 2012). However, in the presence of excess exogenous water, Novozym® 435 activity decreased, as this favored the hydrolysis reaction (Talukder et al., 2009).

In general, there was an increase in the bioconversion yield with each increase in molar equivalent of acyl donor, with a few exceptions. Given that similar yields could be achieved between 1 and 1.5 molar equivalents upon the same reaction time, 1 molar equivalent of acyl donor was selected for future investigations.

Between the reactions catalyzed by Novozym® 435 and lipase from *C. rugosa*, the former showed higher acylation yields (41-100%) in comparison to the latter (<10-41%), using both vinyl esters as acyl donors. The lower bioconversion yields obtained with lipase from *C. rugosa* may be due to its inactivation in the presence of acetaldehyde (Franken et al., 2011; Weber, Stecher, & Faber, 1995). Weber *et al.* (1995) found that lipase from *C. rugosa* lost activity when exposed to this compound, whereas the activity of lipases A and B from *C. antarctica* remained stable. Franken

et al. (2011) reported that this aldehyde-based deactivation could be due to the formation of α,β-unsaturated polyenals from acetaldehyde. These compounds can then go on to form stable Michael-adducts with the enzymes, subsequently deactivating them. These authors found that lyophilizing lipase from *C. rugosa* at a pH below the average pKa of its lysine ε-amino groups helped overcome this limitation. Furthermore, Novozym® 435 is an immobilized lipase, whereas the lipase used from *C. rugosa* is not. The difference in their activity could therefore also lie in the lack of immobilization of the latter, due to the numerous advantages that arise from immobilization. These include, but are not limited to, an increase in performance in non-aqueous solvents, higher catalyst productivity, higher stability, and more (Filho, Silva, & Guidini, 2019).

Ramilijaona *et al.* (2013) have reported the acylation of palmarosa oil components, in particular geraniol, with vinyl esters at a bioconversion yield of 20% to 100% using 0.25–3 equiv. of acyl donors, lipase from *C. rugosa* and 27–168 h reaction time. It is important to note that the amount of geraniol present in palmarosa oil is lower than the amount used in the present study. This may explain the low yields obtained in our study in comparison to those reported by Ramilijaona *et al.* (2013). Another study also successfully acetylated geraniol with vinyl acetate, at a yield of 79%. using concentrated lipase from *C. rugosa* that was enriched and stabilized upon precipitation with acetone (Rosa et al., 2017). Majumder and Gupta (2010) were able to overcome the deactivation of the enzyme by cross-linking the *C. rugosa* lipase with bovine-serum albumin and reported bioconversion yields between 80-83% for reactions between benzyl alcohols and vinyl acetate.

Following our results, Novozym® 435 was selected to gram scale up (10 to 30 g per 100 ml) the transesterification reactions in the presence of 1 molar equivalent of acyl donor (**Table 3.2**). The enzyme concentration was increased from 0.1mg/mL to 2.5mg/mL and proportionally increased with increase in geraniol concentration. The bioconversion yield of geraniol into its esters decreased with increased geraniol concentration. These results could be attributed to mass diffusional limitations, promoted by the limited solvation of substrates in a saturated solvent medium, and/or to the enzyme inhibition by the excess of geraniol and vinyl propionate.

Table 3.2. Effect of geraniol concentration (gram scale) on the bioconversion yield of transesterification reaction catalyzed by Novozym® 435 at 1 molar equivalent of vinyl propionate.

Enzyme Source	Reaction Medium	Acyl Donor	% Geraniol	[Enzyme] (mg/mL)	Reaction Time (h)	Acylation yield %
Novozym® 435	Toluene	VP	1	2.5	175	82 ± 2
			10	25	175	76 ± 17
			30	75	175	75 ± 48

VP, Vinyl Propionate
Reaction conditions: 1 molar equivalent of acyl donor, incubation at 40°C at 150rpm

3.4.2. Acetylation of Geraniol-Enriched Lemon Oils in a Solvent-Free Reaction System

Though these reactions were successful in toluene, the presence of the solvent itself may pose health and environmental concerns. To overcome the obstacle of solvent removal, a solvent-free reaction system was investigated. Since the goal was to enzymatically modify lemon oil, lemon oil was selected to be used as the reaction medium to eliminate the recovery step. This EO was considered a suitable medium since it naturally contained geraniol and offered a nonpolar environment, which is ideal for lipase-catalyzed transesterification (Yadav & Kamble, 2018).

The reaction was successfully carried out (**Table 3.3**) with 2% (v/v) and 5% (v/v) enrichment of geraniol. When no geraniol was added to the lemon oil, no significant reaction had occurred. Indeed, the level of geraniol present in the investigated lemon oil is not high enough for lipase-catalyzed transesterification. Generally, the relative amount of geraniol present in EOs in the *Citrus* family is around <0.05%-0.1% (Choi et al., 2000). At an enrichment of 2% (v/v) geraniol and 5mg/mL Novozym® 435, the transesterification reaction seemed to have reached complete bioconversion at 40h with a maximum yield of 75%. On the other hand, when gram-scaled up, the transesterification of an enrichment of 5% (v/v) geraniol catalyzed by Novozym® 435 achieved complete bioconversion at 15h, with a maximum yield of 89%. At 40h and 175h, there were some minor decreases in yield to 79%, which can be attributed to the low extent of competing reverse reactions. Similar yields can be seen with the use of VA as an acyl donor with a 5% (v/v) geraniol enrichment—yields as high as 89% at 15h, then slightly decreasing over time.

As far as we are aware, no study using EOs as the reaction medium has been found. However, other studies have investigated solvent-free methods of synthesizing geranyl acetate through lipase-catalyzed transesterification. One study managed to acetylate over 90% of geraniol in 6h using ethyl acetate and Novozym® 435 at 55^{o}C (Gryglewicz, Jadownicka, & Czerniak, 2000). The researchers used a molar ratio of 1:2 equivalents of acceptor to donor and added fresh donor after 3h of incubation. Another study successfully acetylated geraniol in a solvent-free system using vinyl acetate as the reaction medium (Xiong et al., 2014). In a reaction catalyzed by lipase from *C. rugosa*, a yield of 40.35% of geranyl acetate was achieved in 3h. Additionally, in the same study, 96.30% of geranyl acetate was produced in 3h when the reaction was catalyzed by lipase sourced from *Pseudomonas fluorescens*.

Table 3.3. Solvent-free acetylation of geraniol-enriched lemon oil.

Lipase Type	Reaction Medium	Acyl Donor	% Geraniol	[Enzyme] (mg/mL)	Reaction Time (h)	Acylation yield %
Novozym® 435	Lemon Oil	VP	0	12.5	40	ND
					175	ND
			2	5	40	75 ± 9
					175	71 ± 7
			5	12.5	15[a]	89 ± 1
					40[b]	84 ± 3
					175	79 ± 3
Novozym® 435	Lemon Oil	VA	5	12.5	15[a]	89 ± 1
					40[a]	88 ± 6
					175	81 ± 7

ND, Not Detected
VP, Vinyl Propionate
VA, Vinyl Acetate
Reaction conditions: 1 molar equivalent of acyl donor, incubation at 40°C at 150rpm

In addition to the bioconversion of geraniol to geranyl propionate with VP in this present study, the reactions also resulted in supplementary side reactions between geraniol and 2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-, a stereoisomer of geranyl acetate found in the lemon oil. This compound acted as an acyl donor, resulting in additional geranyl acetate being formed alongside geranyl propionate (**Table 3.4**). Only slight changes can be seen in the Area % of 2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-, indicating that the reaction highly favored the reaction between geraniol and vinyl propionate. Reactions with VA did not see significant contributions from this side reaction.

These enzymes were found to be insoluble in the reaction media (both toluene and lemon oil), resulting in an easier separation at the end of the reaction, through filtration and centrifugation. This is highly favorable for the food industry, as this allows easy recovery, as well as the option to reuse the lipases, which is highly economical.

Table 3.4. Chemical Profiles of Unmodified and Modified Lemon Oils.

		VABR[a]	VA[b]	VPBR[a]	VP[b]
RT (min)	Constituents	Area %	Area %	Area %	Area %
3.385	7-Oxabicyclo[4.1.0]heptane, 1-methyl-4-(1-methylethenyl)-	0.08	0.32	0.23	0.15
3.412	o-n-Butylhydroxylamine	-	-	0.15	-
3.465	(+)-(E)-Limonene oxide	0.12	0.31	0.37	0.26
3.616	3,7,7-Trimethyl-8-(2-methyl-propenyl)-bicyclo[4.2.0]oct-2-ene	0.11	0.10	-	0.07
3.623	Cyclopentene, 3-ethenyl-	-	-	0.05	-
3.815	6-Octenal, 3,7-dimethyl-, (R)-	0.14	-	0.17	-
3.814	Citronellal	-	0.14	-	0.09
4.006	trans-2-Caren-4-ol	0.03	-	-	0.06
3.997	Ethanone, 1-(1,4-dimethyl-3-cyclohexen-1-yl)-	-	0.03	-	-
4.161	Terpinen-4-ol	-	0.01	0.03	0.06
4.170	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	0.07	-	-	-
4.447	alpha.-Terpineol	0.30	0.28	0.37	0.25
4.543	9,12,15-Octadecatrienoic acid, 2-phenyl-1,3-dioxan-5-yl ester	0.01	-	-	-
4.566	cis-4,7,10,13,16,19-Docosahexaenoic acid, picolinyl ester	-	0.04	-	-
4.839	Decanal	0.38	0.29	0.33	0.26
5.060	2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl)-, cis-	-	0.05	0.03	-
5.309	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	0.17	0.15	0.17	0.10
5.347	Octadecanal, 2-bromo-	-	0.09	-	-
5.497	2,6-Octadienal, 3,7-dimethyl-, (Z)-	9.55	6.34	9.20	5.41
5.979	Geraniol	42.37	4.06	43.05	2.60
6.199	2,6-Octadienal, 3,7-dimethyl-, (E)-	14.65	11.79	13.42	11.42
6.935	2,6-Octadien-1-ol, 3,7-dimethyl-, formate, (E)-	-	0.11	-	0.13
6.989	10-Octadecenal	0.01	-	-	-
6.999	Undecanal	-	-	0.02	-
7.007	Ethanol, 2-(9-octadecenyloxy)-, (Z)-	0.07	-	-	-
7.016	10-Methyl-E-11-tridecen-1-ol propionate	-	-	-	0.06
7.458	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-	0.07	0.04	-	-
7.708	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	-	-	-	0.04
7.736	Ethyl iso-allocholate	-	-	0.04	-
7.835	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, propanoate	-	0.02	-	-
8.119	6-Octen-1-ol, 3,7-dimethyl-, acetate	0.19	0.11	0.13	-
8.364	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	5.04	3.93	4.79	0.59
8.657	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	0.01	0.12	0.13	0.08
8.672	gamma.-Elemene	0.14	-	-	-
8.835	Geranyl acetate	3.28	53.68	2.99	5.15
9.100	Caryophyllene	2.82	1.99	2.46	1.91
9.233	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)-	-	-	0.11	-
9.229	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	0.33	0.17	-	0.19
9.467	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	-	0.03	-	-
9.690	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	5.02	3.86	4.68	3.60
9.848	Humulene	0.31	0.26	0.33	-

Table 3.4. Continued

RT (min)	Constituents	VABR[a] Area %	VA[b] Area %	VPBR[a] Area %	VP[b] Area %
9.850	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	-	-	-	0.25
10.133	6-Octen-1-ol, 3,7-dimethyl-, propanoate	-	-	-	0.08
10.184	Bicyclo[2.2.1]heptane, 2-methyl-3-methylene-2-(4-methyl-3-pentenyl)-, (1S-exo)-	0.16	0.13	0.16	0.20
10.364	(E)-.beta.-Famesene	0.37	0.26	0.31	-
10.379	Neryl (S)-2-methylbutanoate	-	-	-	3.55
10.707	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	-	-	-	0.14
10.712	cis-.beta.-Farnesene	0.26	0.19	0.23	-
10.774	1H-Cyclopropa[a]naphthalene, decahydro-1,1,3a-trimethyl-7-methylene-, [1aS-(1a.alpha.,3a.alpha.,7a.beta.,7b.alpha.)]-	-	0.24	-	-
10.784	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha.)]-	0.47	-	-	-
10.840	2,6-Octadien-1-ol, 3,7-dimethyl-, propanoate, (E)-	-	-	1.64	-
10.845	Butanoic acid, 2-methyl-, 3,7-dimethyl-2,6-octadienyl ester, (E)-	-	0.11	-	-
10.921	Geranyl propionate	-	-	-	51.63
11.288	cis-.alpha.-Bisabolene	0.45	0.32	0.43	0.34
11.380	.beta.-Bisabolene	7.30	5.57	6.79	5.36
11.503	Bioallethrin	0.05	-	-	0.19
11.503	.alpha.-Farnesene	-	0.12	-	-
11.823	1-Cycloheptene, 1,4-dimethyl-3-(2-methyl-1-propene-1-yl)-4-vinyl-	-	0.03	-	-
12.072	.gamma.-Elemene	-	0.02	-	-
12.103	cis-sesquisabinene hydrate	-	-	-	0.02
12.151	Humulene	-	0.07	-	-
12.156	cis-.alpha.-Bisabolene	-	-	0.11	-
12.158	Humulene	-	-	-	0.10
12.594	(-)-Spathulenol	0.53	0.40	0.48	0.36
13.465	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, [S-(Z)]-	0.04	-	-	-
13.168	Tetrapentacontane, 1,54-dibromo-	-	-	-	0.04
13.557	Hexadecane	2.75	1.63	2.98	2.03
13.831	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	-	0.03	-	-
13.831	Ethyl iso-allocholate	-	-	-	0.02
13.834	Formic acid, 3,7,11-trimethyl-1,6,10-dodecatrien-3-yl ester	0.02	-	-	-
14.326	8-Decen-2-one, 9-methyl-5-methylene-	0.02	-	-	0.09
14.338	Ledol	-	0.09	0.10	-
14.424	Benzene, ethoxy-	-	0.04	-	-
14.649	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-tetraenyl)-cyclohexanol	-	-	-	0.00
14.675	Menthol, 1'-(butyn-3-one-1-yl)-, (1R,2S,5R)-	0.06	0.06	-	-

Table 3.4. Continued

RT (min)	Constituents	VA-BR[a] Area %	VA[b] Area %	VP-BR[a] Area %	VP[b] Area %
14.672	4-(2,2-Dimethyl-6-methylenecyclohexyl)butanal	-	-	-	0.05
14.675	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (Z,E)-	-	-	0.06	-
15.106	.alpha.-Bisabolol	0.23	0.18	0.21	0.13
16.384	7,8-Epoxylanostan-11-ol, 3-acetoxy-	-	-	0.03	0.01
20.328	Cholesta-8,24-dien-3-ol, 4-methyl-, (3.beta.,4.alpha.)-	-	-	0.01	-
20.475	Neryl (S)-2-methylbutanoate	-	0.03	-	-
20.558	Norreticuline, N-formyl-	-	-	-	0.03
20.943	Ethyl iso-allocholate	0.02	-	-	-
21.385	7,8-Epoxylanostan-11-ol, 3-acetoxy-	-	-	0.03	-
22.145	Linalyl isobutyrate	-	0.05	-	-
22.177	Neryl (S)-2-methylbutanoate	-	-	-	0.02
23.483	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	-	0.06	-	0.04
25.525	7,8-Epoxylanostan-11-ol, 3-acetoxy-	-	-	0.003	0.01
25.972	Hexanedioic acid, dioctyl ester	0.04	0.03	0.03	-
26.723	7,8-Epoxylanostan-11-ol, 3-acetoxy-	-	-	0.01	-
28.504	Ethyl iso-allocholate	-	-	-	0.02
28.655	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	-	0.31	-	0.24
29.099	3-(1-Cyclohexenyl)-3-ethyl-2,6-piperidinedione	0.50	-	-	-
29.100	.alpha.-Damascone	-	0.48	0.52	0.24
29.364	7H-Furo[3,2-g][1]benzopyran-7-one, 4-[(3-methyl-2-butenyl)oxy]-	0.04	0.06	0.03	0.05
32.779	2,2-Dimethyl-3-(3,7,16,20-tetramethyl-heneicosa-3,7,11,15,19-pentaenyl)-oxirane	0.03	-	-	-
29.798	Trifluoroacetyl-lavandulol	-	0.22	-	-
29.800	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-	-	-	-	0.23
29.855	i-Propyl 9,12,15-octadecatrienoate	-	0.14	-	0.30
32.767	Ethyl iso-allocholate	-	0.02	-	-

[a]VA, Vinyl Acetate; VP, Vinyl Propionate; *BR*, Blank Reaction

[b]Reaction conditions: Lemon oil (reaction medium), Novozym® 435, 1 molar equivalent of acyl donor, 15h incubation, 40^{o}C, 150rpm

3.4.3. Cytotoxicity

Geraniol was cytotoxic to human cell line U-937 above 0.01% (v/v), inclusively. As shown in Fig. 1, the cells were completely lysed at 0.25% (v/v) (**Fig. 3.1A**), 0.05% (v/v) (**Fig. 3.1B**), and partially at 0.01% (v/v) (**Fig. 3.1C**), showing a dose-dependent cytotoxicity. Whereas at 0.008% (v/v) (**Fig. 3.1D**), the cells are healthy and viable. The same can be said of all the modified EOs, regardless of modification. As shown in **Fig. 3.2**, all EOs followed the same pattern of cytotoxicity, therefore lipase-catalyzed transesterification of lemon oils enriched with geraniol does not greatly affect cytotoxicity. Tween®80 also showed a dose-dependent cytotoxicity pattern, where it was no longer cytotoxic under 0.25% (v/v) (**Fig. 3.3**).

It has been reported that clove oil (*Syzygium aromaticum*) followed a dose-dependent cytotoxicity and that it was highly cytotoxic to human fibroblasts and endothelial cells at 0.03% (v/v) (Prashar, Locke, & Evans, 2006). The authors attributed 73% of this cytotoxic effect to the eugenol present in the oil. Another study involving palmarosa, citronella, lemon grass EOs, as well as citral and geraniol reported cytotoxic effects (7.16%-43.94% cell death) in human lymphocytes (Sinha, Jothiramajayam, Ghosh, & Mukherjee, 2014). The authors mentioned that geraniol showed no significant cytotoxicity and that oils containing this compound showed less cytotoxicity than those containing citral.

The mechanism behind the cytotoxicity of EOs and their components could involve apoptosis induced by cellular membrane attack (Prashar et al., 2006). Indeed, Carnesecchi *et al.* (2001) found that geraniol perturbed the cellular membrane function of Caco-2 cells, which then lead to a reduction of protein kinase C and a decreased expression of p44/p42 ERK active forms. The authors directly link this perturbation to the antiproliferation effects of geraniol on Caco-2 cells. Evidence of DNA fragments at high concentrations of EOs reported by Sinha *et al.* (2014) can be attributed to apoptosis/necrosis in human lymphocytes. Geraniol has also been shown to induce cell death in PC-3 prostate cancer cells through the cooperative action of apoptosis and autophagy (Jeon, 2011). The researchers attributed this action to the inhibition of AKT signaling and activated AMPK signaling, which then resulted in mTOR inhibition.

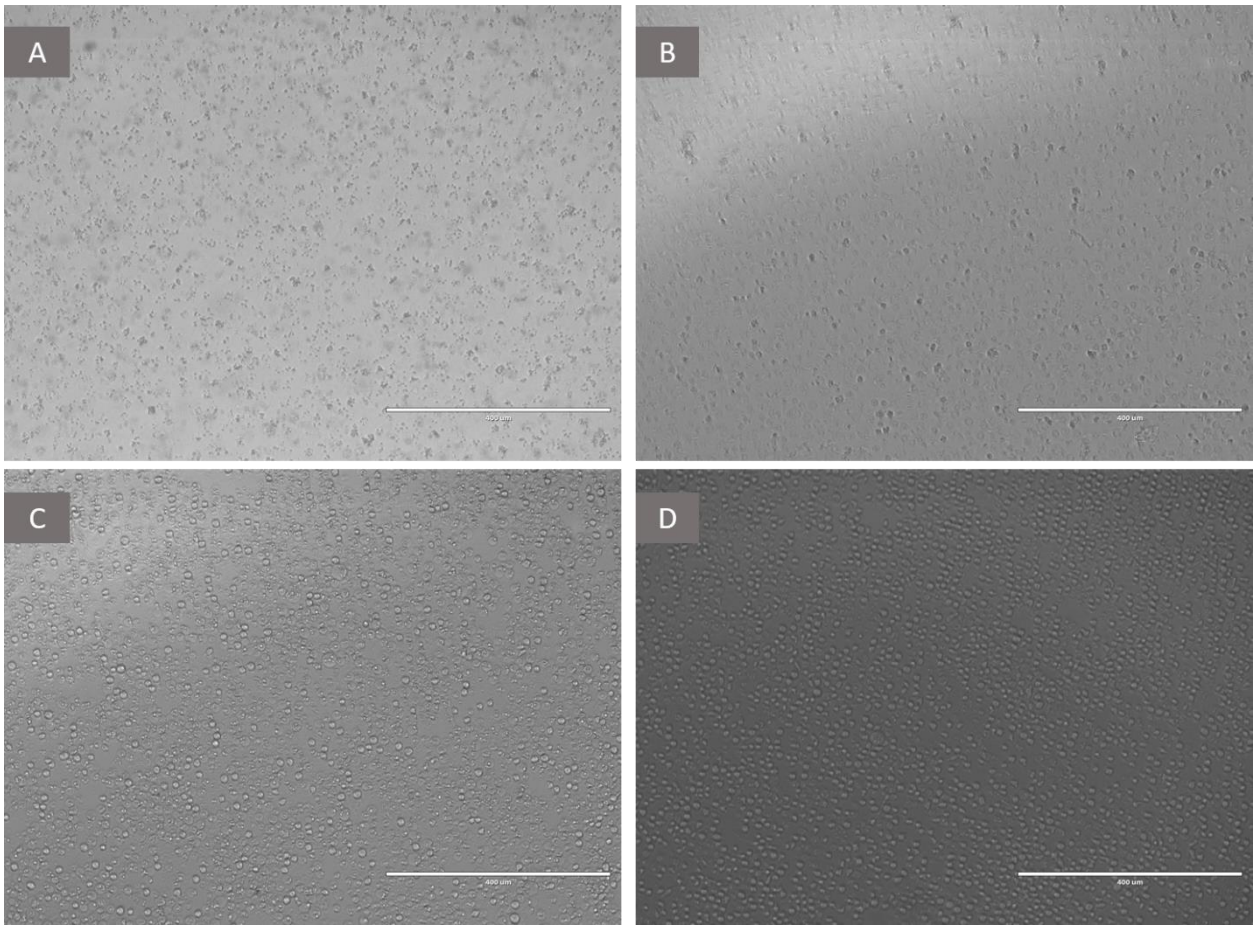


Figure 3.1. Cytotoxicity of geraniol on human macrophage cell line U-937 after 24h treatment with varying concentrations of geraniol. **A:** 0.25% (v/v) geraniol; **B:** 0.05% (v/v) geraniol; **C:** 0.01% (v/v) geraniol; **D:** 0.008% (v/v) geraniol

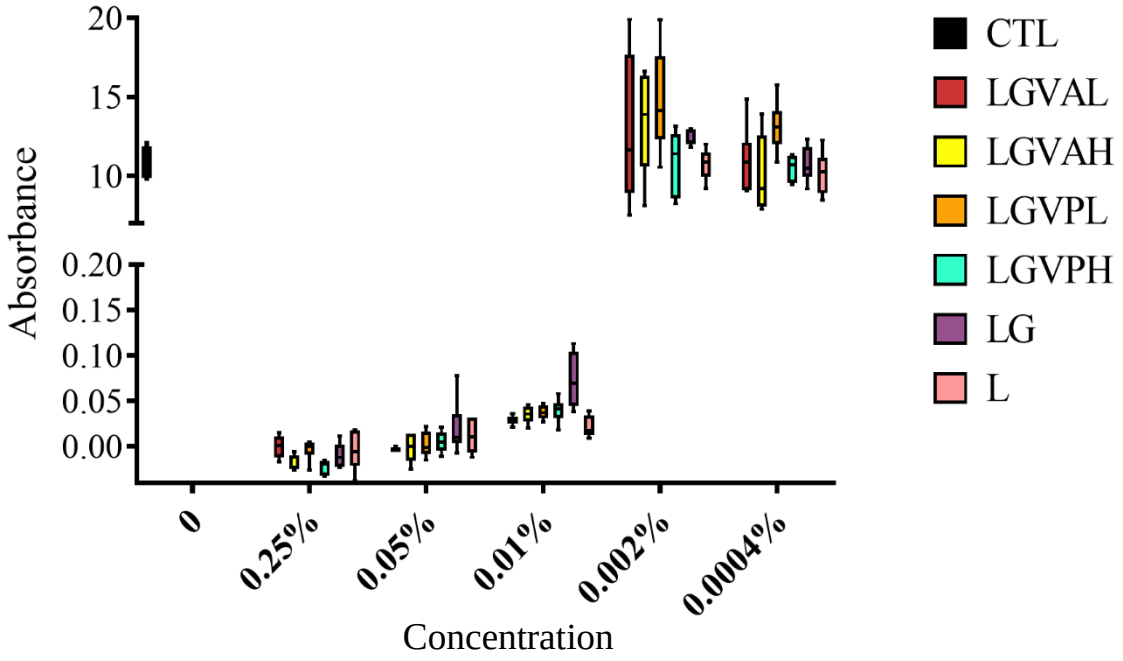


Figure 3.2. Cytotoxicity of unmodified (**LG** ( ); **L** ( )) and modified (**LGVAL** ( ); **LGVAH** ( ); **LGVPL** ( ); **LGVPH** ( )) lemon oils on human macrophage cell line U-937 after 24h treatment at 0.25% (v/v), 0.05% (v/v), 0.01% (v/v), 0.002% (v/v), and 0.0004% (v/v).

CTL ( ): Control; **LGVAL ( )**: lemon oil enriched with 5% geraniol modified with 1 molar equivalent vinyl acetate, low bioconversion; **LGVAH ( )**: lemon oil enriched with 5% geraniol modified with 1 molar equivalent vinyl acetate, high bioconversion; **LGVPL ( )**: lemon oil enriched with 5% geraniol modified with 1 molar equivalent vinyl propionate, low bioconversion; **LGVPH ( )**: lemon oil enriched with 5% geraniol modified with 1 molar equivalent vinyl propionate, high bioconversion; **LG ( )**: lemon oil enriched with 5% geraniol, unmodified; **L ( )**: lemon oil, unmodified

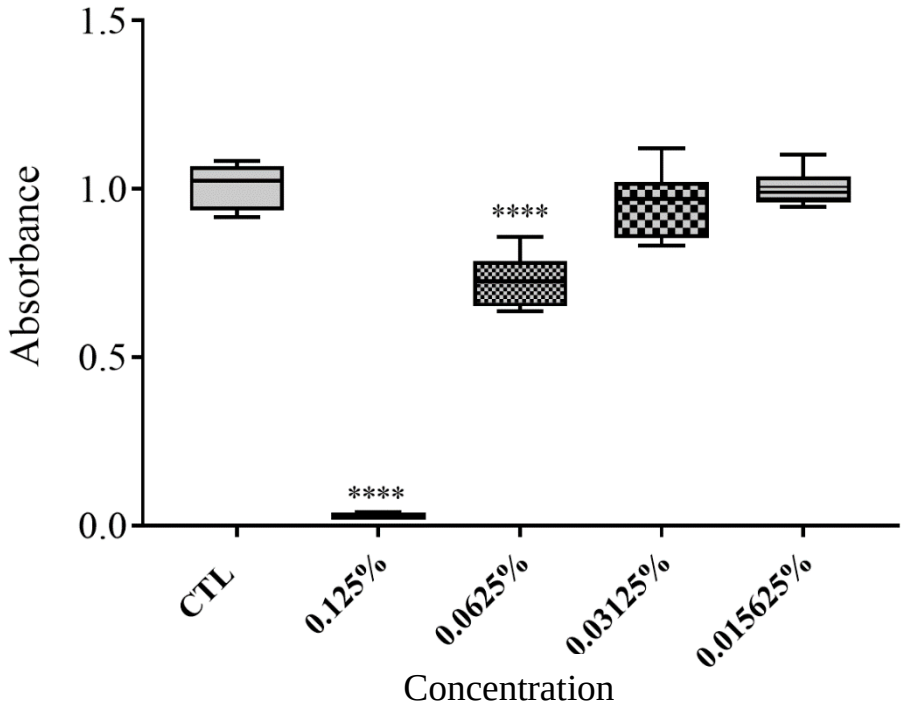


Figure 3.3. Cytotoxicity of Tween®80 at 0.125% (v/v), 0.0625% (v/v), 0.03125% (v/v), and 0.015625% (v/v) on human monocyte cell line U-937. **** $p < 0.0001$

3.4.4. Anti-inflammatory Properties

Following the cytotoxicity test, the following samples were selected to assess their anti-inflammatory properties (**Fig. 3.4**): unmodified lemon oil, unmodified lemon oil enriched with 5% geraniol (v/v), geraniol, and lemon oil enriched with 5% geraniol (v/v) modified to a complete bioconversion with VP (LGVPH). Interleuken-8 (IL-8) was chosen as a marker for anti-inflammatory activity as it is a chemokine implicated in chronic inflammation (Harada, Mukaida, & Matsushima, 1996). Aberrant production of proinflammatory factors such as IL-8 by monocytes and endothelial cells could result in chronic inflammatory conditions. Furthermore, this member of the CXC chemokine family is known to be a neutrophil chemotactic and activating factor (Kownatzki, Kapp, & Uhrich, 1986; Yoshimura et al., 1987) and can activate immune cells, as well as promote angiogenesis (Moore et al., 1998).

Production of IL-8 was significantly ($p < 0.05$) increased in lipopolysaccharide (LPS)-stimulated cells in comparison to non-stimulated cells. The dexamethasone-treated control was also significantly ($p < 0.05$) different from the stimulated cells. All samples exhibited anti-inflammatory activity against LPS-induced inflammation in PMA-differentiated human cell line U-937. There was a significant decrease ($p < 0.05$) in IL-8 levels following LPS-induced inflammation after 24h treatment, when compared to the stimulated cells, with all unmodified oils (non-enriched and enriched with 5% v/v geraniol) at 0.01% (v/v) and under (**Fig. 3.4B-D**). On the other hand, LGVPH at 0.01% (v/v) did not show a significant decrease in IL-8 (**Fig. 3.4A**) when compared to the stimulated cells, which could be due to the higher concentration of IL-8 in one well in comparison to the other repetitions at this concentration. This variation could have arisen from the presence of many cells in that well, thus resulting in a greater production of IL-8 and decreasing the apparent anti-inflammatory activity. However, LGVPH showed a significant decrease in IL-8 at 0.008% (v/v).

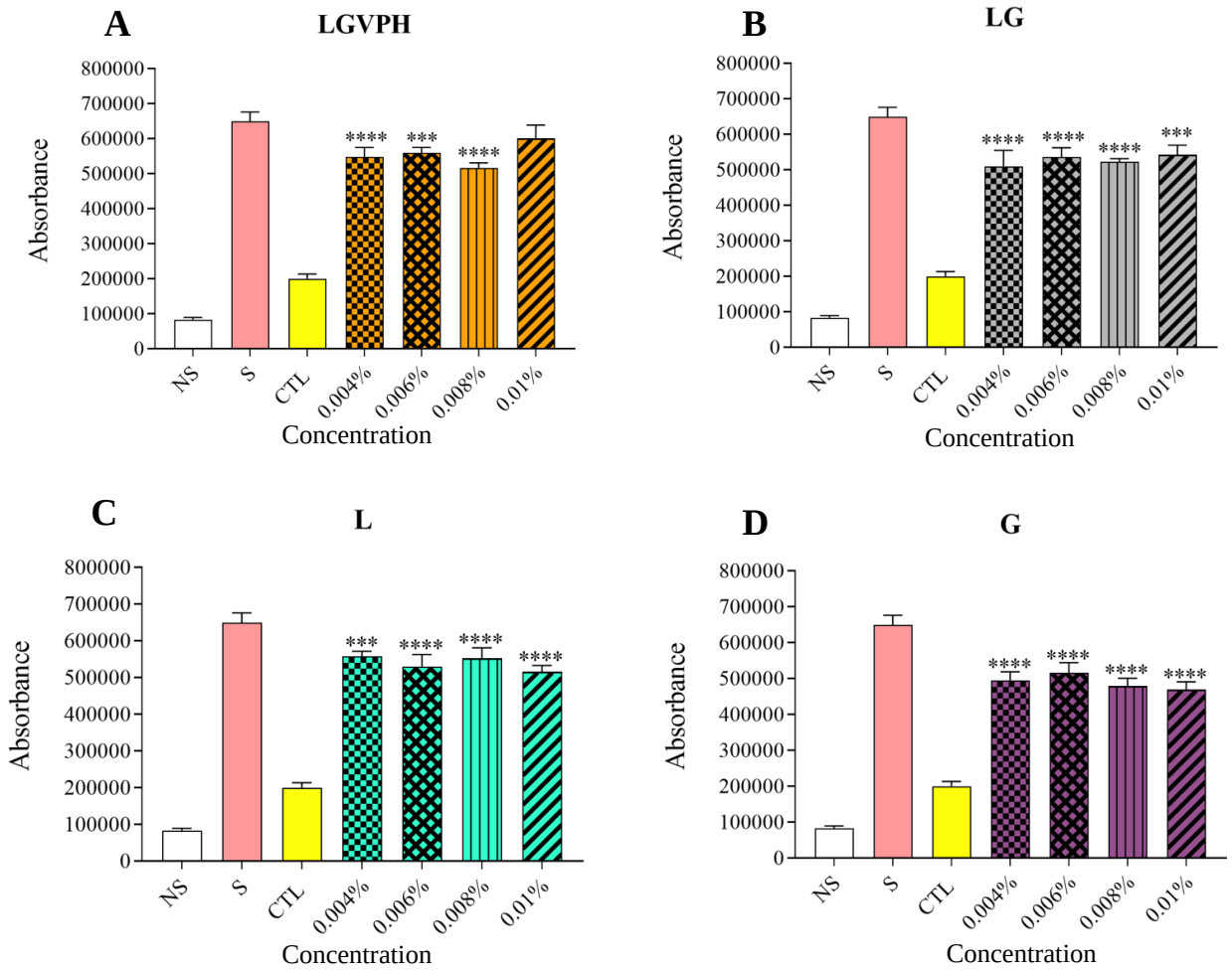


Figure 3.4. Interleuken-8 levels of PMA-differentiated U-937 cells following 24h treatment with LGVPH, LG, L, and G at 0.004%, 0.006%, 0.008%, and 0.01%.

NS: Not stimulated (no LPS); **S:** stimulated (with LPS); **CTL:** control (with LPS, dexamethasone); **LGVPH:** lemon oil enriched with 5% geraniol modified with 1 molar equivalent vinyl propionate, high bioconversion; **LG:** lemon oil enriched with 5%, unmodified; **L:** lemon oil, unmodified; **G:** geraniol, unmodified; ***, p <0.0002; ****, p < 0.0001

Overall, regardless of concentration, these significant differences did not follow a dose-dependent decrease in IL-8. This pattern could also mean that the anti-inflammatory action of the oils and geraniol had reached a plateau and no further increase in concentration could further decrease the inflammation. More work is necessary to determine the reason behind this pattern. Furthermore, there was no significant difference ($p > 0.05$) between the anti-inflammatory activity of modified lemon oil and unmodified lemon oil or geraniol at concentrations below 0.01% (v/v). At 0.01% (v/v), there was a significant difference ($p < 0.05$) in the level of IL-8 when treated with modified lemon oil in comparison to treatment with unmodified lemon oil, as well as geraniol. This suggests that modification does not affect anti-inflammatory activity at lower concentrations, however, at higher concentrations, lemon oil and geraniol exhibit more effective activity when used independently.

Geraniol has been shown to have anti-inflammatory activity in human mast cells (HMC-1) triggered by phorbol 12-myristate 13-acetate plus A23187 (PMACI) (Huang, Yang, Ni, & Xu, 2018). Huang *et al.* (2018) reported a reduction of 30%, 21.6%, and 27.0% in proinflammatory cytokines such as IL-1β, IL-6, and TNF-α, respectively, *in vitro* geraniol concentrations of 160 µmol/L. They also remarked that the same effect was reflected *in vivo* in ovalbumin-induced allergic rhinitis models. The authors attributed this behavior to alterations in MAPK/NF-κB signaling pathways. Another study found that 25µg/mL geraniol reduced pro-inflammatory cytokine levels of iNOS and COX-2 as well as downregulated the levels of TNF-α, IL-1β, and IL-6 in murine RAW 264.7 cells (Jiang et al., 2017). The authors attributed this activity to the inhibition of TLR4-mediated NF-κB and Bcl-2/Bax signalling pathways by geraniol.

EOs from the *Citrus* genus are known to have anti-inflammatory activity and have been used in folk medicine for their antiseptic, anti-inflammatory, and sedative effects (Kummer et al., 2013). A study evaluating the peel of *Citrus limetta* Risso EO showed that at a concentration of 0.1% (v/w), it was able to inhibit pro-inflammatory cytokines TNF-α, IL-6, IL-1β in LPS-induced inflammation *in vitro* and *in vivo* (Maurya, Mohanty, Pal, Chanotiya, & Bawankule, 2018). Another study confirmed the anti-inflammatory properties of another *Citrus* peel EO and reported that it blocked JNK, ERK and NF-κB signaling pathways in LPS-activated macrophages (Kim et al., 2013). The investigation of four *Citrus* EOs revealed that their anti-inflammatory activities were similar to that of pure limonene and attributed their action to the presence of a large quantity of limonene (31.1- 65.7%) in their chemical profiles (Amorim et al., 2016).

3.4.5. Conclusion

Overall, these results demonstrate the feasibility of enzymatic modification of geraniol in lemon EO.

A solvent-free method was developed to enzymatically modify geraniol in lemon EO through lipase-catalyzed transesterification. A solvent-free gram-scale up was successfully carried out with a bioconversion yield as high as 89% with both VA and VP (1 molar equivalent) as acyl donors and lemon oil enriched with 5% (v/v) geraniol. Geraniol, unmodified, and modified lemon oil emulsions exhibited some dose-dependent cytotoxicity towards human macrophage cell line U-937. Unmodified and modified lemon oils showed the same cytotoxic behavior. Their cytotoxicity could be attributed to the presence of geraniol and citral. These compounds could have elicited cell death through cellular membrane attack, inducing apoptosis/necrosis. Additionally, these compounds could have contributed to the perturbation of signaling pathways in the human U-937 cell line in this study.

As for anti-inflammatory activity, IL-8 levels were decreased following 24h treatment with geraniol, unmodified, and modified lemon oil (1 molar equivalent of VP, 5% geraniol v/v) emulsions. This activity, however, was not dose-dependent and therefore suggests that the activity was due to a plateau in anti-inflammatory action of the oils. Further investigations are suggested.

CHAPTER IV

GENERAL SUMMARY AND CONCLUSION

Two *in vitro* methods were used to assess the antioxidant activity of 38 EOs and of their enrichments with individual PPs, mixtures of major PPs, as well as crude extracts. Pimento berry EO (HE-PIM-01) was found to exhibit the highest overall antioxidant capacity (IC_{50} = 0.0816 mg/mg DPPH; ORAC Value = 8174.75 µmol TE/g) and white pine needles (HE-PIN-02) exhibited the lowest (IC_{50} = 652.4518 mg/mg DPPH; ORAC Value = 7.64 µmol TE/g). It was found that phenols were the major contributing components to synergy in the IC_{50} and ORAC values. Monoterpenes or ketones paired with phenols exhibited an antagonistic interactive effect on the anti-scavenging activity, thus decreasing their antioxidant capacity in the DPPH assay, whereas no significant effect was detected in the ORAC assay. There was no correlation found between the IC_{50} and ORAC values, with some EOs exhibiting antioxidant capacities that did not align in both assays, which could have been due to solvent effects, as well as the presence of pro-oxidants.

Enrichments with individual PPs exhibited increasing antioxidant capacity with increasing structural advantages in the phenolic acids or flavonoids used. Rosmarinic acid and quercetin were shown to greatly improve the antioxidant capacity of EOs, whereas *p*-coumaric acid and rutin hydrate improved them the least. Enrichments with mixtures of major PPs and crude extracts showed no antagonistic effects in the DPPH assay. In the ORAC assay, there was a decrease in antagonistic effects when enriching with crude extracts in comparison to mixtures of major PPs, which suggests that interactions of EOs with major and minor components of plant extracts improve the antioxidant capacity of enrichments. EOs enriched with the major PPs of green tea extract showed the lowest IC_{50} values among the 4 enrichments (0.009-0.0681 mg/mg DPPH) and exhibited moderate to high values (6,207.50-13,681.00 µmol TE/g sample) in the ORAC assay. Crude green tea (EX-THE-01) and apple (EX-POM-04) extracts did not exhibit any antagonistic effects in both assays, whereas crude grape seed (EX-RAI-01) and rosemary (EX-ROM-04) extracts equally exhibited antagonistic effects. Predictive models were developed to explain 48% and 50% of the variability in the IC_{50} and ORAC values, respectively. There was a weak to moderate correlation between the expected and observed IC_{50} (R^2 = 0.37) and there was a moderate to strong correlation between the expected and observed ORAC values (R^2 = 0.7).

Following this, the investigation of lipase-catalyzed transesterification of terpenes was carried out and the chemical profiles were assessed with GC-MS. Overall, no modification of eugenol or thymol was observed. However, geraniol was successfully acetylated with Novozym® 435 and lipase from *C. rugosa* using two vinyl esters (VA, VP). Between the reactions catalyzed by Novozym® 435 and lipase from *C. rugosa*, the former showed higher acylation yields (41-100%) in comparison to the latter (<10-41%), using both vinyl esters as acyl donors. With each increase in molar equivalent of acyl donor, there was

an increase in the bioconversion yield, with a few exceptions. Given that similar yields could be achieved between 1 and 1.5 molar equivalents upon the same reaction time, 1 molar equivalent of acyl donor was selected for future investigations.

To overcome the limitation of a solvent removal step, a solvent-free reaction method was developed utilizing lemon oil as the reaction medium. No reaction was observed in the absence of geraniol, however, in the presence of 2% (v/v) and 5% (v/v) geraniol, the reaction was successfully carried out. When gram-scaled up, the transesterification of an enrichment of 5% (v/v) geraniol catalyzed by Novozym® 435 achieved complete bioconversion in 15h, with a maximum yield of 89%. Similar yields were observed with the use of VA as an acyl donor with a 5% (v/v) geraniol enrichment, with yields as high as 89% within 15h.

The cytotoxicity of these newly modified oils was then assessed against human monocyte cell line U-937. The modification of geraniol-enriched lemon oil did not affect the cytotoxicity of the oil, as both modified and unmodified lemon oils showed similar values and patterns. As for the anti-inflammatory properties, lemon oil enriched with 5% geraniol modified with vinyl propionate was assessed alongside unmodified lemon oil and geraniol. Human U-937 cells were differentiated with PMA and subsequently stimulated with LPS to induce an inflammatory response. In the presence of modified and unmodified oils, the cells showed lower levels of IL-8. No significant different ($p > 0.05$) was observed between the anti-inflammatory activities of modified and unmodified lemon oils at lower concentrations. At higher concentrations, however, lemon oil and geraniol exhibited higher anti-inflammatory activity. This effect was not dose-dependent and therefore suggests that the activity could have reached a plateau. Further investigations are suggested.

The understanding of the combinatorial interactions between EOs and plant extracts as well as the feasibility of their enzymatic modification is expected to increase their usage in the food industry as functional ingredients. The investigation of their cytotoxicity and their anti-inflammatory properties is expected to aid the food industry with increasing knowledge regarding their safety and potential health-promoting properties.

CHAPTER V. CONTRIBUTIONS TO KNOWLEDGE AND RECOMMENDATIONS FOR FUTURE STUDIES

Contributions to Knowledge

- A comparative study of the antioxidant capacity of EOs was done with two *in vitro* methods and their correlation was assessed. This contributes to the understanding of how EOs may respond to the assays based on their chemical profiles and classification/type. This study offers recommendations of assays to use to measure antioxidant capacity based on EO classification.
- The investigation of the combinatorial effects between EOs and plant extracts was carried out. Their effects were linked to their chemical profiles and structure-activity relationships were described. To the author's knowledge, no study has characterized the interactions between EOs and plant extracts. This study contributes to the understanding of the underlying interactions between their components and provides a model to predict the effects of EO enrichment on antioxidant capacity.
- For the first time, geraniol was modified in a solvent-free reaction utilizing lemon oil as the reaction medium. This was successfully catalyzed with Novozym® 435 using VA and VP as acyl donors. The newly modified oils were then assessed for their cytotoxicity and anti-inflammatory activity. This study expects to increase the use of EOs in the food industry as functional ingredients by modifying their aromatic profiles. Furthermore, this study also contributes to the knowledge of their potential health-promoting properties.

Recommendations for Future Research

- Investigation of antioxidant properties in different solvents, as well as using other antioxidant assays to obtain a larger picture of synergistic action between the EOs and extracts. Doing so will allow the investigation of synergistic mechanisms, taking into account singlet-oxygen quenching mechanisms, as well as metal-chelating mechanisms.
- An olfactory evaluation of the aromatic profiles of newly modified EOs with perfumers or using gas chromatography-olfactometry is suggested. Such an investigation would be able to reveal whether secondary notes, that were once masked by certain compounds, have now become more apparent or not. It would also shed light regarding the new potential uses for lemon oil in the food industry and which new products this new aromatic profile could accommodate.

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