Universidade do Minho
Escola de Ciências

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Plants extracts: isolation, synthesis of derivatives, nanoencapsulation and biological evaluation for potential application as biopesticides

dezembro de 2021

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Dissertação de Mestrado
Mestrado em Bioquímica Aplicada

Trabalho efetuado sob a orientação do
Professor Doutor António Belmiro Gil Silva Fortes
e da
Professora Doutora Elisabete M. S. Castanheira Coutinho

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With the conclusion of this dissertation, I became more autonomous and confident in my work, qualities that I carry, and I want to improve more and more in the job market.

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Título: Extratos de plantas: isolamento, síntese de derivados, nanoencapsulamento e avaliação biológica para potencial aplicação como biopesticidas

Resumo

Nos últimos anos, o crescente interesse pelas plantas tem sido justificado pelo facto dos seus extratos poderem ser utilizados como uma potencial fonte de compostos para cosmética e farmacologia. Atualmente, os fitoquímicos são também estudados devido ao seu potencial como pesticidas biológicos. Várias plantas, nomeadamente as plantas aromáticas, contêm vários fitoquímicos que podem ser extraídos através de diferentes técnicas. Os óleos essenciais são um grupo diferente de fitoquímicos com comprovada atividade inseticida; no entanto, estes compostos apresentam algumas limitações, sendo facilmente degradados quando expostos a fatores externos, como a luz e o ar.

Os objetivos deste trabalho são a obtenção de extratos vegetais, a síntese de derivados de fitoquímicos e a realização de ensaios de nanoencapsulamento dos compostos mais promissores, permitindo a preservação dos fitoquímicos com atividade inseticida e uma libertação mais direcionada e controlada.

Considerando estes objetivos, a espécie *Syzygium aromaticum* (cravinho) foi submetida a extração por hidrodestilação. O eugenol obtido foi identificado, isolado e utilizado como precursor na síntese de um derivado por reação de epoxidação. O epóxido de eugenol foi depois usado para obter derivados semi-sintéticos de eugenol por reação com diferentes álcoois e aminas para formar alcoxiálcoois e aminoálcoois, respetivamente. Os compostos sintetizados foram caracterizados por RMN (^{1}H e ^{13}C) e espetroscopia de absorção UV-Visível e de infravermelho. Testes preliminares de atividade inseticida foram realizados usando a linha celular *Sf9* (de ovários imaturos de *Spodoptera frugiperda* pupae). Os compostos mais promissores, 4-(3-(*terc*-butoxi)-2-hidroxipropil)-2-metoxifenol e 4-(2-((4-fluorobenzil)oxi)-3-hidroxipropil)-2-metoxifenol, foram submetidos a ensaios de encapsulamento e libertação usando nanossistemas baseados em lipossomas de fosfatidilcolina do ovo e colesterol (7:3). Foram obtidas elevadas eficiências de encapsulamento e a libertação é bem descrita pelos modelos de Weibull e de Korsemeyer-Peppas.

Palavras-chave: biopesticidas, óleos essenciais, atividade inseticida, fitoquímicos, nanoencapsulamento

Title: Plants extracts: isolation, synthesis of derivatives, nanoencapsulation and biological evaluation for potential application as biopesticides

Abstract

In recent years, the growing interest in plants has been justified by the fact that their extracts can be used as a potential source of compounds for cosmetics and pharmacology. Currently, phytochemicals are also studied due to their potential as biological pesticides. Several plants, namely, aromatic plants contain several phytochemicals that can be extracted using different techniques. Essential oils are a different group of phytochemicals with proven insecticidal activity; however, these compounds have some limitations, being easily degraded when exposed to external factors, such as light and air.

The objectives of this work are to obtain plant extracts, to synthesize derivatives of phytochemicals, and to perform nanoencapsulation of the most promising compounds, allowing the preservation of phytochemicals with insecticidal activity and a targeted and controlled release. Considering these objectives, the species *Syzygium aromaticum* (clove) was submitted to extraction by hydrodistillation. The eugenol obtained was identified, isolated, and used as a precursor in the synthesis of a derivative by epoxidation reaction. Eugenol epoxide was then used to obtain semi-synthetic eugenol derivatives by reacting with different alcohols and amines, to form alkoxy alcohols and amino alcohols, respectively. The synthesized compounds were characterized by NMR (^{1}H and ^{13}C) and UV-Visible absorption and infrared spectroscopy. Preliminary tests of insecticidal activity were performed using *Sf9* cell line (from the immature ovaries of *Spodoptera frugiperda* pupae). The most promising compounds, 4-(3-(*tert*-butoxy)-2-hydroxypropyl)-2-methoxyphenol and 4-(2-((4-fluorobenzyl)oxy)-3-hydroxypropyl)-2-methoxyphenol, were subjected to encapsulation and release assays using liposome-based nanosystems of egg phosphatidylcholine/cholesterol (7:3). High encapsulation efficiencies were obtained, and the release profiles were well described by both Weibull and Korsemeyer-Peppas models.

Key words: biopesticides, essential oils, insecticidal activity, phytochemicals, nanoencapsulation

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List of abbreviations

AGS - Adenocarcinoma Gastric Stomach

ATP - Adenosine Triphosphate

CoA - Coenzyme A

Crph - β-caryophyllene

DAHP - 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate

DCM - Dichloromethane

DHQ - 3-dehydroquinic acid

DHS - 3-dehydroshikimic acid

DLS - Dynamic Light Scattering

DMAPP - Dimethylallyl Pyrophosphate

EE - Encapsulation Efficiency

EI - Ethanol Injection

EO – Essential Oil

EPA – Environmental Protection Agency

EPSP - 5-enolpyruvylshikimic acid 3-phosphate

AcOEt - Ethyl acetate

EtOH - Ethanol

Eug – Eugenol

Eug-Ac - Eugenyl Acetate

GC-MS - Gas Chromatography-Mass Spectrometry

HMG-CoA - 3-methylglutaryl-CoA

HPLC - High-Performance Liquid Chromatography

IGR - Insect Growth Regulators

IPP - Isopentenyl Pyrophosphate

IR - Infrared

L-Phe - L-Phenylalanine

L-Trp - L-Tryptophan

L-Tyr - L-Tyrosine

LUV - Large Unilamellar Vesicles

***m*-CPBA** – meta-Chloroperbenzoic Acid

MeOH - Methanol

MLV - Multilamellar Vesicles

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NMR - Nuclear Magnetic Resonance

PEP - Phosphoenolpyruvic acid

PDI - Polydispersity

EP - Petroleum ether

S3P - Shikimic acid 3-phosphate

Sf9 - *Spodoptera frugiperda*

Sn – Nucleophilic substitution

SUV - Small Unilamellar Vesicles

TFH – Thin Film Hydration

TLC – Thin-Layer Chromatography

U.S. – United States

UV - Ultraviolet

Vis – Visible

Introductory note

The numbering of compounds in Chapter 1 is independent of the numbering of compounds in the remaining chapters. The numbering of figures, tables, schemes, and equations is continued throughout the dissertation.

Chapter 1 – State of the art

1.1 Introduction

Food production and human health are greatly affected by insects. One way to control pests is to use synthetic insecticides. Despite being used frequently, the inappropriate utilization of these compounds is related to the development of resistance to pests, human diseases and contamination of food and environment. Consequently, the biological action of natural products with insecticidal activity is a very important alternative that allows to manage in an environmental-friendly way the action of insects and pests, without affecting people's health.[1]

Essential oils (EOs), for example, are a matrix of phytochemicals with efficient biopesticide action, however, their degradation when exposed to external factors, such as light and air, is a barrier to their wide use.[2,3]

One way to overcome the difficulties related to the use of synthetic pesticides and the easy degradation of biopesticides, is to encapsulate these plant extracts, improving their resistance to biological and physicochemical degradation and increasing their effectiveness and controlled release with reduced toxicity.[4,5]

The initial objectives of this master's thesis are the extraction, synthesis, characterization, and biological analysis of plant extracts as biopesticides. Another main objective is to synthesize derivatives of phytochemicals with promising insecticidal activity and to encapsulate them in suitable nanosystems, to promote a more controlled release, as well as to increase the efficacy.

1.2 Biopesticides

There has been a growing interest in studying and evaluating the action of botanical insecticides for pest management due to of insect resistance to the traditional insecticides.[6] Insect resistance is only one of the disadvantages of traditional insecticides; they also have adverse effects on non-target organisms, ecosystems and human health because the long-term applications result in residues accumulating in different environmental components like water, food, air and soil.[7]

For this reason, biopesticides are considered an alternative to synthetic insecticides.[8] These biopesticides are called "green pesticides" and include all natural materials that can reduce the pest population and increase food production.[7]

The United States (U.S.) Environmental Protection Agency (EPA) define that "biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals".[9] The U.S. EPA recognizes three categories of biopesticides: biochemical pesticides, plant-incorporated protectants and biocontrol organisms or microbial pesticides.[9,10] Biochemical pesticides, the focus of this study, contain natural substances that work as pest and diseases control, for example, plant extracts and botanical oils.[10]

The big question is to understand why synthetic pesticides should be replaced by biopesticides. There are some key reasons that justify increasing its production and use: better yields and quality, resistance management, managing residues and safety and biodegradability.[10]

1.3 Phytochemistry

Phytochemicals are chemical compounds, with low molecular weight, biologically active and naturally found in plants. These compounds are called phytochemicals because they are chemicals that protect plant cells from environmental hazards and the risks of pathogenic or insecticidal attacks. In addition to protecting plants from damage and disease, they also contribute to the color, aroma, and flavor of the plant.[11]

Phytochemicals accumulate in different parts of plants: roots, stems, leaves, flowers, fruits, and seeds. However, this distribution varies from plant to plant and depends, for example, on the variety and conditions of cultivation.[11]

1.3.1 Phytochemical metabolic pathways

Over the years of research, more than 100,000 phytochemicals have been cataloged.[12,13] The classification of phytochemicals is done considering the metabolism of plants, being classified as primary or secondary constituents.[11]

Despite the extremely varied characteristics of living organisms, the pathways to synthesize and modify carbohydrates, amino acids, proteins, and nucleic acids are essentially the same in all organisms, with slight variations.[11,12] These bioprocesses, which demonstrate the fundamental unity of all

living matter, are described as primary metabolism and, consequently, the compounds involved in this process are called primary metabolites.[12] In the case of plants, the main plant substances, proteins, fats, and carbohydrates, contribute to energy metabolism and the structure of the plant cell.[13]

In contrast to the primary metabolic pathways, there is a strand of metabolism related to compounds that have a more limited distribution in nature. These compounds, called secondary metabolites, are found only in specific groups of organisms and are an expression of species individuality. They are not necessarily produced in all conditions, but they are essential for plant interactions with the environment and serve as a defense against insects, fungi, and other microorganisms and as growth regulators, pigments, and flavors. The secondary metabolites of plants are quite diverse chemical compounds that are only present in small amounts.[13] It is the area of secondary metabolism that provides most pharmacologically active natural products, which play a vital role in the well-being of the organism that produces them.[12]

Secondary metabolites include phenolic compounds, which comprise phenolic acids, flavonoids and tannins, alkaloids, and terpenoids.[11]

1.3.1.1 Shikimate metabolic pathway

Phenolic compounds and alkaloids are biosynthesized by the shikimate pathway localized in the chloroplast.[14] This pathway produces aromatic amino acids L-phenylalanine (L-Phe), L-tyrosine (L-Tyr), and L-tryptophan (L-Trp) that serve as precursors for these secondary metabolites.[15]

The main aromatic phenolic compounds are synthesized from L-Phe and L-Tyr, and L-Trp is the precursor of alkaloids.[15]

The entire process of biosynthesis of the metabolic pathway of shikimate is shown in figure 1 and consists of sequential enzymatic steps:[14]

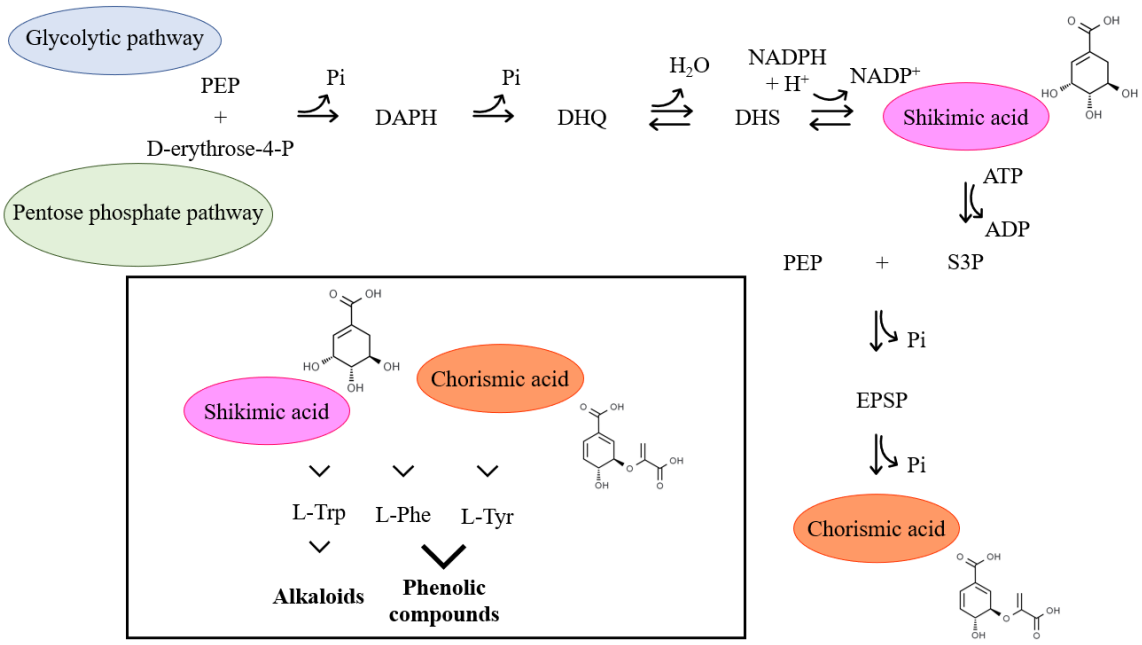


Figure 1. Shikimate pathway. The shikimic and chorismic acids are the common precursors for the synthesis of L-Trp, L-Phe, L-Tyr and diverse phenolic compounds and alkaloids.

1. Begins with an aldol-type condensation of two phosphorylated active compounds, the phosphoenolpyruvic acid (PEP), from the glycolytic pathway, and the carbohydrate D-erythrose-4-phosphate, from the pentose phosphate cycle, to give 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP);
2. DAHP loses phosphate; the enolic type product is cyclized through a second aldol-type reaction to produce 3-dehydroquinic acid (DHQ). The DHQ dehydrates to produce 3-dehydroshikimic acid (DHS);
3. Reduction reaction of DHS with reduced nicotinamide adenine dinucleotide phosphate (NADPH) followed by the activation of shikimic acid with adenosine triphosphate (ATP) to make shikimic acid 3-phosphate (S3P);
4. Addition of PEP to S3P to generate 5-enolpyruvylshikimic acid 3-phosphate (EPSP);
5. The last reaction step of the shikimate pathway is the production of chorismic acid. The shikimic and chorismic acids are the common precursors for the synthesis of L-Phe, L-Tyr, and L-Trp for biosynthesis of phenolic compounds and alkaloids.

The shikimate pathway has special characteristics that are present only in bacteria, fungi, and plants. The absence of the pathway in all other organisms provides the enzymes catalyzing these reactions with potentially useful targets for the development of antibacterial agents and herbicides.[14,15]

1.3.1.2 Mevalonate metabolic pathway

Mevalonic acid is the primary precursor of the terpenoids biosynthesized by plants and the entire process of biosynthesis of the metabolic pathway of mevalonate is shown in figure 2.

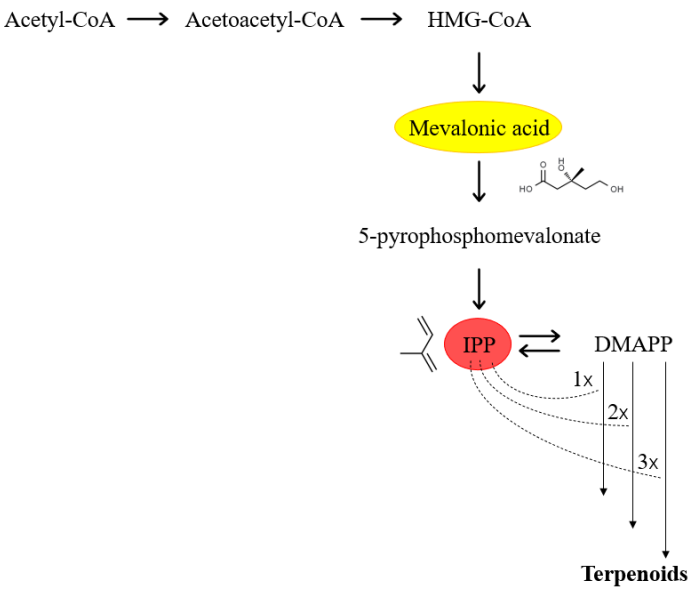


Figure 2. Mevalonate pathway. The mevalonic acid is the precursor for the synthesis of IPP and diverse terpenoids.

Mevalonic acid is derived from acetyl-coenzyme A (CoA) through the intermediate formation of acetoacetyl-CoA and 3-methylglutaryl-CoA (HMG-CoA). Reduction of HMG-CoA gives mevalonic acid. The resulting is sequentially phosphorylated by two separate soluble kinases, to form 5-pyrophosphomevalonate.[16]

Formation of "active isoprene unit", isopentenyl pyrophosphate (IPP) is then catalyzed by pyrophosphomevalonate decarboxylase.[16]

IPP itself is insufficiently reactive to undergo ionization to initiate the condensation to higher terpenoids. Therefore, it is first isomerized to the allylic ester dimethylallyl pyrophosphate (DMAPP).[16]

Higher terpenoids are generated via the action of prenyl transferases, which perform multistep reactions beginning with DMAPP (or a longer allylic pyrophosphate) and IPP to form higher isoprenologs.[16]

1.3.2 Phytochemical classes

1.3.2.1 Phenolic compounds

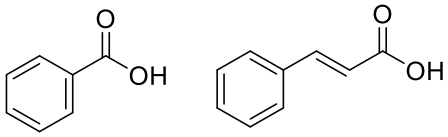
Previous studies reveal that phenolic compounds are the most numerous and structurally diverse constituents present in plants, but their distribution depends on the part of the plant.[11,17]

Plant phenolic compounds are derived from Phe and Tyr, are different in molecular structure and characterized by aromatic hydroxylated rings.[17,18] They can be divided into several subgroups ranging from simple molecules to polymerized compounds.[17] Phenol is considered the simplest class in this group of compounds.[18]

- **Phenolic acids**

The term "phenolic acids" generally means phenols that have a functional group of carboxylic acid.[17]

Naturally occurring acids form a diverse group and contain two distinct carbon structures: benzoic acid **1** and cinnamic acid **2** structures shown in figure 3.[18] The derived acids differ in the degree of hydroxylation and methoxylation of the aromatic ring.[19]



1 **2**

Figure 3. Structure of benzoic acid **1** and cinnamic acid **2**, precursors of the two main classes of phenolic acids.

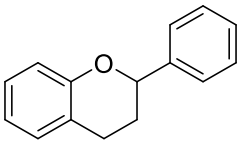
- **Flavonoids**

Flavonoids structure consists of two aromatic rings associated with an oxygenated heterocycle, widely found in fruits and vegetables.[19,20]

In nature, flavonoid compounds are easily recognized as flower pigments in most angiosperm families but they are found in all parts of plants.[20]

Flavonoids **3**, whose basic skeleton is shown in figure 4, are considered primary antioxidants and chelators agents.[17] Its antioxidant activity depends on the position and degree of hydroxylation of the

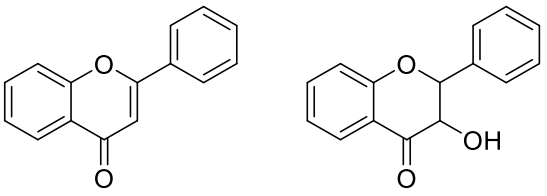
molecule and can be divided into flavones and flavonols, flavanones and flavanols, isoflavones, proanthocyanidins and anthocyanins.[17,19]



3

Figure 4. Basic skeleton structure of flavonoids **3**.

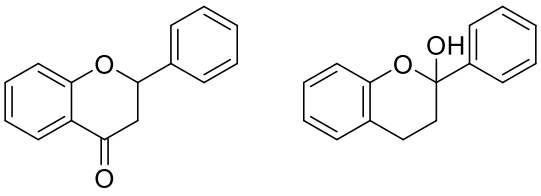
Flavones **4** do not have OH in position 3 and flavonols **5** have a central ring of 3-hydroxypyran-4-one, as shown in figure 5.[19]



4 5

Figure 5. General structure of flavones **4** and flavonols **5**.

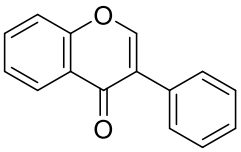
Flavanones **6** have no double bond in position 2,3 of the central ring, whereas flavanols **7** lack a carbonyl group at position 4, as shown in figure 6.[19]



6 7

Figure 6. General structure of flavanones **6** and flavanols **7**.

Isoflavones **8** shown in figure 7 are phytoestrogens present in many leguminous plants.[19,20]



8

Figure 7. General structure of isoflavones **8**.

Proanthocyanidins are oligomeric flavonoids. Anthocyanins are pigments responsible for coloring plants, flowers, and fruits[19,20] and contribute significantly to their antioxidant capacity.[19] General structure of anthocyanin **9** is shown in figure 8.

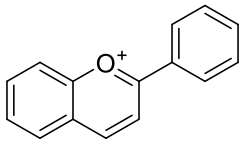


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Figure 8. General structure of anthocyanin **9**.

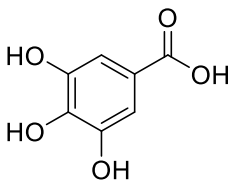
- **Tannins**

Tannins are a heterogeneous group of polyphenolic compounds capable of forming reversible and irreversible complexes, mainly with proteins.[18]

Based on their structural characteristics, it is possible to divide the tannins into four main groups: gallotannins, ellagitannins, complex tannins and condensed tannins, shown in figure 9.[18]

Gallotannins are tannins in which units of gallic acid **10** or its derivatives are linked to several units of polyol-, catechin- or triterpenoid. Ellagitannins **11** are tannins in which at least two units of gallic acid are C-C coupled together and do not contain a glycosidically linked catechin unit. Complex tannins are tannins in which a catechin unit **12** is glycosidically linked to a gallotannin or ellagitannin unit.[11,18]

Condensed tannins are all oligomeric and polymeric proanthocyanidins formed by the binding of C-4 from a catechin with C-8 or C-6 from the next monomeric catechin.[11,18]



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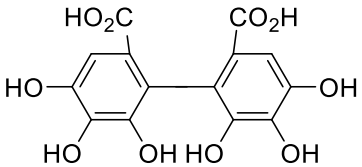

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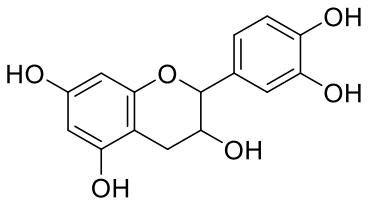


12

Figure 9. Classification of tannins. Gallic acid **10** (base of gallotannins), ellagitannin **11** and a catechin unit **12**.

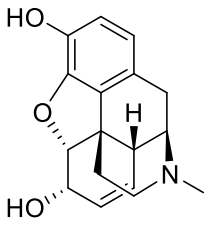
1.3.2.2 Alkaloids

Alkaloids are natural products that contain heterocyclic structures with nitrogen atoms, having a basic character. Alkaloids are numerous and involve a variety of molecular structures, so the best approach is to group them into subgroups, depending on the type of heterocyclic ring system present in each molecule.[18]

Pyrrolidine alkaloids contain the pyrrolidine ring system. Pyridine alkaloids have a pyridine ring system and pyrrolidine-pyridine alkaloids contain the heterocyclic ring system with pyrrolidine-pyridine.[11,18]

Pyridine-piperidine alkaloids contain a pyridine ring system associated with a piperidine ring system. Quinoline alkaloids have a basic quinoline heterocyclic ring system.[11]

Isoquinoline alkaloids contain isoquinoline in the heterocyclic ring system. Examples of isoquinoline alkaloids are opium alkaloids, such as morphine **13** shown in figure 10.[11,18]



13

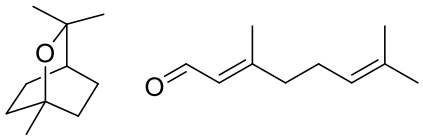
Figure 10. Isoquinoline alkaloid morphine **13**.

1.3.2.3 Terpenoids

Among the secondary metabolites of plants, terpenoids are the most structurally diverse group. They act as phytoalexins in the direct defense of the plant or as signals in the indirect defense responses, which involve herbivores and natural enemies. Some plants produce fewer volatile terpenes that protect some plants from being eaten by animals.[18]

This class includes natural products derived from five-carbon isoprene units. Most terpenoids have multicyclic structures that differ due to functional groups and basic carbon skeletons. Terpenes are abundant in nature, mainly in plants, as constituents of EOs.[11,18] Hemiterpenoids consist of a single isoprene unit. The only hemiterpene is isoprene itself, but isoprene derivatives that contain oxygen are also classified as hemiterpenoids.[11]

Monoterpenoids have two isoprene units. They can be linear or contain cyclic rings. Some examples are geranyl pyrophosphate, eucalyptol **14**, citral **15**, camphor, limonene and pinene, as shown in figure 11. Sesquiterpenes have three units of isoprene and diterpenes are composed of four isoprene units and are derived from geranyl-geranyl pyrophosphate. Triterpenes consist of six units of isoprene and are found, for example, in wheat germ and olives.[11]



14 **15**

Figure 11. Monoterpenoids eucalyptol **14** and citral **15**.

Tetraterpenoids contain eight isoprene units that can be acyclic like lycopene, monocyclic like gamma-carotene or bicyclic like alpha- and beta-carotenes.[11]

1.3.3 Essential oils

EOs are a matrix of lipophilic and highly volatile secondary plant metabolites, with a molecular weight below of 300 kDa, that can be physically separated from other plant/membranous components or tissues.[2] They can be found in aromatic plants and play an important role in plant defense system against microorganisms, insects, herbivores and allelopathic interactions.[7] Many of different, but structurally related, components have been identified in EOs. Each oil in turn can be composed of only a few up to a complex mixture of substances.[2] Some previous studies have reported that EO components can be assigned as lipophilic terpenoids, phenylpropanoids, or short-chain aliphatic hydrocarbon derivatives of low molecular weight, with the first being the most frequent and characteristic constituents, around 90%.[2,3] Besides that, previous reports have shown that the toxicity of EOs against a variety of insects is related to terpenoids.[2,3,7]

Degradation of EOs depends on several factors. For this reason, external factors such as temperature, light, and accessibility to atmospheric oxygen must be considered after extraction and storage. In addition, EO composition, compound structures, and the presence of impurities may also affect stability.[2,3]

1.3.4 Structure-activity relationship

Establishing clear functionality and structure-activity relationships in relation to the effect of phytochemicals on biological systems is difficult. This is because some classes of phytochemicals have more than one biological function.[11] They have important biological activities but in plant, the important roles of phytochemicals include protection against harmful environmental conditions, protection against pathogens and herbivores, feeding deterrence and attraction of pollinators and seed dispersers.[21] Table 1 summarizes some biological properties of these classes of phytochemicals in health.

Table 1. Biological activities of different classes of phytochemicals.

Secondary Metabolites	Role in health care
Phenolic acids	Anticarcinogenic[13,17], Antimicrobial[13], Antioxidant[11,13], Antithrombotic[13], Immunomodulatory properties[13], Anti-inflammatory[11,13,17], Influence on blood pressure[13], Modulate blood glucose levels[13], Increases bile secretion[11], Antiulcer[11], Cytotoxicity[11], Antidepressant[11]
Flavonoids	Anticarcinogenic[13,20], Antimicrobial[11,13,20], Antioxidant[11,13,20], Antithrombotic[13], Immunomodulatory properties[13], Anti-inflammatory[11,13,20], Influence on blood pressure[13], Modulate blood glucose levels[13], Anti-mutagenic[20], Cytotoxicity[11]
Tannins	Anticarcinogenic[13], Antimicrobial[13], Antioxidant[11,13], Antithrombotic[13], Immunomodulatory properties[13], Anti-inflammatory[11,13], Influence on blood pressure[13], Modulate blood glucose levels[13], Anti-diarrhea[11], Antiseptic[11]
Alkaloids	Anticarcinogenic[11,18], Antimicrobial[18], Sedative[18], Neuropharmaceuticals[18], Antihypertensive[11], Antiarrhythmic[11], Antimalarial[11], Analgesic[11]
Terpenoids	Anticarcinogenic[11,13], Antimicrobial[11,13,18], Cholesterol-lowering effect[13], Anti-rheumatic[18], Anti-malarial[11,18], Hepaticidal[11,18], Antiulcer[11]

1.4 Essential oils and plant families with insecticidal activity

The EOs can be applied as biopesticides and they can be act as contact insecticide, causing changes in the pathways of biochemical metabolism of the insects, repellents and in insect growth regulators (IGR) activity.[7]

Some examples of terpenes found in EOs with insecticidal activity are linalool, thymol, eugenol (Eug), β-thujon, menthol, methyl thujate, α-terpineol, carvacrol, limonene, α-pinene, citronellol, geraniol, menthone, myrcene, pulegone, camphor, citral and 1,8-cineole.[7,22] Some of these compounds are the most abundant constituents of some of the most common aromatic plants.[22]

Previous studies have confirmed that EOs obtained from the plant families like Asteraceae, Myrtaceae, Apiaceae, Lamiaceae and Rutaceae have insecticidal activity.[7,22] Table 2 shows examples of EOs with insecticidal activity with the respective insecticidal constituents, family and botanical source.

Table 2. Examples of insecticidal EOs with the respective insecticidal constituents, family and botanical source.[22]

Plant oil or derivative	Plant EO	Major constituent(s) (% by weight)	Plant Family	Botanical Source
Rosemary oil	Rosemary	1,8-cineole, α-pinene, Camphor, β-pinene	Lamiaceae	*Rosmarinus officinalis*
Peppermint oil	Peppermint	Menthol, Menthone	Lamiaceae	*Mentha piperita*
Clove oil, Eug	Clove	Eugenol	Myrtaceae	*Syzygium aromaticum*
Cinnamon oil	Cinnamon	Cinnamaldehyde	Lauraceae	*Cinnamomum* spp.
Thyme oil	Thyme	Thymol, *p*-cymene, γ-terpinene	Lamiaceae	*Thymus vulgaris*
Lemongrass oil	Lemongrass	Geraniol, Citral	Poaceae	*Cymbopogon citratus*
Eucalyptus oil	Eucalyptus	1,8-cineole	Myrtaceae	*Eucalyptus globulus*
Orange oil	Orange	*d*-limonene	Rutaceae	*Citrus sinensis*
Thujone-rich oil	Absinthe	α-thujone, β-thujone	Asteraceae	*Artemisia absinthium*

1.4.1 Essential oils secretion

EOs are biosynthesized, accumulated, and stored in specialized secretory glands. There are two types of secretory glands: those located on the surface of the plant with exogenous secretion and those located inside the plant in internal organs with endogenous secretion. Some of them are also accumulated in the cytoplasm of some secretory cells in one or more plant organs.[4]

External secretion tissue is located outside of the plant and includes:

- Epidermal papillae: they are conical epidermal cells which secrete essences that are generally encountered in flower petals;[4]
- Glandular trichomes: they develop from epidermal cells. They are biosynthesis and accumulation sites of EOs.[23] The synthesized EO is accumulated in a pocket between secretory cells and a common cuticle;[4]
- Non glandular trichomes: they are bristles having similar structure to glandular trichomes.[4]

Internal secretion tissue is located inside the plant and includes:[4]

- Secretory canals: they are small canals which sometimes extend over the entire length of the plant and the walls of which are formed of secreting cells;
- Schizogenous pockets: it is an intercellular space, often spherical, which is filled by EOs droplets synthesized by the cells which border it;
- Cells with intracellular secretion: they are isolated cells specialized in the accumulation and secretion of EOs inside their vacuoles.

Table 3 shows the families of plants with insecticidal activity and the respective characteristic EOs secretion tissue.

Table 3. Plants with insecticidal activity and respective characteristic EOs secretion tissue.[4]

Plant Family	Secretory structure	Organ plant
Lamiaceae	Glandular trichomes	Leaves
Rutaceae	Schizogenous pockets	Epicarp of fruit
Myrtaceae	Schizogenous pockets	Epicarp of fruit
Apiaceae	Secretory canals	Stem
Lauraceae	Cells with intracellular secretion	Stem

1.4.2 Plant species

1.4.2.1 *Syzygium aromaticum*

Syzygium aromaticum is a median size (8-12 m) tropical evergreen tree of the family Myrtaceae that is indigenous to the Maluku islands in east Indonesia but has been farmed in different places worldwide.[24,25] The clove tree is composed of leaves and buds, that is the commercial part of the tree.[24]

Strong of aroma and hot and pungent in taste cloves are the dried flower buds of the clove tree (*Syzygium aromaticum*).[25] Figure 12 shows the *Syzygium aromaticum* tree, the leaves and buds and the dried flower buds.

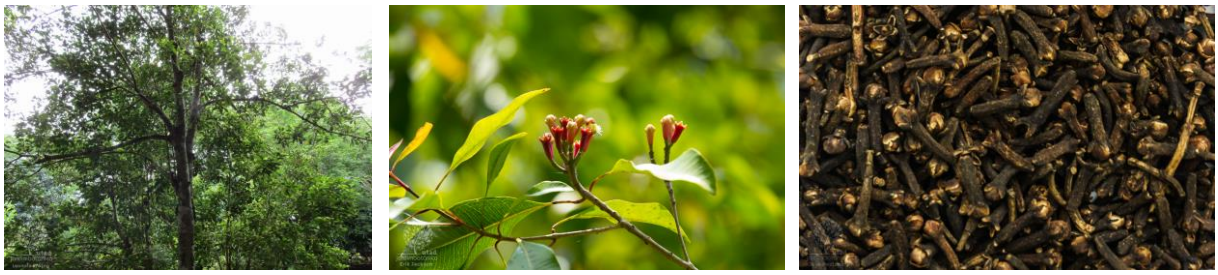
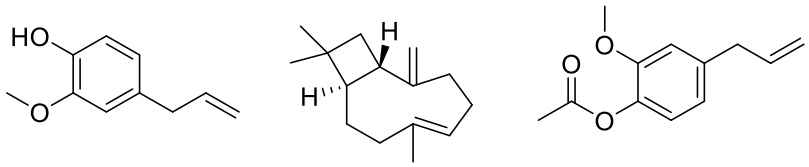


Figure 12. *Syzygium aromaticum*. From left to right: tree (by Leonora (Ellie) Enking from UTAD Botanical Garden, Digital Flora of Portugal), leaves and buds (by Erik Jackson from UTAD Botanical Garden, Digital Flora of Portugal) and dried flower buds (by José Pestana from UTAD Botanical Garden, Digital Flora of Portugal).

Clove represents one of the major vegetal sources of phenolic compounds as flavonoids, hydroxybenzoic acids, hydroxycinnamic acids and hydroxyphenyl propens.[24]

Clove essential oil obtained by hydrodistillation (concentrations up to 18%)[24] of dried flower buds[26] is composed mainly (around 89%)[24] of the phenylpropanoid of the allyl-phenol type[27], Eug, which is 4-allyl-2-methoxy phenol **16**, the sesquiterpene β-caryophyllene **17** (Crph) and the monoterpene ester, eugenyl acetate (Eug-Ac), which is 4-allyl-2-methoxyphenyl acetate **18**.[28] The chemical structures are shown in figure 13.



16 17 18

Figure 13. Major compounds of clove essential oil. 4-allyl-2-methoxy phenol **16**, β-caryophyllene **17** and 4-allyl-2-methoxyphenyl acetate **18**.

4-Allyl-2-methoxy phenol **16** is a pale yellow oil with clove odor.[27] It was reported numerous applications in the pharmaceutical, food, agricultural, and cosmetics industries[29], it showed promising antimicrobial, antioxidant, antiviral[30], and anti-inflammatory effects.[31]

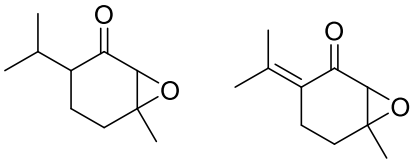
1.4.2.2 *Mentha suaveolens* Ehrh.

Mentha suaveolens Ehrh., showed in figure 14, is an aromatic herb with fruit and a spearmint flavor. This plant is native to Southern and Western Europe.[32] It is generally found along streams, bogs and humid places.[33]

Like *Syzygium aromaticum*, *Mentha suaveolens* Ehrh. is also useful in numerous applications in the pharmaceutical and agricultural industries[32], it showed promising applications in skin diseases[34], for example, and is also known by its anti-inflammatory, cytotoxic and antifungal effects.[35–37]

Figure 14. *Mentha suaveolens* Ehrh.. (by Isabel Garcia-Cabral from UTAD Botanical Garden, Digital Flora of Portugal).

Analysis of the essential oil extracted from different populations of this plant collected in different regions, revealed the presence of high concentrations of oxides. These include piperitone oxide **19** and piperitenone oxide **20** (PO) as mains components.[33,35,38] The chemical structures of these oxides are shown in figure 15.



19 20

Figure 15. Major compounds of *Mentha suaveolens* Ehrh. essential oil. Piperitone oxide **19** and piperitenone oxide **20**.

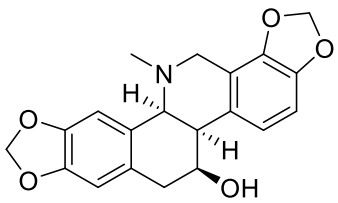
1.4.2.3 *Chelidonium majus*

Chelidonium majus, showed in figure 16, commonly known as greater celandine, is a perennial herb that is widely spread throughout the world, including Europe, Asia, Northwest Africa and North America.[39]

Figure 16. *Chelidonium majus*. (by RockerBOO from UTAD Botanical Garden, Digital Flora of Portugal).

The *Chelidonium majus* plant has been widely studied in the medical field due to its diverse application in biological activities.[39] Its insecticidal activity has also been the object of considerable interest and investigation, since the results show that the alkaloids present in this plant are responsible for the larvicidal and insecticidal activity against some insects.[40]

The plant's orange latex contains more than 20 isoquinoline alkaloids, which have already been isolated and chemically identified. The main secondary metabolites of *Chelidonium majus* are benzylisoquinoline alkaloids, including, for example, chelidonine **21**, whose structure is shown in figure 17, protoberberines and protopines.[40]



21

Figure 17. Structure of the alkaloid chelidonine **21** of the *Chelidonium majus* plant.

1.5 Synthesis of eugenol analogues

The broad spectrum of biological activity makes 4-allyl-2-methoxy phenol **16** a target molecule for structural modifications to produce substances with higher biological activity.[27] Structural modifications from 4-allyl-2-methoxy phenol **16**, can be carried out on the hydroxyl group and the double bond[27] and some of derivatives of 4-allyl-2-methoxy phenol **16** were reported as new potential botanical insecticides[41], being effective on a wide variety of domestic arthropod pests.[27,42]

Some of these studies have demonstrated that a structural modification of EOs can increase the biocidal effect of these phytochemicals, increasing their insecticidal activity.[43,44]

Following previous work in our lab[45], in which some eugenol derivatives showed high potential as biopesticides in assays using the *Sf9* insect cell line, a completely new series of eugenol alkoxy- and some new amino alcohols derivatives was obtained and evaluated for insecticidal activity. Besides that the synthesis of β-alkoxy alcohol is one of the important reactions due to its wider application in the synthesis of potent insecticidal penifulvins bicyclic backbones and also for the direct synthesis of α-alkoxy ketones.[46,47] β-amino alcohols are important organic compounds of considerable use in medicinal chemistry, amino acids, and chiral auxiliaries.[48]

In the present work, semisynthetic 4-allyl-2-methoxy phenol **16** derivatives were synthetized through different approaches with the main objective to use them as potential insecticides.

1.6 Extraction methods

EOs are obtained from the plant's raw material by various extraction methods that can be classified into two categories: conventional methods and innovative methods. The growth in research into new methods is due to the need for innovative and more efficient extraction methods.[4]

Conventional methods can be divided in three main classes: hydrodistillation, solvent extraction (Soxhlet extraction) and cold pressing.

1.6.1 Hydrodistillation

In this technique the plant material is immersed directly in water inside the flask which is boiled. The extraction device includes a heating source with a flask in which distilled water and plant material are placed. The apparatus also includes a condenser and a decanter to collect the condensate and separate the EO from the water, respectively as shown in figure 18.[4]

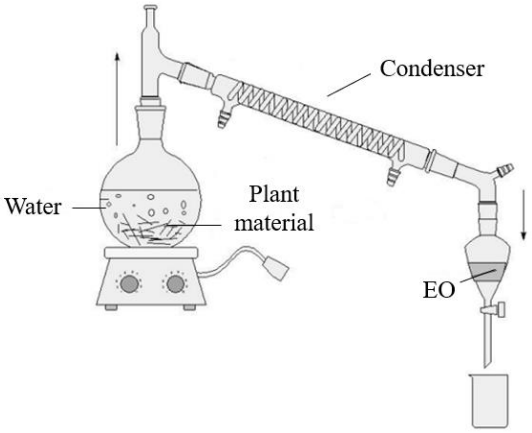


Figure 18. Hydrodistillation extractor. Adapted from Vinatoru *et al.*[49]

At atmospheric pressure, during the extraction process, the water and EO molecules form a heterogeneous mixture. This mixture is distilled simultaneously as if it were a single compound in a process called co-distillation. The advantage of water is that it is immiscible with most terpenes in EOs and, therefore, after condensation, EOs can be easily separated from water by decantation.[3,4]

This technique has, however, some drawbacks like long extraction time that create artifacts and chemical alterations of terpenic molecules by prolonged contact with boiling water.[4]

1.6.2 Soxhlet extraction

Soxhlet extraction is widely used for many types of solid samples, especially biological and environmental samples. The experimental Soxhlet extraction device consists of a distillation flask, sample holder or thimble, siphon and condenser as shown in figure 19.[50]

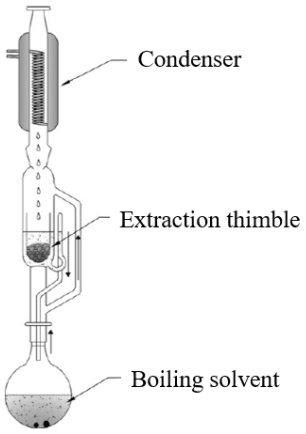


Figure 19. Conventional Soxhlet extractor. Adapted from Zygler *et al.*[50]

A Soxhlet extraction procedure usually involves the following steps:[50]

1. The sample material is packed in cellulose tube and placed in the thimble;
2. Vapors of a fresh solvent, produced in a distillation flask, pass through the thimble containing the material to be extracted and are liquefied in the condenser;
3. When the liquid reaches the overflow level in the thimble, a siphon aspirates the solution, and the liquid falls back into the distillation flask, carrying the extracted solutes into the bulk liquid;
4. The separation of solute from solvent takes place in the distillation flask. Then solute is left in the flask and fresh solvent vapors pass back into the solid bed of sample material. The operation is repeated until complete extraction is achieved.

This technique has the advantage of being possible to obtain large amounts of extract that can be collected with much less solvent. On the other hand, extracts obtained by organic solvent contain residues that compromises the safety of products extracted by this technique.[4]

1.6.3 Cold pressing

Cold pressing is the traditional technique to extract EOs from citrus fruit zest. During extraction, oil pockets break and release volatile oils. This oil is removed mechanically by cold pressing yielding a watery emulsion. Oil is recovered subsequently by centrifugation.[3,4]

1.7 Characterization of essential oils

The methods used to characterize the EOs depend mainly on the number of molecular species, which is closely related to the extraction procedure used, so choosing the appropriate extraction method is particularly important. Most methods used in the analysis of EOs are based on chromatographic procedures.[7]

Some techniques for analysis and identification of EOs are Gas Chromatography-Mass Spectrometry (GC-MS)[7], High-Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR), Ultraviolet-Visible (UV-Vis) absorption spectroscopy and Infrared (IR) spectroscopy.

1.7.1 Gas Chromatography-Mass Spectrometry

GC-MS is the analysis technique of choice for smaller and volatile molecules such as hydrocarbons, alcohols, and aromatics.[51]

GC-MS begins with the gas chromatograph, where the sample is volatized. This effectively vaporizes the sample (the gas phase) and separates its various components using a capillary column packed with a stationary (solid) phase. The compounds are propelled by an inert carrier gas. As the components become separated, they elute from the column at different times.[51]

Once the components leave the GC column, they are ionized by the mass spectrometer using ionization sources. Ionized molecules are then accelerated through the instrument mass analyzer. It is here that ions are separated based on their different m/z ratios.[51]

The final steps of the process involve ion detection and analysis, with compound peaks appearing as a function of their m/z ratios. Using computer libraries of mass spectra for different compounds, researchers can identify and quantify unknown compounds and analytes.[51]

1.7.2 High-Performance Liquid Chromatography

HPLC is a modern application of liquid chromatography.[52] Liquid chromatography is a separation technique in which the mobile phase is a liquid, where sample ions or molecules are dissolved. The sample with the mobile liquid will pass through the column, which is packed with a stationary phase composed of irregularly or spherically shaped particles. Due to the differences in ion-exchange, adsorption, partitioning, or size, different solutes will interact with the stationary phase in different degrees, and therefore the separation of compounds can be achieved and the transit time of the solutes through the column can be determined by utilizing these differences.[53]

The principle of HPLC is the same as that of liquid chromatography but guarantees a high sensitivity.[52]

1.7.3 Nuclear Magnetic Resonance

The NMR technique is based on the interaction between matter and electromagnetic forces and can be observed by subjecting a sample simultaneously to two magnetic fields: one stationary and the other varying at a certain radio frequency. Using this combination, energy is absorbed by the sample, and this absorption can be observed as a change in the signal developed by a radio frequency detector and, later by a radio frequency amplifier.[52]

Two techniques of NMR are commonly used: ^{1}H NMR and ^{13}C NMR. The local environment around a given nucleus in a molecule will slightly perturb the local magnetic field exerted on that nucleus and

affect its exact transition energy. This dependence of the transition energy on the position of an atom in a molecule makes NMR spectroscopy extremely useful for determining the structure of molecules.[52]

All of these characteristics make NMR one of the most powerful techniques for studying the structure of both organic and inorganic species.[52]

1.7.4 Ultraviolet-Visible absorption spectroscopy

UV-Vis absorption spectroscopy is applied to the evaluation of samples that may contain aromatic compounds such as benzene derivatives or to a wide variety of UV absorbing compounds. Allows the identification and quantification of organic and inorganic compounds.[54]

UV-Vis spectroscopy is a type of absorption spectroscopy in which UV-Vis light is absorbed by the molecule. Absorption of the UV-Vis radiation result in the excitation of the electrons from lower to higher energy levels.[54]

1.7.5 Infrared spectroscopy

IR spectroscopy is the measurement of the wavelength and intensity of the absorption of mid-infrared light by a sample.[55]

The wavelength of IR absorption bands is characteristic of specific types of chemical bonds, and infrared spectroscopy finds its greatest utility for identification of organic molecules.[55]

1.8 Nanoencapsulation systems

The application of nanotechnology in encapsulation of EOs can offer many advantages such as protection of active ingredients of EOs from degradation due to external factors that can compromise their biological activity.[4]

Nanoparticles have properties that can increase the efficiency of agrochemicals and make the pesticide delivery process a "smart" process.[56] In this way, they can be delivered in a more controlled and targeted manner.[5]

In agriculture, the development of new plant protection formulations has long been a highly active field of research because such problems associated with common pesticides must be overcome.[57] Nanomaterials loading pesticides have exhibited useful properties such as stiffness, permeability, thermal stability, and biodegradability. Nanocarrier materials spread evenly over the leaves and in the

soil surface, so they can be easily absorbed by chewing insects or they are absorbed into the lipid cuticular wax layers of insects via a physiosorption process. The large surface area of nanopesticides increases the affinity to the target species/groups and reduces the amount of pesticide required for pest control.[5]

1.8.1 Nanoencapsulation materials

Nanoencapsulation is the coating of various substances within another material at various sizes in the nano-range. The encapsulated material is commonly referred to as the core material. The encapsulation material is known as the external phase, shell, coating, or membrane.[5]

Nanoencapsulation of pesticides involves the formation of pesticide-loaded particles having a certain diameter. According to the definition of nanoparticle, this size range should be 1–100 nm, but there is some flexibility about the particle size in a colloidal system such as in pesticide formulations. Definition of nanoparticles can be based also on their application in medicine or agriculture where their size may be larger than 100 nm.[5]

Several nanomaterials have already been used to encapsulate pesticides, forming different types of nanomaterials, for example, microparticles, lipid nanoparticles, liposomes, micelles, nanoemulsions and polymer-based nanoemulsions.[5]

1.8.1.1 Liposome-based systems

Liposome-based systems have been reported as potential delivery systems for bioactive substances with good encapsulating efficiency and low toxicity.[58,59] They have great potential to encapsulate the hydrophilic, hydrophobic, and lipophilic active ingredients and they regularly facilitate the dispersion of hydrophobic biopesticides in aqueous solutions and absorption of the bioactive compounds through the cuticle of the insect body.[5]

Liposomes are systems formed by one or several phospholipids bilayers defining one or several aqueous compartments. Colloidal structures are formed by the arrangement of lipids, most commonly phospholipids in an aqueous solution. When phospholipids are placed in an aqueous medium, they self-assemble to defend hydrophobic tails from water molecules, whereas the hydrophilic heads maintain a close association with aqueous phase. This amphiphilic nature is the key factor for developing vesicle structure.[4,5]

Liposomes can be classified depending on their size and lamellarity[4] as shown in figure 20.

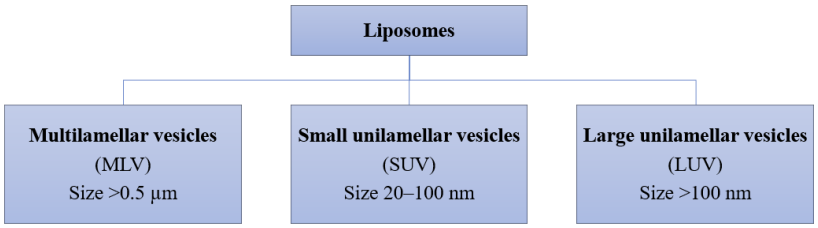


Figure 20. Classification of liposomes according to structural parameters: size and lamellarity.

Using these encapsulation nanosystems, both the water-soluble and -insoluble active ingredients of pesticides can be delivered simultaneously.[4,5] Different methods have been used to encapsulate EOs in liposome-based systems. In this work, the focus is on of ethanol injection (EI) and lipid thin film hydration (TFH) methods.

EI method is commonly used for liposome production scale-up. The main relevance of the ethanol injection method lies on the possibility to obtain small liposomes with an uniform distribution by simply injecting an ethanolic lipid solution in water, as shown in figure 21, without extrusion or sonication.[60]

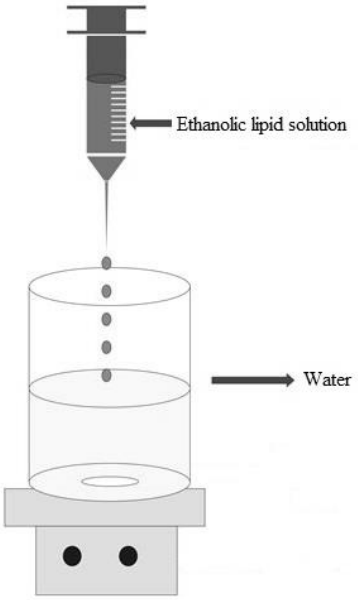


Figure 21. Ethanol injection method. Adapted from Ramanujam *et al.*[61]

Lipid TFH method, shown in figure 22, is used to form MLV and consists in three steps:[4]

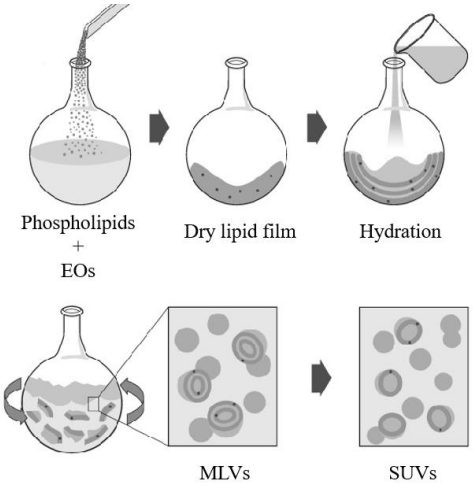


Figure 22. Lipid film hydration method. Adapted from Asbahani *et al.*[4]

1. Phospholipids and EOs are dissolved in an organic phase;
2. A thin phospholipid film of stacked bilayers is obtained at the bottom of the flask after rotative evaporation of the organic solvent under pressure;
3. This dry film is hydrated with an aqueous phase under agitation, which allows spontaneous formation of MLV. However, this method gives large vesicles with heterogeneous size distribution and lamellarity.

Different approaches are used to obtain liposomes suspensions with homogeneous and reduced size. The basic principle is the conversion of MLVs into SUVs. Sonication – which consists in ultrasonic wave application that provides enough energy to disrupt MLVs - and extrusion – which consists of the passage through a track-etched polycarbonate membrane with pores of different diameters (performed several times) – are the common methods used.[4]

1.8.2 Characterization of nanoparticles

1.8.2.1 Nanosystems size

Particle size is an important parameter of a sample and these measurements can also be used to characterize, monitor the stability of a formulation, and provide a measure of quality control in a manufacturing process.[62]

One of the techniques commonly used is Dynamic Light Scattering (DLS), employed to measure the size distribution of samples, including nanoparticles such as emulsions and liposomes. The velocity due to Brownian motion of a spherical particle suspended in a liquid with a known viscosity at a constant temperature is proportional to the particle diameter according to the Stokes-Einstein equation. By solving the equation, algorithms can be used to determine the particle size distribution.[62]

DLS is a simple measurement: a laser illuminates the sample in a cuvette and light that is scattered by particles in solution is collected by a detector. By inputting the refractive index and the viscosity of the diluent or sample medium, particle size, size distribution and zeta potential can be determined.[62]

The zeta potential of slurry particles is often used as a metric to gauge the particles' stability in the colloidal sense. Particles of like charge will have a lower propensity to agglomerate as their absolute zeta potential increases and, as a rule of thumb, slurries with particle zeta potentials > ±30 mV are considered colloidally stable.[63] Table 4 shows the relationship between colloidal stability and ranges of zeta potential.

Table 4. Colloidal stability for ranges of zeta potential.[63]

Zeta potential (mV)	Colloidal stability
0 to ±5	Rapid agglomeration
±10 to ±30	Incipient stability
±30 to ±40	Moderate stability
±40 to ±60	Good stability
> ±60	Excellent stability

1.8.2.2 Encapsulation efficiency and release studies

The encapsulation efficiency (EE%) of a compound in a nanosystem, can be determined through UV-Vis absorption or fluorescence measurements, using previously obtained calibration curves (absorbance or fluorescence intensity as function of concentration). The systems containing the encapsulated extracts or derivatives are subjected to centrifugation and the filtrate containing the non-encapsulated extract can be analyzed by UV-Vis absorption/fluorescence spectroscopy and the concentration is determined through the calibration obtained for each of the extracts/compounds. It is also important to carry out extracts release assays in each nanoencapsulation system in different periods of time.

Chapter 2 – Materials and methods

The EO of *Syzygium aromaticum* was extracted using the extraction technique of hydrodistillation. The characterization of extracted compound and synthesized compounds was done using some characterization techniques previously mentioned, NMR, UV-Vis absorption and IR spectroscopy.

Nuclear magnetic resonance spectra were determined in the Bruker Avance III 400 apparatus at a frequency of 400 MHz for ^{1}H and 100.6 MHz for ^{13}C, using the solvent peak as an internal reference, at 25 °C. Chemical shifts are reported in ppm, using as reference the value δ_H Me_4Si = 0 ppm, and coupling constants (*J*) appear in Hz. The assignment of ^{1}H and ^{13}C signals was performed by comparing chemical shifts, multiplicity of peaks and *J* values. The deuterated solvents used were chloroform–d_1 (99.8%) and methanol-d_4 (99.8%) from Euriso-top. The materials used for a preliminary characterization were thin layer chromatography (TLC) 0.20 mm thick silica gel plates, provided by Macherey-Nagel.

Purification of compounds was performed by column chromatography with Acros Organics 60 A gel silica (0.035-0.070 mm).

For the characterization of compounds, ultraviolet/visible absorption spectra (200-600 nm) were obtained on a Shimadzu UV/2501PC spectrophotometer. IR spectra were obtained at a frequency (500-4000 cm^{-1}) on the FTLA2000 detector.

The cell line chosen for biological assays was the *Sf9* cell line, which was originally established from the immature ovaries of *S. frugiperda* pupae. This cell line has already been used and is suggested for assays of insecticidal activity, due to its instability in the presence of reactive oxygen species that cause oxidative damage in cells and, consequently, apoptosis.[64]

To increase the insecticidal efficiency of the extracts and avoid their degradation, nanoencapsulation was performed in liposomal systems. The nanoencapsulation systems tested were liposomes. Regarding their preparation, liposomes were prepared using the EI and TFH methods, both described above. Then, the characterization was performed using the DLS technique. EE (%) were determined and release studies were carried out.

2.1 Preparation and extraction of plant material

2.1.1 Materials and chemical reagents

For the execution of the extraction of plant material from the *Syzygium aromaticum* plant, the hydrodistillation method was used. The following materials were used: heating blanket, 500 mL round bottomed flask, condenser, and an Erlenmeyer flask for collection. The solvent used in the 4-allyl-2-methoxy phenol **1** extraction was dichloromethane (DCM, 99.8%), 1M sodium hydroxide and 1M hydrochloric acid.

2.1.2 Experimental procedures

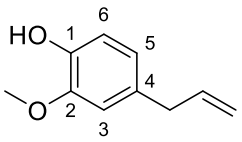
The hydrodistillation extraction technique was the method selected to obtain plant extracts from the *Syzygium aromaticum* plant. After assembling the hydrodistillation, the clove (25 g) was placed in the round bottomed flask and distilled water (250 mL) was added. The extraction time was 2 hours, with the addition of small amounts of water periodically.

After the extraction time, the amount of distillate obtained was approximately 150 mL. The extraction of the desired product from the distillate was carried out using DCM. The 150 mL of distillate was placed in a 500 mL separating funnel and the extraction was done with DCM (2 x 20 mL), combining the DCM extracts and discarding the aqueous phase.

After extraction, the drying phase was carried out, adding anhydrous magnesium sulfate. After drying, the solvent was filtered into a 50 mL round bottomed flask. The extract obtained with DCM was subjected to solvent evaporation in a rotary evaporator until a yellowish transparent oily residue was obtained. The ^{1}H NMR spectrum confirmed the presence of 4-allyl-2-methoxy phenol (eugenol) **1** and 4-allyl-2-methoxyphenyl acetate, in lesser quantity.

To obtain a pure 4-allyl-2-methoxy phenol **1** sample, the extraction and purification procedure was optimized. After extraction with DCM, 1M NaOH (2 x 20 mL) was added to the organic phase. To the obtained aqueous phase, 1M HCl (40 mL) was added until pH = 1 was obtained. Then, this solution was placed in the separating funnel and the pure 4-allyl-2-methoxy phenol **1** was extracted again with DCM (2 x 20 mL). The drying phase was carried out with anhydrous magnesium sulfate and the solvent was evaporated.

After evaporation of the solvent, 4-allyl-2-methoxy phenol **1** was stored in a refrigerator (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.



1

η = 14% (w/w)

^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 6.89 (d, *J* = 8.8 Hz, 1H, H-6), 6.73-6.71 (m, 2H, H-3 and H-5), 6.03-5.96 (m, 1H, C*H*=CH_2), 5.60 (broad s, 1H, OH), 5.14-5.08 (m, 2H, CH=C*H*$_2$), 3.89 (s, 3H, OCH_3), 3.36 (d, *J* = 6.8 Hz, 2H, C*H*$_2$Ph) ppm.

2.2 Synthesis of 4-allyl-2-methoxy phenol 1 derivatives

2.2.1 Materials and chemical reagents

In the epoxidation reaction of 4-allyl-2-methoxy phenol **1** and subsequent extraction, the following chemical reagents were used: *meta*-chloroperbenzoic acid (*m*-CPBA, 70% purity), sodium sulfite 10% (w/v) in aqueous soluton and sodium hydrogen carbonate in saturated aqueous solution. The solvent used in the 2-methoxy-4-(oxiran-2-ylmethyl)phenol (eugenol epoxide) **2** extraction was DCM (99.8%).

For the synthesis of 4-allyl-2-methoxy phenol **1** analogues that involved dry solvents, the distillation method was used. The following materials were used: heating blanket, reflux condenser, flask and a balloon to create a nitrogen atmosphere and a round bottomed flask for collection. The solvent was placed on the heating blanket and was refluxed for approximately 1 hour. After that time, the required volume of solvent was collected.

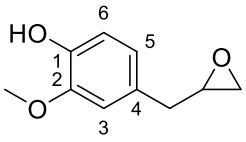
In all synthesis reactions, the reaction round bottomed flasks used were previously dried using liquid nitrogen, for approximately 1 hour. All the material used in these reactions was also previously dried in the oven and stored in the desiccator.

For the synthesis of 4-allyl-2-methoxy phenol **1** derivatives, an epoxidation reaction was carried out and the product obtained, 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2**, was used as a precursor to all the syntheses of the nine derivatives obtained. Seven alcohols, namely methanol, ethanol, 3-bromopropan-

1-ol, *tert*-butanol, 3-butyn-2-ol, 4-fluorobenzyl alcohol, phenol, and two amines, 3-bromoaniline and *p*-anisidine were used to react with the precursor.

2.2.2 Experimental procedures

2.2.2.1 Synthesis of 2-methoxy-4-(oxiran-2-ylmethyl)phenol 2



2

In a reaction flask containing *m*-CPBA (0.750 g, 4.35 mmol, 1 equiv) dissolved in DCM (10 mL) while stirring in an ice bath (at 0 °C), 4-allyl-2-methoxy phenol **1** (0.500 g, 3 mmol, 1 equiv), dissolved in DCM (10 mL) was added, dropwise, following a known procedure.[65] After stirring for 1 hour, additional *m*-CPBA (0.750 g, 4.35 mmol, 1 equiv) was added, the reaction was kept stirring for 24 hours at room temperature, and its evolution was monitored by ^{1}H NMR ($CDCl_3$). To the final product, DCM (20 mL) and 10% Na_2SO_3 aqueous solution (2 x 20 mL) were added, and the organic phase was collected. The collected organic phase was washed with saturated aqueous solution of $NaHCO_3$ (2 x 20 mL). The organic phase was dried over anhydrous magnesium sulfate and the remaining solvent was evaporated to a thick dark orange oily residue (0.337 g, 1.87 mmol).

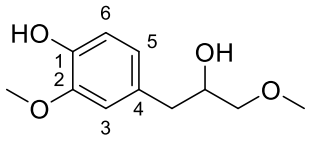
η = 67% (w/w)

R_f = 0.27 (DCM)

^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 6.87 (d, *J* = 7.6 Hz, 1H, H-6), 6.77 (d, *J* = 2 Hz, 1H, H-3), 6.75 (dd, *J* = 8 Hz and 2 Hz, 1H, H-5), 5.54 (broad s, 1H, OH), 3.90 (s, 3H, OCH_3), 3.16-3.12 (m, 1H, C*H*-oxirane), 2.82-2.79 (m, 3H, CH_2Ph and CH_2-oxirane), 2.55 (dd, *J* = 4.8 Hz e 2.8 Hz ,1H, CH_2-oxirane) ppm.

^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 146.46 (C-2), 144.39 (C-1), 129.03 (C-4), 121.64 (C-5), 114.32 (C-6), 111.54 (C-3), 55.90 (OCH_3), 52.67 (*C*H-oxirane), 46.79 (*C*H_2-oxirane), 38.37 (*C*H_2Ph) ppm.

2.2.2.2 Synthesis of 4-(2-hydroxy-3-methoxypropyl)-2-methoxyphenol 3a



3a

After distilling the dry solvent and drying the reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.131 g, 0.73 mmol, 1 equiv) dissolved in methanol (MeOH) (CH_4O) (5 mL), boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$) (0.103 g, 0.73 mmol, 1 equiv) was added. The reaction mixture was left stirring under a nitrogen atmosphere, at 0 °C (ice bath with salt), for 2 hours. The progress of the reaction was monitored by ^1H NMR ($CDCl_3$) and TLC (AcOEt/EP 1:1). The MeOH was evaporated, and the reaction mixture was dissolved in DCM (5 mL) and distilled water (5 mL) was added. The organic phase was extracted with DCM (2 x 5 mL).

After the extraction of the organic phase, the drying phase was carried out, adding anhydrous magnesium sulfate. After drying, the solvent was filtered. The extract obtained with DCM was subjected to solvent evaporation in a rotary evaporator.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using AcOEt/EP (1:1) as eluent.

The obtained purified compound **3a** was a thick yellow oily residue (0.089 g, 0.42 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 68% (w/w)

R_f = 0.30 (AcOEt:EP 1:1)

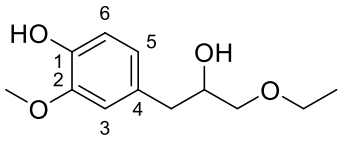
UV-Vis (EtOH) = λ_{max} (log ε): 280 (3.63) nm.

IR (DCM) ν_{max} = 3412, 2933, 1602, 1516, 1463, 1453, 1431, 1369, 1154, 1124, 1035, 964, 911, 733 cm^{-1}

^1H NMR δ_H ($CDCl_3$, 400 MHz): 6.86 (d, *J* = 7.6 Hz, 1H, H-6), 6.75 (d, *J* = 1.6 Hz, 1H, H-3), 6.71 (dd, *J* = 8 Hz and 2 Hz, 1H, H-5), 4.01-3.96 (m, 1H, C*H*OH), 3.89 (s, 3H, OCH_3), 3.44-3.40 (m, 1H, CH_2O), 3.40 (s, 3H, OCH_3), 3.40-3.28 (m, 1H, CH_2O), 2.73 (d, *J* = 6.8 Hz, 2H, CH_2Ph) ppm.

^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 146.46 (C-2), 144.24 (C-1), 129.66 (C-4), 121.93 (C-5), 114.34 (C-6), 111.80 (C-3), 75.94 (CH_2O), 71.37 (CHOH), 59.06 (OCH_3), 55.85 (OCH_3), 39.47 (*C*H_2Ph) ppm.

2.2.2.3 Synthesis of 4-(3-ethoxy-2-hydroxypropyl)-2-methoxyphenol 3b



3b

After distilling the dry solvent and drying the reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.109 g, 0.60 mmol, 1 equiv) dissolved in ethanol (EtOH) (C_2H_6O) (5 mL) was added boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$) (0.085 g, 0.60 mmol, 1 equiv). The reaction mixture was left stirring under a nitrogen atmosphere, at room temperature, for 2 hours. The progress of the reaction was monitored by ^{1}H NMR and TLC (AcOEt/EP 1:1). The EtOH was evaporated, and the reaction mixture was dissolved in DCM (5 mL) and distilled water (5 mL) was added. The organic phase was extracted with DCM (2 x 5 mL).

After the extraction of the organic phase, the drying phase was carried out, adding anhydrous magnesium sulfate. After drying, the solvent was filtered. The extract obtained with DCM was subjected to the solvent evaporation in a rotary evaporator until a dark oily residue was obtained.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using AcOEt/EP (1:1) as eluent.

The obtained purified compound **3b** was a white solid (0.023 g, 0.10 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 21% (w/w)

R_f = 0.37 (AcOEt:EP 1:1)

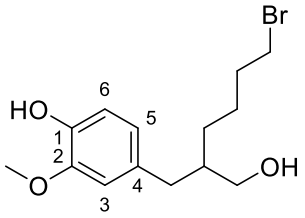
UV-Vis (EtOH) = λ_{max} (log ε): 280 (2.22) nm.

IR (DCM) ν_{max} = 3395, 3279, 3055, 2998, 2976, 2920, 2859, 1601,1522, 1462, 1441, 1375, 1310, 1267, 1216, 1159, 1106, 1087, 1069, 1037, 907, 740 cm^{-1}

^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 6.86 (d, *J* = 8 Hz, 1H, H-6), 6.76 (d, *J* = 1.6 Hz, 1H, H-3), 6.71 (dd, *J* = 7.6 Hz and 1.6 Hz, 1H, H-5), 5.56 (broad s, 1H, OH), 4.02-3.96 (m, 1H, C*H*OH), 3.88 (s, 3H, OCH_3), 3.57-3.50 (m, 2H, OC*H*$_2$$CH_3$), 3.45-3.30 (m, 2H, CH_2O), 2.73 (d, *J* = 6.8 Hz, 2H, C*H*$_2$Ph), 1.22 (t, *J* = 14 Hz, 3H, OCH_2C*H*$_3$) ppm.

^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 146.44 (C-2), 144.20 (C-1), 129.77 (C-4), 121.94 (C-5), 114.31 (C-6), 111.80 (C-3), 73.78 (CH_2O), 71.45 (CHOH), 66.72 (OCH_2CH_3), 55.85 (OCH_3), 39.52 (CH_2Ph), 15.13 (OCH_2CH_3) ppm.

2.2.2.4 Synthesis of 4-(2-(3-bromopropoxy)-3-hydroxypropyl)-2-methoxyphenol 3c



3c

To a dried reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.231 g, 1.28 mmol, 1 equiv) dissolved in DCM (7 mL) was added 3-bromopropan-1-ol (C_3H_7BrO) (0.178 g, 1.28 mmol, 1 equiv.) and boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$) (0.182 g, 1.28 mmol, 1 equiv). The reaction mixture was left stirring and under a nitrogen atmosphere, at room temperature, for 2 hours. The progress of the reaction was monitored by ^1H NMR ($CDCl_3$) and TLC (AcOEt/EP 1:1). The reaction mixture was dissolved in DCM (5 mL) and distilled water (5 mL) was added. The organic phase was extracted with DCM (2 x 5 mL).

After the extraction of the organic phase, the drying phase was carried out, adding anhydrous magnesium sulfate. After drying, the solvent was filtered. The extract obtained with DCM was subjected to the solvent evaporation in a rotary evaporator.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using AcOEt/EP (1:1) as eluent.

The obtained purified compound **3c** was a thick brown oily residue (0.035 g, 0.11 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 15% (w/w)

R_f = 0.33 (AcOEt:EP 1:1)

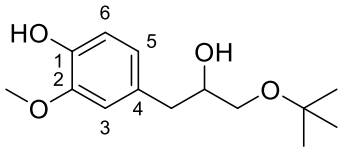
UV-Vis (EtOH) = λ_{max} (log ε): 279 (2.22) nm.

IR (DCM) ν_{max} = 3398, 2927, 2854, 2361, 1741, 1659, 1603, 1515, 1464, 1452, 1431, 1365, 1271, 1236, 1210, 1154, 1122, 1105, 1034, 936, 858, 815, 797, 737 cm^{-1}

^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 6.85 (d, *J* = 8.4 Hz, 1H, H-6), 6.73 (s, 1H, H-3), 6.70 (dd, *J* = 9 Hz and *J* = 2 Hz, 1H, H-5), 5.51 (broad s, 1H, OH), 3.90 (s, 3H, OCH_3), 3.73-3.42 (m, 7H, CHO, OC*H*$_2$CH$_2$C*H*$_2$Br, C*H*$_2$OH), 2.83 (dd, *J* = 14 Hz and 6.4 Hz, 1H, C*H*$_2$Ph), 2.70 (dd, *J* = 14 Hz and 6.8 Hz, 1H, C*H*$_2$Ph), 2.09-2.02 (m, 2H, $OCH_2CH_2CH_2Br$) ppm.

^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 146.46 (C-2), 144.14 (C-1), 129.87 (C-4), 121.99 (C-5), 114.29 (C-6), 111.83 (C-3), 81.62 (CHO), 67.02 (O*C*H$_2$CH$_2$CH$_2$Br), 63.67 (CH_2OH), 55.93 (OCH_3), 38.14 (*C*H$_2$Ph), 32.82 ($OCH_2CH_2CH_2Br$), 30.62 ($OCH_2CH_2CH_2Br$) ppm.

2.2.2.5 Synthesis of 4-(3-(*tert*-butoxy)-2-hydroxypropyl)-2-methoxyphenol 3d



3d

To a dried reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.149 g, 0.83 mmol, 1 equiv) dissolved in *tert*-butanol ($C_4H_{10}O$) (5 mL) was added boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$) (0.117 g, 0.82 mmol, 1 equiv). The reaction mixture was left stirring under a nitrogen atmosphere, at room temperature, for 24 hours. The progress of the reaction was monitored by ^{1}H NMR and TLC (AcOEt/EP 1:1). The *tert*-butanol was evaporated, and the reaction mixture was dissolved in DCM (5 mL) and distilled water (5 mL) was added. The organic phase was extracted with DCM (2 x 5 mL).

After the extraction of the organic phase, the drying phase was carried out, adding anhydrous magnesium sulfate. After drying, the solvent was filtered. The extract obtained with DCM was subjected to the solvent evaporation in a rotary evaporator.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using AcOEt/EP (1:1) as eluent.

The obtained purified compound **3d** was a thick yellowish transparent oily residue (0.018 g, 0.07 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 12% (w/w)

R_f = 0.54 (AcOEt:EP 1:1)

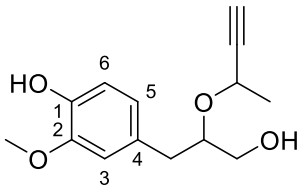
UV-Vis (EtOH) = λ_{max} (log ε): 280 (3.24) nm.

IR (DCM) ν_{max} = 3381, 2972, 2929, 2872, 1736, 1601, 1517, 1464, 1451, 1430, 1365, 1312, 1269, 1237, 1155, 1122, 1077, 1035, 939, 911, 873, 842, 820, 801, 756, 738 cm^{-1}

^1H NMR δ_H ($CDCl_3$, 400 MHz): 6.85 (d, *J* = 8 Hz, 1H, H-6), 6.77 (d, *J* = 1.6 Hz, 1H, H-3), 6.72 (dd, *J* = 8 Hz and 2 Hz, 1H, H-5), 5.53 (broad s, 1H, OH), 3.94-3.89 (m, 1H, C*H*OH), 3.88 (s, 3H, OCH_3), 3.38-3.24 (m, 2H, CH_2O), 2.73 (d, *J* = 7.2 Hz, 2H, CH_2Ph), 1.20 (s, 9H, $OC(CH_3)_3$) ppm.

^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 146.38 (C-2), 144.09 (C-1), 130.12 (C-4), 121.91 (C-5), 114.24 (C-6), 111.77 (C-3), 73.19 (O*C*(CH_3)$_3$), 71.79 (CHOH), 64.91 (CH_2O), 55.84 (OCH_3), 39.54 (*C*H_2Ph), 27.57 OC(*C*H_3)$_3$) ppm.

2.2.2.6 Synthesis of 4-(2-(but-3-yn-2-yloxy)-3-hydroxypropyl)-2-methoxyphenol 3e



3e

To a dried reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.195 g, 1.08 mmol, 1 equiv) dissolved in DCM (7 mL) was added 3-butyn-2-ol (C_4H_6O) (0.076 g, 1.08 mmol, 1 equiv.) and boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$) (0.153 g, 1.08 mmol, 1 equiv). The reaction mixture was left stirring and under a nitrogen atmosphere, at room temperature, for 2 hours. The progress of the reaction was monitored by ^1H NMR ($CDCl_3$) and TLC (AcOEt/EP 1:1). The reaction mixture was dissolved in DCM (5 mL) and distilled water (5 mL) was added. The organic phase was extracted with DCM (2 x 5 mL).

After the extraction of the organic phase, the drying phase was carried out, adding anhydrous magnesium sulfate. After drying, the solvent was filtered. The extract obtained with DCM was subjected to the solvent evaporation in a rotary evaporator.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using AcOEt/EP (1:1) as eluent.

The obtained purified compound **3e** was a thick brown oily residue (0.015 g, 0.06 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 8% (w/w)

R_f = 0.56 (AcOEt:EP 1:1)

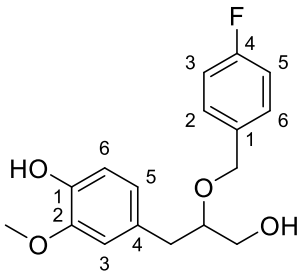
UV-Vis (EtOH) = λ_{max} (log ε): 281 (2.34) nm.

IR (DCM) ν_{max} = 3425, 3287, 2936, 2360, 1721, 1603, 1515, 1464, 1452, 1431, 1370, 1326, 1271, 1237, 1210, 1154, 1123, 1098, 1034, 955, 859, 818, 795, 739 cm^{-1}

^1H NMR δ_H ($CDCl_3$, 400 MHz): 6.85 (d, *J* = 8 Hz, 1H, H-6), 6.73-6.71 (m, 2H, H-5 and H-3), 5.52 (broad s, 1H, OH), 4.06-4.01 (m, 1H, OC*H*(CH_3)CCH), 3.89 (s, 3H, OCH_3), 3.88-3.84 (m, 1H, CHO), 3.70-3.52 (m, 2H, C*H*$_2$OH), 2.76 (dd, *J* = 13.6 Hz and 6.8 Hz, 1H, C*H*$_2$Ph), 2.68 (dd, *J* = 14 Hz and 6.4 Hz, 1H, C*H*$_2$Ph), 2.44 (d, *J* = 2 Hz, 1H, OCH(CH_3)CC*H*), 1.37 (d, *J* = 6.4 Hz, 3H, OCH(C*H*$_3$)CCH) ppm.

^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 146.37 (C-2), 144.19 (C-1), 129.76 (C-4), 122.01 (C-5), 114.33 (C-6), 111.84 (C-3), 84.41 (OCH(CH_3)*C*CH), 80.33 (CHO), 72.97 (OCH(CH_3)C*C*H), 64.69 (O*C*H(CH_3)CCH), 64.54 (CH_2OH), 55.90 (OCH_3), 37.13 (CH_2Ph), 22.34 (OCH(*C*H_3)CCH) ppm.

2.2.2.8 Synthesis of 4-(2-((4-fluorobenzyl)oxy)-3-hydroxypropyl)-2-methoxyphenol 3f



3f

To a dried reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.176 g, 0.98 mmol, 1 equiv) dissolved in DCM (7 mL) was added 4-fluorobenzyl alcohol (0.123 g, 0.98 mmol, 1 equiv.) and boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2$) (0.138 g, 0.98 mmol, 1 equiv). The reaction mixture was left stirring and under a nitrogen atmosphere, at room temperature, for 4 hours. The progress of the reaction was monitored by ^1H NMR ($CDCl_3$) and TLC (AcOEt/EP 1:1). The reaction mixture was dissolved in DCM (5 mL) and distilled water (5 mL) was added. The organic phase was extracted with DCM (2 x 5 mL).

After the extraction of the organic phase, the drying phase was carried out, adding anhydrous magnesium sulfate. After drying, the solvent was filtered. The extract obtained with DCM was subjected to the solvent evaporation in a rotary evaporator.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using AcOEt/EP (1:1) as eluent.

The obtained purified compound **3f** was a thick light brown oily residue (0.033 g, 0.11 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 19% (w/w)

R_f = 0.42 (AcOEt:EP 1:1)

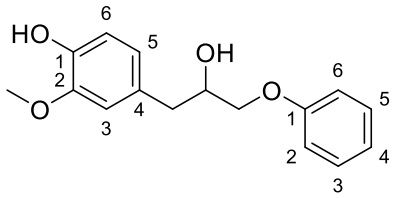
UV-Vis (EtOH) = λ_{max} (log ε): 282 (3.12) nm.

IR (DCM) ν_{max} = 3416, 3054, 2936, 2874, 1603, 1513, 1464, 1452, 1431, 1366, 1270, 1222, 1155, 1123, 1099, 1035, 853, 824, 737 cm^{-1}

^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 7.26-7.21 (m, 2H, H-2 *p*-F-Ph and H-6 *p*-F-Ph), 7.04-6.99 (m, 2H, H-5 *p*-F-Ph and H-3 *p*-F-Ph), 6.85 (d, *J* = 8 Hz, 1H, H-6), 6.72-6.69 (m, 2H, H-5 and H-3), 5.55 (broad s, 1H, OH), 4.49 (s, 2H, OC*H*$_2$-*p*-F-Ph), 3.84 (s, 3H, OCH_3), 3.71-3.48 (m, 1H, C*H*$_2$OH), 3.60-3.49 (m, 2H, C*H*$_2$OH and CHO), 2.85 (dd, *J* = 13.6 Hz and 6.8 Hz, 1H, C*H*$_2$Ph), 2.75 (dd, *J* = 14 Hz and 6.4 Hz, 1H, C*H*$_2$Ph) ppm.

^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 161.13 (C-4 *p*-F-Ph), 146.38 (C-2), 144.17 (C-1), 133.99 (C-1 *p*-F-Ph) 129.86 (C-4), 129.57 (C-2 *p*-F-Ph), 129.49 (C-6 *p*-F-Ph), 122.00 (C-5), 115.37 (C-5 *p*-F-Ph), 115.16 (C-3 *p*-F-Ph) 114.30 (C-6), 111.91 (C-3), 80.98 (CHO), 71.27 (O*C*H$_2$-*p*-F-Ph), 63.80 (CH_2OH), 55.83 (OCH_3), 37.18 (CH_2Ph) ppm.

2.2.2.5 Synthesis of 4-(2-hydroxy-3-phenoxypropyl)-2-methoxyphenol 3g



3g

To a dried reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.255 g, 1.42 mmol, 1 equiv) dissolved in DCM (7 mL) was added phenol (0.133 g, 1.41 mmol, 1 equiv.) and boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$) (0.201 g, 1.42 mmol, 1 equiv). The reaction mixture was left stirring and under a nitrogen atmosphere, at room temperature, for 51 hours. The progress of the reaction was monitored by ^1H NMR ($CDCl_3$) and TLC (AcOEt/EP 1:1). The reaction mixture was dissolved in DCM (5 mL) and distilled water (5 mL) was added. The organic phase was extracted with DCM (2 x 5 mL).

After the extraction of the organic phase, the drying phase was carried out, adding anhydrous magnesium sulfate. After drying, the solvent was filtered. The extract obtained with DCM was subjected to the solvent evaporation in a rotary evaporator.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using AcOEt/EP (1:1) as eluent.

The obtained purified compound **3g** was a thick brown oily residue (0.108 g, 0.39 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 42% (w/w)

R_f = 0.48 (AcOEt:EP 1:1)

UV-Vis (EtOH) = λ_{max} (log ε): 278 (3.13) nm.

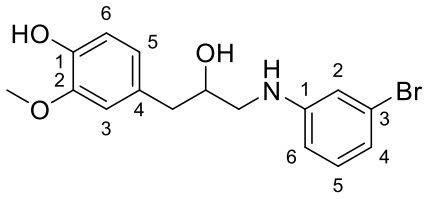
IR (DCM) ν_{max} = 3421, 3059, 2937, 2844, 1728, 1598, 1587, 1515, 1493, 1464, 1453, 1431, 1366, 1270, 1237, 1171, 1154, 1124, 1034, 1079, 955, 915, 819, 796, 754, 737 cm^{-1}

^1H NMR δ_H ($CDCl_3$, 400 MHz): 7.32-7.27 (m, 2H, H-3 O-Ph and H-5 O-Ph), 6.99-6.94 (m, 3H, H-4 O-Ph, H-2 O-Ph and H-6 O-Ph), 6.85 (d, *J* = 8 Hz, 1H, H-6), 6.75 (d, *J* = 2 Hz, 1H, H-5), 6.72 (d, *J* = 8Hz and 2 Hz, 1H, H-3), 5.53 (broad s, 1H, OH), 4.55-4.49 (m, 1H, C*H*OH), 3.84 (s, 3H, OCH_3), 3.81-3.71

(m, 2H, C*H*$_2$OPh), 2.99 (dd, *J* = 14 Hz and 5.6 Hz, 1H, C*H*$_2$Ph), 2.90 (dd, *J* = 14 Hz and 7.2 Hz, 1H, C*H*$_2$Ph) ppm.

^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 157.79 (C-1 O-Ph), 146.42 (C-2), 144.30 (C-1), 129.64 (C-3 O-Ph and C-5 O-Ph), 129.04 (C-4), 122.08 (C-5), 121.42 (C-4 O-Ph), 116.24 (C-2 O-Ph and C-6 O-Ph), 114.36 (C-6), 112.03 (C-3), 79.63 (CHOH), 63.55 (CH_2O), 55.89 (OCH_3), 36.33 (CH_2Ph) ppm.

2.2.2.9 Synthesis of 4-(3-((3-bromophenyl)amino)-2-hydroxypropyl)-2-methoxyphenol 4a



4a

To a dried reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol (0.180 g, 1 mmol, 1 equiv) **2** dissolved in EtOH (5 mL) was added 3-bromoaniline (0.189 g, 1.10 mmol, 1.1 equiv). The reaction mixture was left stirring, at 50 °C, for 26 hours. The progress of the reaction was monitored by ^{1}H NMR ($CDCl_3$) and TLC (DCM). The EtOH was subjected to the solvent evaporation in a rotary evaporator.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using DCM/MeOH (99:1) as eluent.

The obtained purified compound **4a** was a thick dark brown oily residue (0.086 g, 0.24 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 48% (w/w)

R_f = 0.62 (DCM/MeOH 95:5)

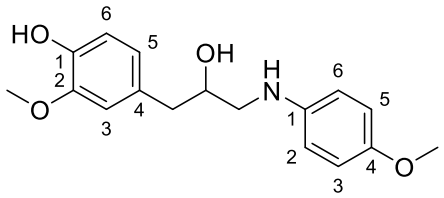
UV-Vis (EtOH) = λ_{max} (log ε): 279 (3.52) and 251 (3.92) nm.

IR (DCM) ν_{max} = 3505, 3401, 3061, 2935, 2847, 1746, 1596, 1515, 1483, 1464, 1452, 1431, 1368, 1324, 1269, 1237, 1208, 1153, 1123, 1069, 1034, 985, 934, 911, 843, 818, 796, 764, 736 cm^{-1}

^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 7.01 (t, *J* = 8 Hz, 1H, H-5 *m*-Br-Ph), 6.88 (dd, *J* = 8.4 and 1.6 Hz, 1H, H-6), 6.83 (dd, *J* = 8 Hz and 1.6 Hz, 1H, H-4 *m*-Br-Ph), 6.75 (t, *J* = 2.4 Hz , 1H, H-2 *m*-Br-Ph), 6.74-

6.70 (m, 2H, H-3 and H-5), 6.53 (dd, J = 8.1 and 2.4 Hz, 1H, H-6 *m*-Br-Ph), 4.06-4.00 (m, 1H, C*H*OH), 3.87 (s, 3H, OCH_3), 3.27 (dd, *J* = 12.8 e 7.2 Hz, 1H, CH_2NH), 3.06 (dd, *J* = 12.6 and 7.6 Hz, 1H, CH_2NH), 2.82 (dd, *J* = 13.6 and 5.2 Hz, 1H, CH_2Ph), 2.73 (dd, *J* = 14 and 5 Hz, 1H, CH_2Ph) ppm. ^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 149.37 (C-1 *m*-Br-Ph), 146.65 (C-2), 144.46 (C-1), 130-46 (C-5 *m*-Br-Ph), 129.13 (C-4), 123.23 (C-3 *m*-Br-Ph), 121.92 (C-5), 120.50 (C-4 *m*-Br-Ph), 115.69 (C-6), 114.56 (C-3), 112.04 (C-2 *m*-Br-Ph), 111.74 (C-6 *m*-Br-Ph), 71.06 (CHOH), 55.88 (OCH_3), 48.94 (CH_2NH), 41.18 (*C*H_2Ph) ppm.

2.2.2.10 Synthesis of 4-(2-hydroxy-3-((4-methoxyphenyl)amino)propyl)-2-methoxyphenol 4b



4b

To a dried reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.131 g, 0.73 mmol, 1 equiv) dissolved in EtOH (7 mL) was added *p*-anisidine (0.098 g, 0.80 mmol, 1.1 equiv). The reaction mixture was left stirring, at 50 °C, for 30 hours. The progress of the reaction was monitored by ^{1}H NMR ($CDCl_3$) and TLC (DCM). The EtOH was subjected to the solvent evaporation in a rotary evaporator.

After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using DCM/MeOH (99:1) as eluent.

The obtained purified compound **4b** was a thick dark brown oily residue (0.028 g, 0.09 mmol) and was stored in the freezer (0-5 °C), avoiding any contact with environmental factors, to preserve the integrity of the sample.

η = 21% (w/w)

R_f = 0.35 (DCM/MeOH 95:5)

UV-Vis (EtOH) = λ_{max} (log ε): 286 (2.62) and 239 (3.19) nm.

IR (DCM) ν_{max} = 3462, 3373, 3222, 3058, 2934, 2934, 1735, 1619, 1591, 1572, 1512, 1480, 1446, 1369, 1266, 1235, 1163, 1123, 1033, 863, 768, 680 cm^{-1}

^{1}H NMR δ_H ($CDCl_3$, 400 MHz): 6.86 (d, *J* = 8 Hz , 1H, H-6 NH-*Ph*-OMe), 6.80-6.78 (m, 2H, H-2 NH-*Ph*-OMe and H-5 NH-*Ph*-OMe), 6.75-6.69 (m, 4H, H-3 NH-*Ph*-OMe, H-6, H-3 and H-5), 4.11-4.04 (m, 1H, C*H*OH), 3.87 (s, 3H, NH-Ph-OC*H*$_3$), 3.76 (s, 3H, OCH_3), 3.26 (dd, *J* = 12.8 Hz and 8.2 Hz, 1H, C*H*$_2$NH), 3.06 (dd, *J* = 12.8 Hz and 8.4 Hz, 1H, C*H*$_2$NH), 2.87-2.72 (m, 2H, C*H*$_2$Ph) ppm.
^{13}C NMR δ_C ($CDCl_3$, 100.6 MHz): 153.39 (C-4 NH-*Ph*-OMe), 146.57 (C-2), 144.39 (C-1), 129.36 (C-4), 121.95 (C-5), 115.90 (C-6), 114.91 (C-3 NH-*Ph*-OMe and C-5 NH-*Ph*-OMe), 114.36 (C-3), 111.80 (C-2 NH-*Ph*-OMe and C-6 NH-*Ph*-OMe), 70.81 (CHOH), 55.90 (OCH_3), 55.76 (NH-Ph-O*C*H_3), 51.55 (CH_2NH), 41.17 (*C*H_2Ph) ppm.

2.2.2.11 Synthesis attempt of 4-(2-hydroxy-3-((4-methoxyphenyl)amino)propyl)-2-methoxyphenol

To a dried reaction flask under a nitrogen atmosphere, containing 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** (0.180 g, 1 mmol, 1 equiv) dissolved in EtOH (5 mL) was added 4-aminophenol (0.149 g, 1.36 mmol, 1 equiv). The reaction mixture was left stirring, at 60 °C, for 30 hours. The progress of the reaction was monitored by ^{1}H NMR ($CDCl_3$) and TLC (DCM). The EtOH was subjected to the solvent evaporation in a rotary evaporator.
After evaporation of the solvent, the crude mixture was purified by column chromatography on silica gel using DCM/MeOH (99:1) as eluent but it was not possible to isolate the pure compound.

2.3 Biological assays

The tests of the biological activity of compounds **3a-g** and **4a-b**, performed in *Sf9* cells, were carried out at REQUIMTE, Faculty of Pharmacy of the University of Porto. All studies were performed with the same compound concentration (100 μg/mL), so that direct comparison of all results was possible. These results are presented based on the loss of cell viability, as a percentage (%).

Cell viability assays allow analyzing cell proliferation, determining metabolically active cells in a cell culture, and are often performed to identify molecules that have effects on cell proliferation or toxic effects that may eventually induce cell death.[66]

2.4 Nanoencapsulation studies

2.4.1 Materials and chemical reagents

Egg yolk phosphatidylcholine (Egg-PC) and cholesterol (Ch) were obtained from Sigma-Aldrich. Absorption spectra were measured in a Shimadzu UV-3600 Plus UV-Vis-NIR spectrophotometer and the fluorescence spectra were measured in a Fluorolog 3 Horiba Jobin-Yvon spectrofluorometer. The compound-loaded liposomes' mean hydrodynamic diameter, size distribution (polydispersity (PDI)) and zeta potential were measured with a DLS equipment Litesizer 500 Anton Paar at 25 °C, using a solid-state laser of 648 nm and 40 mW.

2.4.2 Experimental procedures

2.4.2.1 Liposomes preparation

Liposomes were prepared by both the EI[67] and TFH[68] methods, using a lipid mixture of Egg-PC:Ch in the ratio 7:3, with a total lipid concentration of 1×10^{-3} M. In the EI method[67], the liposomes were prepared by a slow injection of an ethanolic solution of lipids and compound mixture to an aqueous buffer solution under vortexing.

For TFH method[68], a lipid film of the Egg-PC:Ch mixture was obtained from the evaporation of a lipid solution in chloroform under an ultrapure nitrogen stream. The compound solution was added, and, after evaporation, the film was hydrated with the aqueous buffer solution, followed by bath sonication and vortexing.

2.4.2.2 Size measurements, encapsulation efficiencies and release assays

For size and zeta potential measurements, three independent measurements were performed for each sample of the liposomes obtained by the two different methods.

Dilutions of the selected compounds solutions were carried out to determine the calibration curve (fluorescence *vs.* concentration) and calculate the EE (%). Loaded nanosystems were subjected to centrifugation at 3000 rpm for 10 min using Amicon® Ultra centrifugal filter units 100 kDa. Then the supernatant (containing the non-encapsulated compound) was removed out and its fluorescence was

measured, allowing the determination of compound concentration using a calibration curve previously obtained in the same solvent.

Three independent measurements were performed for each system. The EE (%), was obtained through equation 1:

$$\text{EE (\%)} = (\text{Total amount} - \text{Amount of non-encapsulated compound})/(\text{Total amount}) \times 100$$

Equation 1. Encapsulation Efficiency (%).

Release assays to phosphate buffer (pH = 7.4) were performed during 24 h, using Amicon® Ultra centrifugal filter units 100 kDa as dialysis membranes. The loaded liposomes solutions were maintained at 25 °C and were kept covered. The Weibull model was used to study the transport mechanism in compound release, being used for the comparison of release profiles from matrix systems. For that, the compound fraction accumulated (m) in solution on time t was fitted to the Weibull equation 2:

$$m = 1 - \exp\left[- (t - T_i)^b / a \right]$$

Equation 2. Weibull equation.

where a is the scale parameter that defines the timescale of the process, T_i is a localization parameter representing the latency time of release mechanism (taking zero many times), and b denotes the form parameter characterizing the type of curve. According to b value, the curve can be exponential, for $b = 1$; sigmoid (ascendant curvature limited by an inflection point), for $b > 1$; and parabolic (high initial slope and a consistent exponential profile), for $b < 1$.[69]

The Korsemeyer-Peppas model was also used to describe the compound release kinetics from the liposomes through equation 3:

$$M_t / M_\infty = K \cdot t^n$$

Equation 3. Korsmeyer-Peppas equation.

where M_t / M_∞ represents the fraction of release drug, K is the release constant, n the transport exponent (dimensionless) and t is the time.

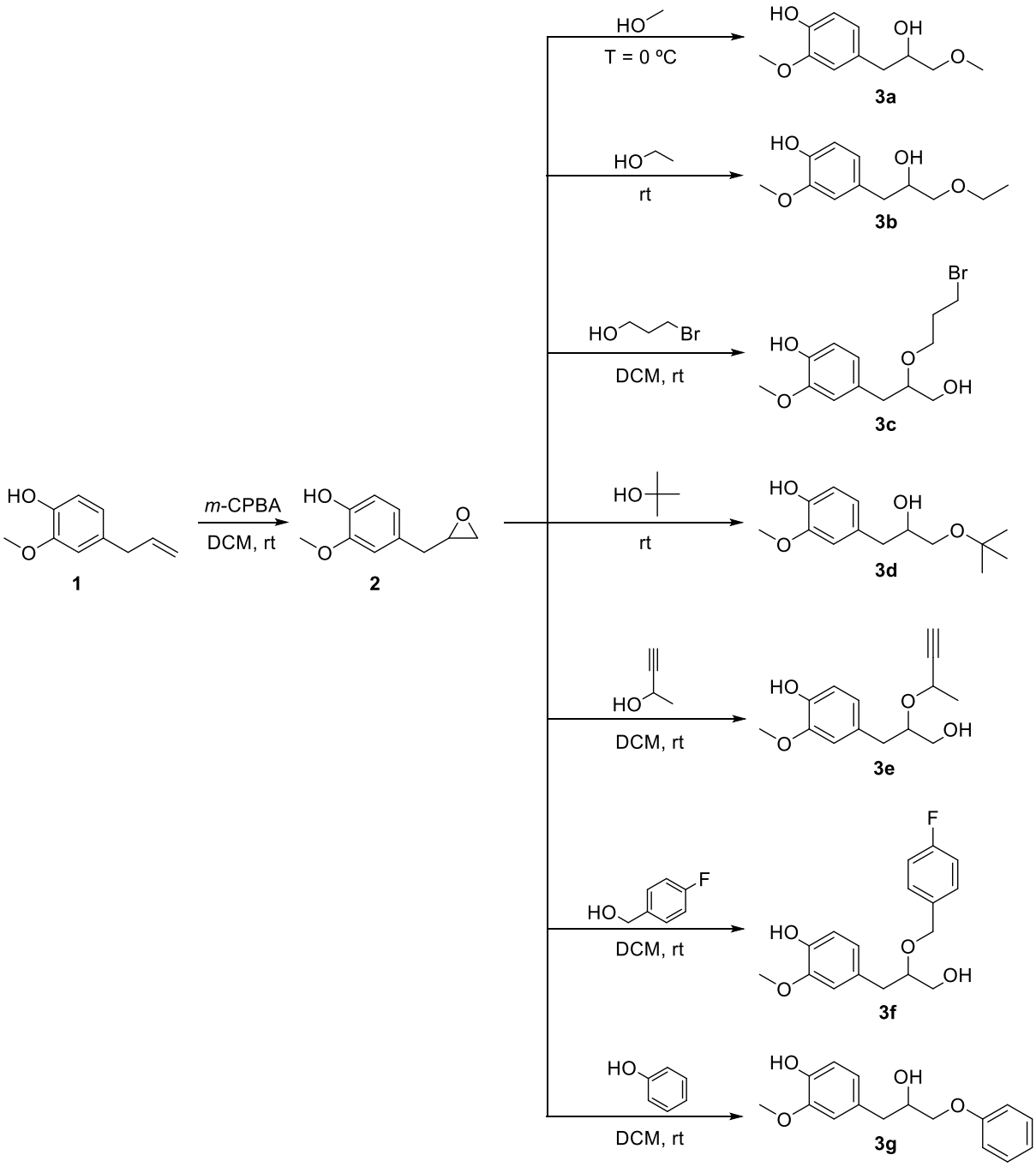
Chapter 3 – Results and discussion

3.1 Synthesis of eugenol alkoxy alcohols derivatives

2-Methoxy-4-(oxiran-2-ylmethyl)phenol **2** was obtained through the epoxidation reaction of 4-allyl-2-methoxy phenol **1** (conventional designation eugenol), conventional designation of 4-allyl-2-methoxy phenol. Compounds **3a-g** were obtained, with different alcohols, having alkyl chains of different sizes and/or presence of a terminal halogen, non-linear chains and with the presence of a triple bond, a methanol derivative in which one of the H atoms of the methyl group was replaced by fluorophenyl and an unsubstituted aromatic ring.

By reaction of 4-allyl-2-methoxy phenol **1** with *m*-CPBA, in DCM, after 24 hours, 2-methoxy-4-(oxiran-2-ylmethyl)phenol **2** was obtained. Starting from compound **2**, in the presence of a Lewis acid and in different reaction times, using methanol, ethanol, 3-bromopropan-1-ol, *tert*-butanol, 3-butyn-2-ol, 4-fluorobenzyl alcohol and phenol, the corresponding alkoxy alcohols were obtained, namely 4-(2-hydroxy-3-methoxypropyl)-2-methoxyphenol **3a**, 4-(3-ethoxy-2-hydroxypropyl)-2-methoxyphenol **3b**, 4-(2-(3-bromopropoxy)-3-hydroxypropyl)-2-methoxyphenol **3c**, 4-(3-(*tert*-butoxy)-2-hydroxypropyl)-2-methoxyphenol **3d**, 4-(2-(but-3-yn-2-yloxy)-3-hydroxypropyl)-2-methoxyphenol **3e**, 4-(2-((4-fluorobenzyl)oxy)-3-hydroxypropyl)-2-methoxyphenol **3f** and 4-(2-hydroxy-3-phenoxypropyl)-2-methoxyphenol **3g**, respectively.

Reactions were monitored by TLC and/or ^{1}H NMR and, in all compounds **3a-g**, it was necessary to carry out a purification by column chromatography. These eugenol epoxide derivatives were obtained as oils and a solid and with yields ranging from 8 to 68% (scheme 1).



Scheme 1. Synthesis of alkoxy alcohols **3a-g** derived from eugenol epoxide.

The structure of compounds **3a-g** was confirmed by ^{1}H and ^{13}C NMR. In the corresponding spectra of ^{1}H NMR the aromatic signals appear, for compounds **3a-e** and **3g**, H-6 as duplets and appear integrated in a multiplet for compound **3f**, H-3 as duplets (for compounds **3a-b**, **3d** and **3g**) and as a singlet for compound **3c**, H-5 as double duplets (for compounds **3a-d**) and as a duplet (for compound **3g**) or H-3 and H-5 as multiplets (for compounds **3e-f**) (δ 7.21-6.85 ppm, H-6; δ 7.04-6.70 ppm, H-3

and H-5). The OH group proton directly linked to the aromatic ring appears, for compounds **3b-g**, as broad singlet (δ 5.53 ppm, OHPh) and the methoxy group protons directly linked to the aromatic ring appears as a singlet for all **3a-g** compounds (δ 3.87 ppm, OCH_3). Protons of the methylene group directly attached to the aromatic ring appear as duplets (for compounds **3a-b** and **3d**) (δ 2.73 ppm, CH_2Ph) or two double duplets (for compounds **3c** and **3e-g**) (δ 2.99-2.68 ppm, CH_2Ph).

The proton from the C*H*OH group (compounds **3a-b**, **3d** and **3g**) and from the CHO group (compounds **3c** and **3e**) appear as multiplets or integrated in multiplets (for compound **3f**) (δ 4.55-3.89 ppm, C*H*OH); (δ 3.88-3.42 ppm, CHO).

The methylene protons of the CH_2O group (compounds **3a-b**, **3d** and **3g**) appear as multiplets (δ 3.81-3.24 ppm, CH_2O) and the methylene protons of the C*H*$_2$OH group appear integrated in a multiplet (for compounds **3c** and **3f**) (δ 3.73-3.42 ppm, C*H*$_2$OH) and as a multiplet (for compound **3e**) (δ 3.70-3.52 ppm, C*H*$_2$OH).

The protons of the chains present in alkoxy groups are showed, namely the OCH_3 (**3a**) as a singlet (δ 3.40 ppm); OC*H*$_2$$CH_3$ (**3b**) as a multiplet (δ 3.57-3.50 ppm) and OCH_2C*H*$_3$ (**3b**) as a triplet (δ 1.22 ppm); OC*H*$_2$$CH_2C$*H*$_2$Br (**3c**) appear integrated in a multiplet (δ 3.73-3.42 ppm) and OCH_2C*H*$_2$$CH_2$Br (**3c**) also as a multiplet (δ 2.09-2.02 ppm); $OC(CH_3)_3$ (**3d**) as a singlet (δ 1.20 ppm); OC*H*(CH_3)CCH (**3e**) as a multiplet (δ 4.06-4.01 ppm), OCH(CH_3)CC*H* (**3e**) as a duplet (δ 2.44 ppm) and OCH(C*H*$_3$)CCH (**3e**) also as a duplet (δ 1.37 ppm); aromatic protons of *p*-F-Ph (**3f**) as multiplets (δ 7.25-6.99 ppm) and OC*H*$_2$-*p*-F-Ph (**3f**) as a singlet (δ 4.49 ppm); and aromatic protons of O-Ph (**3g**) as multiplets (δ 7.32-6.94 ppm).

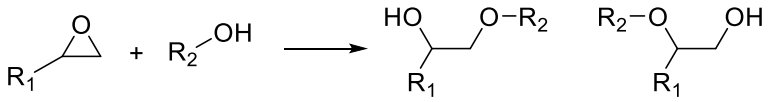
In the ^{13}C spectra of compounds **3a-g**, signals from aromatic carbons appear (δ 112.03-146.46 ppm); Furthermore, there also present the carbon of the methoxy group directly attached to the aromatic ring (δ 55.87 ppm, OCH_3); the carbon from the methylene group directly attached to the aromatic ring (δ 38.19 ppm, CH_2Ph); carbon from the CHOH group (compounds **3a-b**, **3d** and **3g**) (δ 73.56 ppm, CHOH) and from the CHO group (compounds **3c** and **3e-f**) (δ 80.98 ppm, CHO); the carbon from the CH_2O group (compounds **3a-b**, **3d** and **3g**) (δ 69.55 ppm, CH_2O) and from the CH_2OH group (compounds **3c** and **3e-f**) (δ 64.00 ppm, CH_2OH).

The carbons of the cyclic chains present in alkoxy groups are shown, namely the OCH_3 (**3a**) (δ 59.06 ppm), O*C*H_2CH_3 (**3b**) (δ 66.72 ppm) and OCH_2*C*H_3 (**3b**) (δ 15.13 ppm), O*C*$H_2CH_2CH_2$Br (**3c**) (δ 67.02 ppm), OCH_2*C*H_2CH_2Br (**3c**) (δ 32.82 ppm) and OCH_2CH_2*C*H_2Br (**3c**) (δ 30.62 ppm); O*C*(CH_3)$_3$ (**3d**) (δ

73.19 ppm) and OC(CH_3)$_3$ (**3d**) (δ 27.57 ppm); OCH(CH_3)CCH (**3e**) (δ 84.41 ppm), OCH(CH_3)CCH (**3e**) (δ 72.97 ppm), OCH(CH_3)CCH (**3e**) (δ 64.69 ppm) and OCH(CH_3)CCH (**3e**) (δ 22.34 ppm); aromatic carbons of *p*-F-Ph (**3f**) (δ 115.16-161.13 ppm) and OCH_2-*p*-F-Ph (**3f**) (δ 71.27 ppm); and aromatic carbons of O-Ph (**3g**) (δ 116.24-157.79 ppm).

The IR spectra of compounds **3a-g** displayed stretching bands characteristics of the hydroxyl groups from 3381 to 3425 cm^{-1}.

Epoxides are very interesting chemical intermediates due to their versatility. They are compounds that can be processed to produce many products.[70] They are commonly transformed through ring opening reactions with different nucleophiles such as water[71], alcohols[46,72] and amines[48] to produce a wide range of functional products. Alcohol ring-opening products are commonly found in compounds relevant to the pharmaceutical industry. The main challenge for these reactions is selectivity, due to the polymerization tendency of epoxides, since ring opening can occur with nucleophile attack on carbon in position 1 or 2 (scheme 2).[70]



Scheme 2. Possible products of a ring-opening reactions of epoxide.

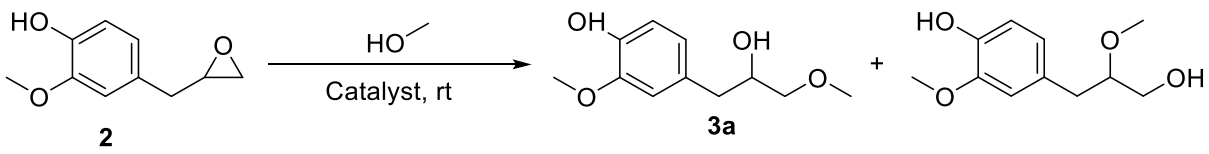
In general, ring-opening reactions of epoxides can be catalyzed under basic or acidic conditions. Under basic conditions, a nucleophilic substitution S_N2-type reaction occurs, and nucleophile attack typically occurs at the least hindered carbon. Under acidic conditions, epoxide ring opening resembles an S_N1 reaction, i.e., the nucleophile tends to attack the more highly substituted carbon atom of the epoxide ring, which carries more of the positive charges and resembles a stable carbocation species. Therefore, the formation of a product is highly favored under acidic conditions.[72]

In early attempts to open the epoxide ring, acidic conditions were created to facilitate product formation. These conditions were tested with methanol and ethanol and drops of hydrochloric acid were added as the reaction was being monitored. After the reaction work-up, it was realized that the desired product was not formed. The next step was to test the same method, with the same alcohols but increasing the temperature to 50 °C. However, the increase in temperature also did not contribute to the formation of the expected product.

This result is due to the low nucleophilicity of the alcohols, which makes the preparation of pure compounds of β-alkoxy alcohols under moderate reaction conditions a very difficult task in organic synthesis. The epoxide ring opening reaction with alcohols for the synthesis of the corresponding β-alkoxy alcohols is a widely studied reaction. However, due to the low nucleophilicity of alcohols, strong acidic or basic catalysts are needed for the reaction.[46]

The ring opening reactions are commonly catalyzed using strong acids, Lewis bases, or Lewis acids.[70] Lewis acids are significant reagents and/or catalysts in synthetic organic reactions, and those most commonly found in organic synthesis are BF_3, $ZnCl_2$, $AlCl_3$, $BF_3 \cdot OEt_2$ and $SnCl_4$.[73] Lewis acid catalysts have received considerable attention in various organic transformations, namely ring opening and epoxide rearrangement.[73,74]

In the presence of Lewis acid as catalyst, at room temperature, and in the presence of ethanol, *tert*-butanol and phenol, the typical S_N2-type reaction occurred, where the nucleophile attack occurs at the least hindered carbon **3b**, **3d** and **3g** (scheme 1). However, in the reaction with methanol, in the presence of the catalyst and at room temperature, two isomers were formed, 4-(2-hydroxy-3-methoxypropyl)-2-methoxyphenol **3a** through a Sn_2-type reaction and 4-(3-hydroxy-2-methoxypropyl)-2-methoxyphenol through a Sn_1-type. That is, both types of Sn reaction occurred (scheme 3).



Scheme 3. Synthesis of products of both types of S_N reaction using methanol.

To make the nucleophile attack more selective, the temperature at which the reaction takes place was reduced to 0°C. By monitoring the reaction, after 2 hours, it was found that only one reaction of the S_N2-type occurred. Through this result it is possible to state that temperature can affect the selectivity of this type of reactions.

In the case of compounds **3c** and **3e-f**, it is possible, through the NMR spectrum of ^1H and ^{13}C, to verify that a S_N1-type reaction has occurred. This can be observed in the spectra through the chemical shift of the CH proton and carbon. According to theoretical predictions made on software ChemDraw Professional 18.1, when an S_N1-type reaction occurs with these reagents, the CH carbon proton has a chemical shift smaller than in cases where a S_N2-type reaction occurs, being slightly lower than the

chemical shift of the protons of the OCH_3 group (3.87 ppm). In the case of CH carbon, the chemical shift, when a S_N2-type reaction occurs, is less than 80 ppm, and when a S_N1-type reaction occurs, is slightly greater than 80 ppm.

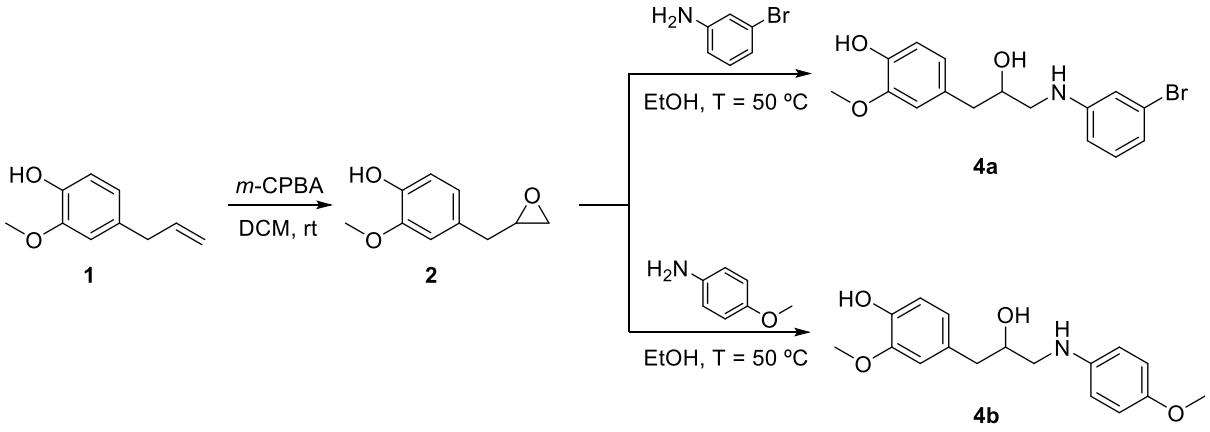
In addition to the low nucleophilicity of alcohols, which can be increased through catalysts, there are other factors that affect the selectivity and yield of reactions, namely solvents[46], prolonged reaction time[73], concentration and different Lewis acids[46,48,70,72,74], temperature and the alcohol used[72,73]. Thus, it is important to understand factors controlling catalytic activity and selectivity for these reactions.

3.2 Synthesis of eugenol amino alcohols derivatives

Compounds **4a-b** were obtained with two different aromatic amines, possessing the bromine atom as substituent in the *meta*-position or a methoxy at the *para*-position, respectively.

Also starting from compound **2**, with heating at 50 °C for 26-30 hours, using 3-bromoaniline and *p*-anisidine, the corresponding amino alcohols were obtained, namely 4-(3-((3-bromophenyl)amino)-2-hydroxypropyl)-2-methoxyphenol **4a** and 4-(2-hydroxy-3-((4-methoxypheny)amino)propyl)-2-methoxyphenol **4b**, respectively.

Reactions were monitored by TLC and/or ^{1}H NMR and, in all compounds **4a-b**, it was necessary to carry out a purification by column chromatography. These eugenol epoxide derivatives **4a-b** were obtained as oils with yields of 48% and 21% respectively (scheme 4).



Scheme 4. Synthesis of alkoxy alcohols **4a-b** derived from eugenol epoxide **2**.

The structure of compounds **4a-b** was confirmed by ^{1}H and ^{13}C NMR. In the corresponding spectra of ^{1}H the aromatic signals appear, for compounds **4a-b**, H-3 and H-5 appear integrated in a multiplet

(for compound **4a**) (δ 6.74-6.70 ppm) and H-6 (for compound **4a**) as a double duplet (δ 6.88 ppm) or H-5, H-6, and H-3 (for compound **4b**) integrated in a multiplet (δ 6.75-6.69 ppm). The methoxy group protons directly linked to the aromatic ring appears as a singlet for **4a-b** compounds (δ 3.81 ppm, OCH_3). Protons of the methylene group directly attached to the aromatic ring appear as two double duplets (for compound **4a**) (δ 2.82-2.73 ppm, CH_2Ph) and as a multiplet (for compound **4b**) (δ 2.87-2.72 ppm, CH_2Ph).

The proton from the C*H*OH group appears as multiplets (δ 4.11-4.00 ppm, C*H*OH) and the methylene protons of the CH_2NH group appear as two double duplets (δ 3.27-3.06 ppm, CH_2NH) for **4a-b** compounds.

The protons of the aromatic chains present in amino groups are shown, namely the aromatic protons of *m*-Br-Ph (**4a**): H-5 as a triplet (δ 7.01 ppm), H-4 as a double duplet (δ 6.83 ppm), H-2 as a triplet (δ 6.75 ppm) and H-6 as a double duplet (δ 6.53 ppm); and aromatic protons of NH-Ph-OMe (**4b**): H-6 as a duplet (δ 6.86 ppm), H-2 and H-5 as a multiplet (δ 6.80-6.78 ppm) and H-3 integrated in a multiple (δ 6.75-6.69 ppm) and NH-Ph-OC*H*$_3$ (**4b**) as a singlet (δ 3.87 ppm).

In the ^{13}C spectra of compounds **4a-b**, signals from aromatic carbons appear (δ 114.36-146.65 ppm); Furthermore, there are also present the carbon of the methoxy group directly attached to the aromatic ring (δ 55.89 ppm, OCH_3); the carbon from the methylene group directly attached to the aromatic ring (δ 41.18 ppm, CH_2Ph); carbon from the CHOH group (δ 70.94 ppm, CHOH); the carbon from the CH_2NH group (δ 50.25 ppm, CH_2NH).

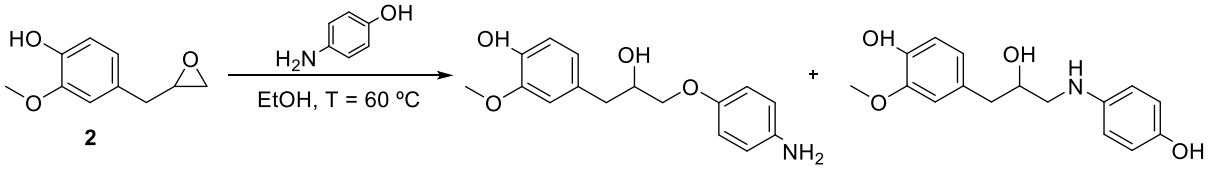
The carbons of the aromatic chains present in amino groups are shown, namely the aromatic carbons of *m*-Br-Ph (**4a**) (δ 111.74-149.37 ppm) and; NH-*Ph*-OMe (**4b**) (δ 111.80-153.39 ppm), as well as of its substituent NH-Ph-O*C*H$_3$ (**4b**) (55.76 ppm).

The IR spectra of compounds **4a-b** displayed stretching bands characteristics of the hydroxyl groups and amine functions from 3373 to 3505 cm^{-1}.

In the case of reactions of epoxide with amines, the method used was different. One of the most widely used pathways for the synthesis of β-amino alcohols is the direct aminolysis of epoxides at high temperature with excess amine.[75] However, in recent years, several methods using promoters or catalysts in different organic solvents have been reported.[76,77] The preparation of β-amino alcohols in water is an option as using water instead of organic solvents has become more important due to environmental considerations in recent years in order to practice green and sustainable chemistry.[48] In our lab, a procedure similar to that used by Azizi and Saidi[48] was adopted previously, using water as

solvent and temperature at 50 °C, with a slight excess of amine. Despite the reaction taking place, the work-up was found to be difficult because it was hard to remove water from the reaction mixture. In view of this difficulty, ethanol was used as solvent, keeping the temperature at 50 °C and a slight excess of amine. At this point, we thought there might be a risk that the ethanol would react with the epoxide and form an alkoxy alcohol. The NMR spectra show that only the amino alcohol was formed, and this reinforces the results previously obtained with the reactions with alcohols without catalyst.

In the synthesis attempt of compound 4-(2-hydroxy-3-((4-methoxyphenyl)amino)propyl)-2-methoxyphenol, the same procedure used in the previous reactions with amines was used, but in this case, we also wanted to test which of the nucleophilic groups would has an advantage in the nucleophilic attack, NH_2 (4-(2-hydroxy-3-((4-hydroxyphenyl)amino)propyl)-2-methoxyphenol) or OH (4-(3-(4-aminophenoxy)-2-hydroxypropyl)-2-methoxyphenol) (scheme 5).



Scheme 5. Synthesis of two possible products with 4-aminophenol.

By predicting the NMR spectrum of the two possible structures through ChemDraw Professional 18.1 software, it is possible to verify that the main difference is in the chemical shift value of the CH_2-O or CH_2-N carbon, depending on the product formed, as shown in figure 23. In case there is a nucleophilic attack through the NH_2 group, this chemical shift value is between 3.06-3.31 ppm; if a nucleophilic attack through the OH group occurs, this chemical shift value is between 3.98-4.20 ppm.

Although it was not possible to completely isolate and purify the compound, it is possible to observe in the NMR spectrum of the major fraction that the nucleophilic attack occurred through the NH_2 group on the least hindered carbon (δ 2.96-3.11 ppm).

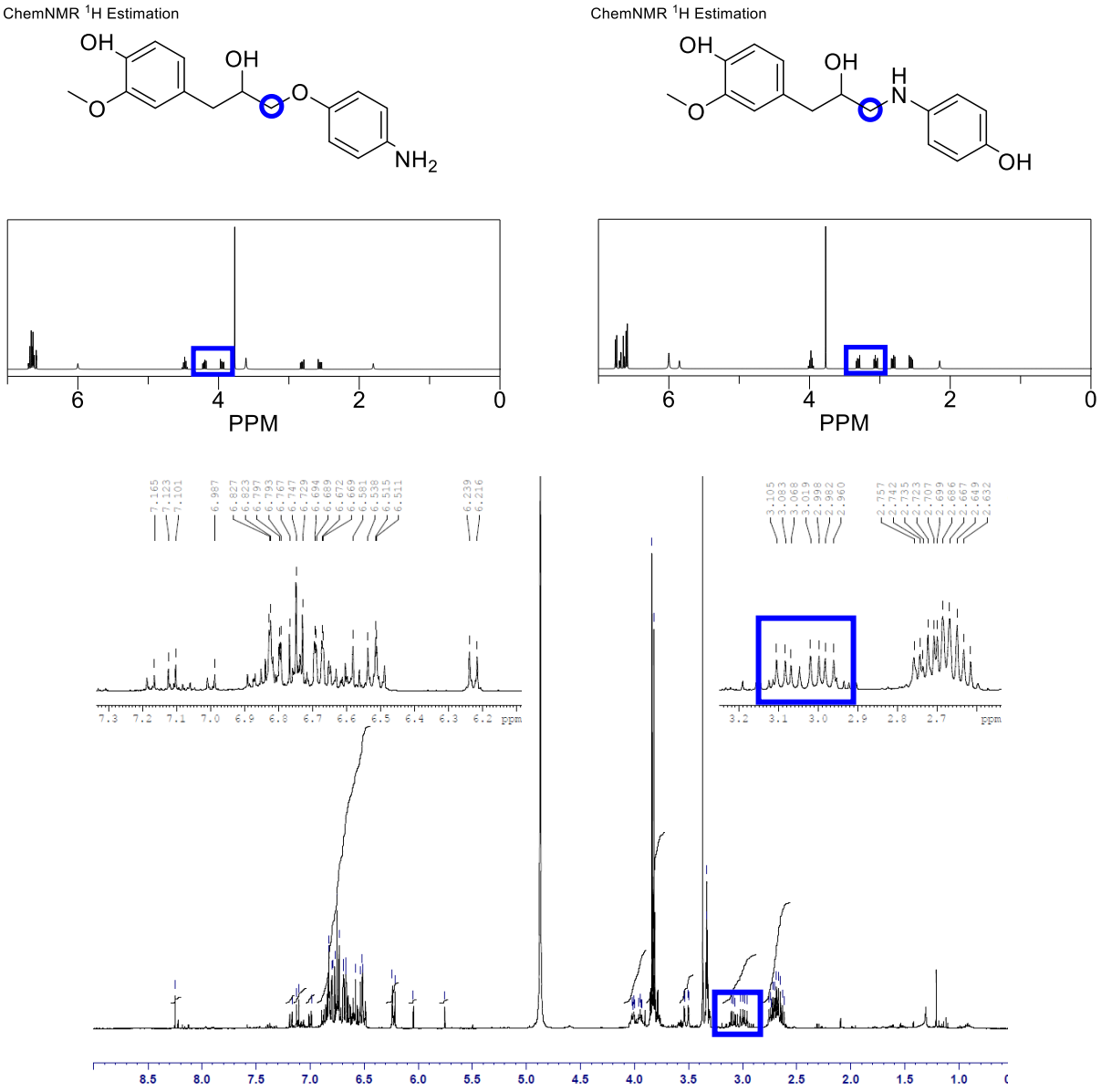


Figure 23. Prediction of the chemical shift value of the CH_2-O or CH_2-N carbon of the two possible products that could be formed (top) and the spectrum obtained after the reaction (bottom). Marked in blue (δ 3.11-2.96 ppm) are protons whose chemical shift value changes if one compound or another is formed.

This result is explained by the fact that the nucleophilicity of alcohols is lower than that of amines.[46]

3.3 Biological evaluation

The study of the insecticidal activity of the synthesized compounds **3a-g** and **4a-b** was carried out in two-dimensional (2D) cultures of *Sf9* cells, which are derived from ovary cells of *Spodoptera frugiperda*.

In this evaluation, it is expected that the compound presents a low percentage for cell viability, that is, the lower the percentage of associated viability, the greater the loss of viability, the compound possessing greater insecticidal activity, being more toxic to *Sf9* cells.

The starting reagents of this group of compounds, eugenol **1** and eugenol epoxide **2**, show a percentage of loss of cell viability of about 15% in *Sf9*. When compared with their alkoxy alcohol derivatives synthesized **3a-g**, the **3c-f** derivatives show greater activity, reflecting a higher percentage of cell viability loss, between 17 and 40%. Figure 24 shows the cell viability plot, in percentage, of eugenol **1**, eugenol epoxide **2** and their alkoxy alcohol derivatives **3a-g** under study.

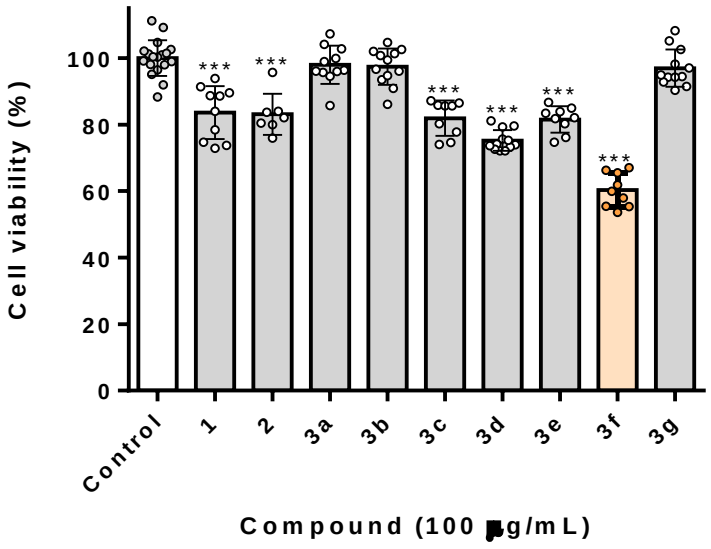


Figure 24. Cell viability, in percentage, of eugenol **1**, eugenol epoxide **2** and derivatives **3a-g**. Results correspond to the mean ± SD of at least three independent assays. *** $p < 0.001$

Among all eugenol-derived alkoxy alcohols synthesized, compound **3f**, whose structure includes a group of methanol derivative in which one of the H atoms of the methyl group was replaced by fluorophenyl, presents the highest toxicity in *Sf9*, that is, a higher percentage of cell viability loss (approximately 40%) when compared to the cell viability obtained with the remaining derivatives. Compound **3d**, whose structure includes a non-linear alkyl chain with three terminal methyl groups, has a loss of cell viability of approximately 25%. The remaining compounds **3c** and **3e**, which have greater activity than the starting compounds **1** and **2**, show a loss of cell viability of approximately 17%, that is, the toxicity in *Sf9* is very low.

Considering the synthesized amino alcohol derivatives **4a-b**, and comparing with the starting reagents of this group of compounds, eugenol **1** and eugenol epoxide **2**, the derivative **4a** has greater activity,

reflecting in a higher percentage of cell viability loss of approximately 23%. Figure 25 shows the cell viability plot, in percentage, of eugenol **1**, eugenol epoxide **2**, their amino alcohol derivatives **4a-b** under study, and two compounds with similar structure that had already been synthesized in our laboratory.[78] Their structures are also shown in figure 25.

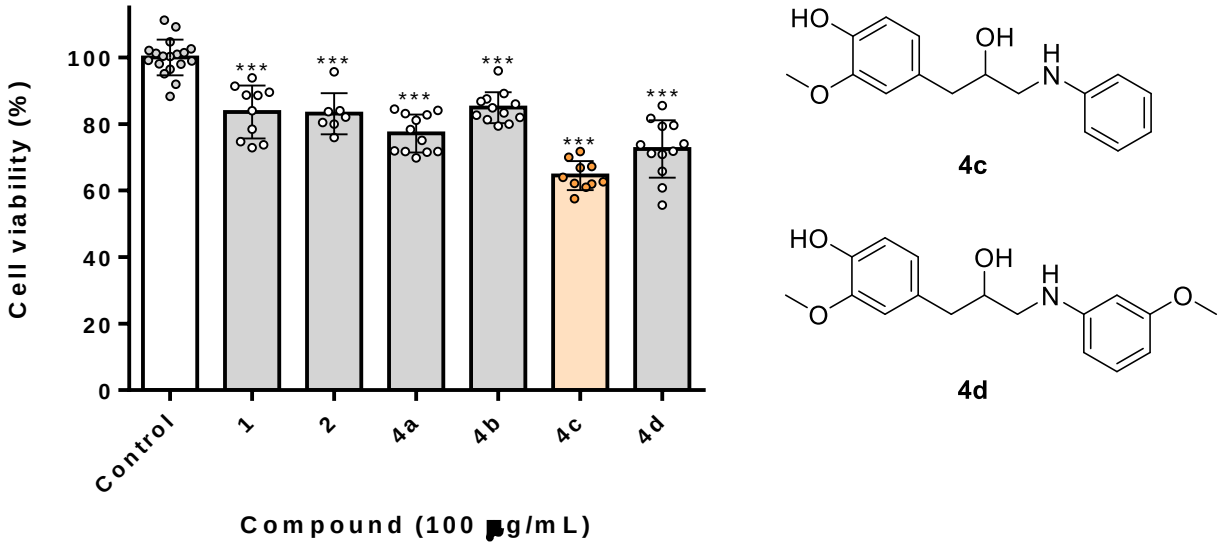


Figure 25. Cell viability, in percentage, of eugenol **1**, eugenol epoxide **2** and derivatives **4a-d**. Results correspond to the mean ± SD of at least three independent assays. *** $p < 0.001$

Directly comparing compounds **4a** and **4c**, whose structure differs in the presence of a halogen in the *meta* position, it is verified that the presence of the bromine atom hinders the insecticidal action of compound **4a**, reducing the loss of cell viability by 12% when compared to the loss of cell viability of compound **4c** which is approximately 35%.

Comparing compounds **4b** and **4d**, whose structure differs in the position of the methoxy group, it is verified that by changing methoxy from the *meta* position (compound **4d**) to the *para* position (compound **4b**), the viability is reduced by about 15%. That is, the methoxy group in the *para* position hinders the insecticidal action of compound **4b**.

3.4 Nanoencapsulation studies

The most active new compounds against *Sf9* cells, compounds **3d** and **3f**, were encapsulated in Egg-PC:Ch (7:3) liposomes, prepared by two methods, EI[67] and TFH[68].

The absorption and fluorescence properties of compounds **3d** and **3f** in ethanol were studied. The normalized absorption and emission spectra are displayed in figure 26. Both compounds are fluorescent molecules exhibiting a maximum emission around 340 nm.

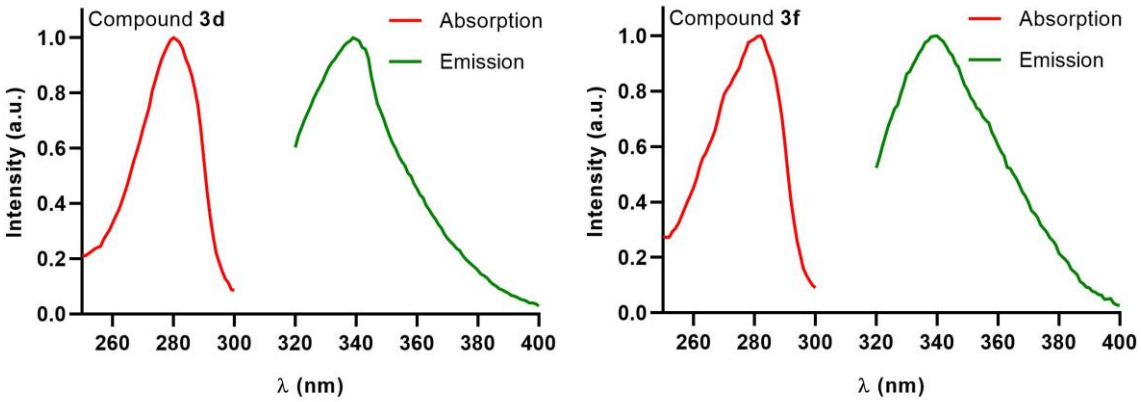


Figure 26. Normalized absorption and emission (λ_{exc} = 290 nm) spectra of compounds **3d** (left) and **3f** (right) in ethanol.

Hydrodynamic sizes, PDI and zeta potential were measured by DLS (table 5). The liposomes prepared by the EI method have a smaller size than the ones prepared by TFH, but both methods originate small polydispersity values. Considering the hydrodynamic size values, SUVs (size around 100 nm) were formed through the EI method and MLVs (size below 500 nm) through the TFH method. This result emphasizes the need of performing extrusion when using TFH method. Considering the zeta potential values, the liposomes from both methods are considered neutral (zeta potential below ±5 mV).

Table 5. Size (hydrodynamic diameter), PDI and zeta potential of Egg-PC:Ch (7:3) liposomes determined by DLS. (SD: standard deviation from three independent measurements).

System	Size ± SD (nm)	PDI ± SD	Zeta potential ± SD (mV)
Liposomes (EI)	114.44 ± 1.82	0.27 ± 0.01	0.97 ± 0.29
Liposomes (TFH)	484.23 ± 43.53	0.29 ± 0.02	3.03 ± 0.12

Considering the encapsulation efficiencies (table 6), both methods have proven to be suitable for both compounds, with EE (%) values above 93%. However, the TFH method reveals greater EE (%) for both compounds **3d** and **3f**, with values higher than 95% and 99%, respectively.

Table 6. EE (%) of the compounds with the best insecticidal activity in liposomes (SD: standard derivation of three independent measurements).

Compound	System	EE ± SD (%)
3d	Liposomes (EI)	93.52 ± 0.75
	Liposomes (TFH)	95.99 ± 0.17
3f	Liposomes (EI)	97.89 ± 0.56
	Liposomes (TFH)	99.54 ± 0.15

The release of the encapsulated compounds **3d** and **3f** was followed for 24h at room temperature towards buffer of pH = 7.4. The release profiles were fitted to the Weibull model[69] (fitted curves in figure 27) and the results are shown in table 7.

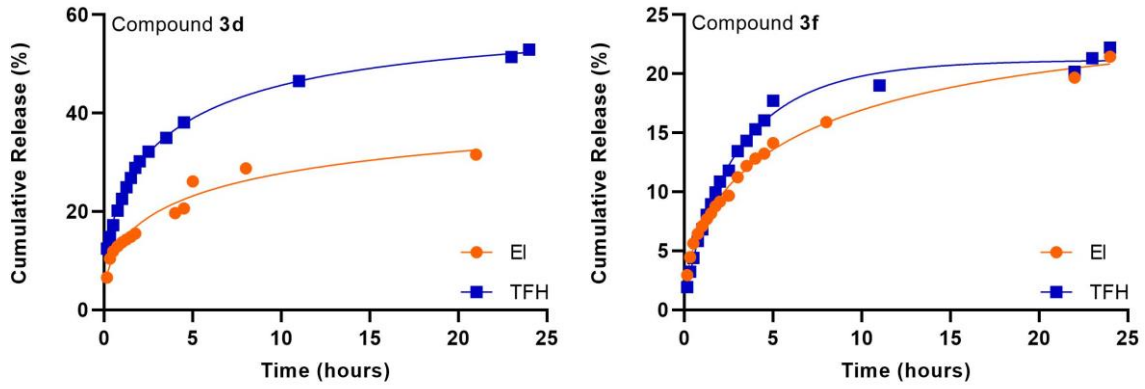


Figure 27. Release profiles of compounds **3d** and **3f** and fitting to Weibull model.

Table 7. Release parameters of the Weibull model (equation 2) fitted to the release profiles. R^2 is the coefficient of determination.

Compound	System	Y_{max}	b	a	R^2
3d	Liposomes (EI)	41.65	0.44	0.40	0.96
	Liposomes (TFH)	57.49	0.48	0.52	0.99
3f	Liposomes (EI)	24.90	0.53	0.34	0.99
	Liposomes (TFH)	21.19	0.83	0.40	0.99

The coefficients of determination (R^2) above 0.96 show that Weibull model is suitable to describe the release of compounds **3d** and **3f** from liposomes prepared by both methods. According to

Papadopoulou *et al.,* the parameter *b* can be related to the diffusion mechanism; accordingly, if $b > 1$, the compound transport is a complex release mechanism; if $b \leq 0.75$ is a Fickian diffusion (in either fractal or Euclidian spaces), and if $0.75 < b < 1$ the mechanism is a combination of Fickian diffusion and Case II transport.[69] Thus, according to the Weibull model, the release mechanism of compound **3d** from liposomes of both methods and of compound **3f** from liposomes obtained by EI is Fickian diffusion, and the release mechanism of compound **3f** from liposomes obtained by TFH is a combination of Fickian diffusion and Case II transport.

From the fitted release curves, it is also possible to verify that from liposomes prepared by the TFH method, the maximum amount of **3d** compound released after 24 hours is greater (57%) than the maximum amount released by the liposomes of the EI method (42%), being also a faster release.

Regarding compound **3f**, the maximum amount of compound released from liposomes of the two methods is similar after 24 hours (EI = 25%; TFH = 21%), but the release is also faster from liposomes obtained through the TFH method.

The release profiles were also fitted to the Korsmeyer-Peppas model[79] (fitted curves in figure 28) and the results are showed in table 8.

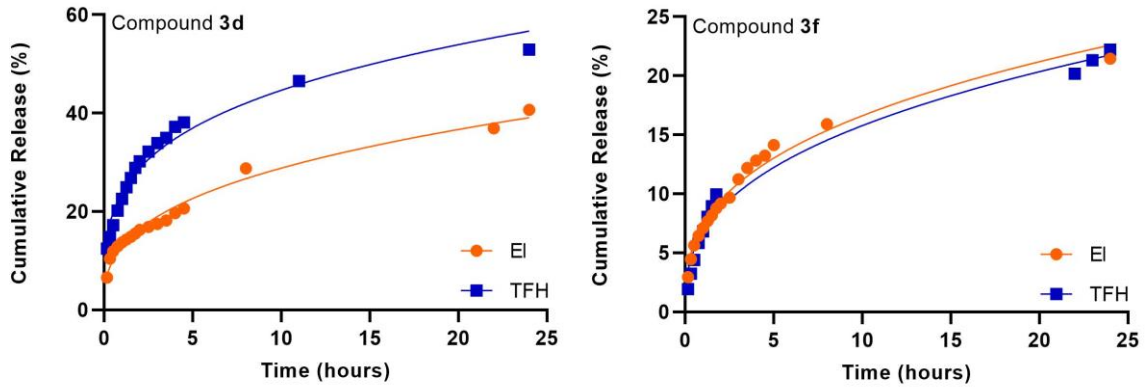


Figure 28. Release profiles of compounds **3d** and **3f** and fitting to Korsmeyer-Peppas model.

Table 8. Release parameters of the Korsmeyer-Peppas (equation 3) model fitted to the release profiles. R^2 is the coefficient of determination.

Compound	System	k (min^{-1})	n	R^2
3d	Liposomes (EI)	12.94	0.35	0.98
	Liposomes (TFH)	23.85	0.27	0.97
3f	Liposomes (EI)	7.40	0.35	0.98
	Liposomes (TFH)	6.78	0.37	0.98

The coefficients of determination (R^2) above 0.97 show that also Korsmeyer-Peppas model is suitable to describe the release of compounds **3d** and **3f** from both liposome methods. According to Wu *et al.* the parameter n is directly related to the release mechanism of the compound; accordingly, if $n > 1$, the release is controlled by swelling and material relaxation; if $0.89 < n < 1$ indicates a relaxation-controlled mechanism; if $0.45 < n < 0.89$ indicates a combination of diffusion and erosion in drug release (non-Fickian release); and when $n < 0.45$, the release mechanism is diffusion-controlled (Fickian release).[79] Thus, according to the Korsmeyer-Peppas model, the release mechanism of compounds **3d** and **3f** is diffusion-controlled (Fickian release).

Considering the values of the R^2, the Weibull model is more suitable to describe the release profiles of compound **3d** from the liposomes obtained by the TFH method ($R^2 = 0.99$) and of compound **3f** from the liposomes prepared by the two methods ($R^2 = 0.99$). However, to describe the release profile of compound **3d** from the liposomes of the EI method ($R^2 = 0.98$), the Korsmeyer-Peppas model is more suitable.

Chapter 4 – Concluding and future remarks

Plant extracts and phytochemicals have been extensively studied, and their pesticidal activity has been demonstrated. Essential oils are a matrix of phytochemicals with this characteristic most evident. The nanoencapsulation of these biopesticides is a great innovation in this project, whose objective is to realize what benefits can bring to the pesticide action in different aspects such as solubility, targeted action, and preservation.

With the accomplishment of the experimental work leading to this master's dissertation, the essential oil of the plant *Syzygium aromaticum* was extracted and several derivatives of eugenol, alkoxy and amino alcohols compounds were synthesized. All synthesized compounds were submitted to tests of biological activity in *Sf9*, in comparison with the corresponding precursor, to evaluate their application as biopesticides. The two biosynthetic compounds that showed greater insecticidal activity in *Sf9* namely **3d** (loss of cell viability of, approximately, 25%) and **3f** (loss of cell viability of, approximately, 40%)) were subjected to encapsulation in lipid nanosystems and release studies. For both compounds, the EE (%) was higher than 93%, for the two methods of liposomes preparation. In release assays, it was observed that liposomes from the two methods do not completely release the compounds after 24 hours. For compound **3d**, there is a greater release from liposomes prepared by the TFH method, and a slower release from liposomes obtained by the EI method. For compound **3f**, there is a slightly larger release from the liposomes of the EI method, this method also being the most suitable for slower release.

The results obtained in the nanoencapsulation tests indicate that liposomal systems allow high EE (%) of compounds with insecticidal activity and provide different release rates considering the encapsulation method. This represents a basis for future development of bioinsecticide nanoformulations using natural extracts and derivatives with promising insecticidal activity.

There are studies that indicate that eugenol induces cell apoptosis and functions as an anti-cancer drug in several types of tumors, such as malignant melanoma cells, osteosarcoma cells, mast cells and prostate cancer cells. Previous studies also suggested that AGS cell growth is inhibited by extracts of EOs. These results show that EOs have anti-proliferative properties in gastric cancer cells and could be a future perspective in their treatment. Has been also studied the tumor suppressive role and potential mechanisms of eugenol in lung cancer adenocarcinoma A549 cells. For this reason, the AGS and A549 cell lines have also been included in current biological “screening” assays.

As future approach, the compounds should be evaluated in relation to their biological activity, before and after encapsulation, to compare their potential with non-encapsulated materials. New lipid and non-lipid formulations should also be tested, to obtain a broader study in the field of nano-insecticides.

Biological assays can also be performed on keratinocytes with the compounds that showed better results in *Sf9* to allow the assessment of their toxicity in mammals. New cell lines must also be tested to value eugenol.

Regarding chemical synthesis, the conditions under which each of the reactions with the alcohols are carried out can be studied and revised, to increase the selectivity and yield of each of the alkoxy alcohols obtained. Furthermore, chemical synthesis of derivatives of isolated compounds can be supported and enhanced by molecular modeling studies.

Thus, it is intended to contribute to the development of derivatives of compounds of botanical origin that work as promising alternatives to currently available synthetic insecticides.

Chapter 5 - References

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