Encapsulation of essential oil obtained by distillation methods from Azorean *Cryptomeria japonica* foliage in two different seasons and its impact in antimicrobial, neuroprotective and toxicity effects against *Artemia salina*

State of the Art

1. Overview on antimicrobial resistance and dementia – Two important research topics worldwide

1.1. Antimicrobial resistance (AMR): drug resistance in bacteria and in fungi

- WHO recognizes AMR one of the top global public health and development threats.[1]

  Antimicrobial drugs (e.g., antibiotics, antifungals, antiparasitics, and antivirals) are used to treat, prevent, or control infectious diseases in humans, animals and plants. The AMR occurs when bacteria, fungi, parasites, and viruses no longer respond to antimicrobial drugs, being a natural process that happens over time through genetic changes in pathogens; unfortunately, its emergence and spread are accelerated by human activity, mainly the misuse and overuse of antimicrobials in humans, animals, and plants. As a result, these antimicrobial drugs become ineffective and, consequently, infections become difficult or impossible to treat, increasing the risk of disease spread, severe illness, disability and death.[1] For example, it is estimated that bacterial AMR was directly responsible for 1.27 million global deaths in 2019 and contributed to 4.95 million deaths.[2] In addition to death and disability, AMR has significant economic costs. The World Bank estimates that AMR could result in US$ 1 trillion additional healthcare costs by 2050, and US$ 1 trillion to US$ 3.4 trillion gross domestic product losses per year by 2030.[3]

  The emergence of new resistant microbial strains, and re-emergence of known pathogens, to current synthetic antibiotics/fungicides, combined with a decline in the development of new antibiotics/fungicides drugs, presents a major problem for both public and animal health. AMR also poses a constant threat for food safety management systems worldwide, being plant diseases recognized as one of the biggest challenges. In fact, several important crops are susceptible to infection by pathogenic bacteria and/or fungi, with significant impact on foodstuff loss worldwide[4–8]. Moreover, global regulations concerning pesticide residue levels

in fruits and vegetables have become increasingly stricter over the years for human health and environmental protection purposes.[9]

- Hence, there is a need to explore the use of eco-friendly and more effective antibacterial/antifungal agents, such as ESSENTIAL OILS (EOs). In fact, the EO antimicrobial activity is attributed to several distinct mechanisms, preventing the development of resistance in microorganisms.[10–14]

1.2. Dementia: Alzheimer's disease (AD)

- WHO recognizes dementia as a public health priority worldwide,[15] since, nowadays, it is more and more usual. In fact, at present, around 50 million people worldwide are estimated to suffer from dementia, and this number is expected to increase to 152 million by 2050,[16] as a result of the increase in life expectancy.[17] Dementia affects individuals, their families, and the economy, with global costs estimated at about US$1 trillion annually.[16] AD, a multifactorial disorder that affects, mainly, older people, is the most common form of dementia and may contributes to 60–70% of cases. After decades of investigation, there is currently no cure for AD. Therefore, this disease remains the subject of major ongoing research.[15,17]

  The symptoms of AD include deficient cholinergic function, memory loss, cognitive decline, neuronal cell death and behavioural disturbances. Patients with advanced AD have physiological and psychological problems, cannot live normally and usually die of complications such as infection.[15,18] Some major risk factors associated with AD include: ageing; genetic predisposition, such as carriers of the ε4 allele of the apolipoprotein E (ApoE) gene (*APOE*); cardiovascular disease risk factors; lifestyle and psychosocial factors; and family medical history.[18]

  The *APOE* is associated with the late-onset AD (LOAD), the most usual AD form that occurs after the age of 65. The other form to develop AD, called early-onset (EOAD), since it occurs on people younger than 65 years old, is associated with genetic mutations, generally in three genes, the amyloid precursor protein (APP) gene (*APP*) and the presenilin 1 and 2 genes (*PSEN1* and *PSEN2*), which are involved in the production of the amyloid-β (Aβ) peptides in the brain. Besides extracellular senile plaques of Aβ protein, other major AD clinical indications (CIs) are intracellular hyperphosphorylated tau neurofibrillary tangles (NFTs), uncommon neuroinflammatory response and neuronal cell apoptosis and death caused by oxidative stress.[17]

Although the mechanisms of action of the above-mentioned four genes in AD pathogenesis have been studied extensively, the ones involved in the AD progression are still not clear, suggesting that the AD is driven by a complex combination of genetic and other risk factors (e.g., biological and environmental).[19] Owing to that complexity, involving several mechanisms which may work altogether through interaction between genetic, molecular, and cellular events, an efficacious treatment for AD remains to discover, in fact, the action against it has concerned mostly the reduction of the major CIs of the disease,[17] identified above.

So, as AD is such a complex neurodegenerative disease caused by various pathways, a single-target drug approach is unlikely to be effective. It is currently believed that the best approach for AD is a therapy capable of modulating multiple target pathways. In this context, two promising strategies are: (i) a combination therapy and (ii) a multitarget-directed ligands (MTDLs) therapy.[20–23] For example, cholinergic deficit and neuroinflammation are considered major contributing factors for AD. Thus, compounds which have activity against acetylcholinesterase enzyme (AChE) and anti-inflammatory properties are multi-target compounds to combat AD[17].

- The most promising compounds to combat AD are natural products, because they can interact with a wide range of molecular targets[17,24–27] and present a higher resemblance to biosynthetic intermediates or endogenous metabolites than do synthetic compounds[28]. The findings of literary investigation indicate promising outcomes concerning the diverse ESSENTIAL OILS (EOs) that have been examined in research on AD.[29–31]

2. Overview on *Cryptomeria japonica* (Thunb. ex L. f.) D. Don (Cupressaceae) biomass residues valorisation by production of EO

2.1. Essential oils – From "obsolescence to opportunity"

- It is well known that EOs (i) play a key role in plant protection in Nature and (ii) have also been used therapeutically since ancient times,[32,33] due to its high effectiveness. Thus, nowadays, there has been a renewed interest in the usage of this "generally recognized as safe status (GRAS)" product,[10,33] traditionally obtained by distillation processes, such as hydrodistillation (HD) and steam distillation (SD) methods.

In fact, the global EO production has increased substantially in the last few decades, which can be attributed to several key factors, including: (i) the wide range of biological and pharmacological (including antimicrobial and neuroprotective effects) activities exhibited by EOs, and hence, its tremendous use in several industries, namely, food, agrochemical, textile,

perfumery, traditional medicine, and complementary therapies (such as aromatherapy), as well as cosmetic and hygiene applications, (ii) the increasing consumers' demand for safe, eco-friendly and effective natural products, and (iii) the great interest from the scientific community in the discovery of novel drugs or botanical pesticides from natural resources, such as the EOs.[33–35]

Nevertheless, only a few crude EOs and their components (EOCs) or derivatives thereof have been approved as drugs or botanical pesticides. Thus, the potential therapeutic and/or pesticidal properties of EOs is still under-scrutinized.[36] Particularly, these natural products (i) can be valuable sources of antimicrobial agents (with application in the fight against human and animal infectious diseases or in green plant protection and food preservation),[11–14,37,38] and (ii) have a huge potential for being novel, multi-target and low toxicity anti-AD drugs,[17,29–31] due to their broad-spectrum bioactivities (e.g., anticancer, antimicrobial, antioxidant and anti-inflammatory).[17,38–40]

- However, it should be highlighted that the chemical composition/biological activities, as well as the quantity of an EO and, consequently, its specific commercial applications and price, can be influenced by both endogenous and exogenous factors, such as: plant's species, chemotype, developmental stage and tissue; geographical localization; harvest time and procedure used for distillation.[41]

2.2. *Cryptomeria japonica* – a rich source of EOs

- *C. japonica*, native to Japan, is a large evergreen, long-lived, monoecious conifer tree that pollinates from February to April, and the seeds mature in October. It was introduced in the Azores in the mid-19th century and, currently, is the most important commercial forestry tree in the Azores (Portugal), representing 60% of the total wood-producing forest area[42].

  Due to their timber exploitation, a huge amount of Azorean *C. japonica* biomass residues, particularly *C. japonica* foliage (CJF), is generated from the wood industry and forest operations, which can cause several environmental problems. Hence, currently, CJF is the main source of biomass for Azorean *C. japonica* EO production, which only has applications in fragrance industry and aromatherapy.[42]

- However, this CJF-EO remain a relatively underutilized resource, compared to its potential application in integrated pest management programs or its use in the development of natural health products, since it has shown some biological properties, namely molluscicidal, antimicrobial and antioxidant activities, among others.[43–46]

3. Contribution to fostering innovation and sustainability in the *C. japonica*´s EO industry

While the CJF-EO does exhibit biological properties, it is also characterized by high volatility and hydrophobicity, stemming from its significant content of monoterpene hydrocarbons, particularly α-pinene.[46] Therefore, for utilize this EO in food preservation, pest control or medicine, it seems necessary to employ nanotechnology tools. The incorporation of CJF-EOs into nanosized encapsulation systems can significantly improve their physical properties, and thus promote their functional activities, in comparison to free EOs. Moreover, nanoencapsulation can also be used to protect EOs from degradation; to improve their solubility in aqueous media; to mask unpleasant taste and aroma; and to release them in the desired target.[47–49]

Recently, EOs-encapsulated from other species, like *Thymus vulgaris*, have been seen as potential antimicrobial agents against drug-resistant bacteria[47] and for enhancing antifungal activity against phytopathogenic fungi.[50]

Thus, CJF-EO will be incorporated into nanosized encapsulation systems, for the first time, employing cellulose acetate (CA), which is an environmentally friendly and biodegradable polymer.

This strategy will diversify the local market supply of *C. japonica* EOs, namely in health, food, and pest control industries. Simultaneously, new greener products, EOs-encapsulated, will be produced in an economically and environmentally sustainable manner, without competing for land areas.

Objectives

Azorean CJF-EO exhibit antimicrobial properties against a variety of microorganisms, including pathogenic bacteria and fungi, as extensively published by our research group. However, this EO is characterized by instability, high volatility, and hydrophobicity, stemming from its significant content of monoterpene hydrocarbons, particularly α-pinene. Therefore, the aim of this study is to improve Azorean CJF-EO physical properties, for the first time, through encapsulation techniques. The application of these techniques accomplishes several goals, such as, prevent degradation of the EO, allowing for longer storage, better dispersion in aqueous mediums, and act as a controlled release mechanism of the bioactive molecules, which may translate into a better biological activity than the unencapsulated EO.

Next, the efficacy of Azorean CJF-EO encapsulation will be evaluated in terms of its biological activity using the disc diffusion and microdilution methods against important pathogenic bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus, S. epidermidis*, *Escherichia coli*, *Enterobacter cloacae*, and *Serratia marcescens*). On the other hand, agar dilution method will be employed against phytophatogenic fungi *Thielaviopsis paradoxa, Penicillium digitatum*). Additionally, it will be assessed synergistic, additive, and antagonistic interactions. Additionally, other activities would be tested, such as anticholinesterase and anti-inflammatory activity, with a potential application in health. Encapsulation of Azorean CJF-EO may diversify the local market supply of *C. japonica* EOs, namely in health, food and pest control industries.

Project Description

A. Plant material collection, handling, and preservation (duration 1 month)

The plant material, namely *Cryptomeria japonica* foliage (CJF), will be provided by local wood producers (Marques S.A., S.Miguel, Azores, Portugal). Then, CJF will be immediately brought to a laboratory in the University of the Azores to be stored at −20 °C until further usage in the hydrodistillation (HD) process. A portion of CJF will be dried at room temperature for moisture calculation. A voucher specimen will be deposited in the Herbarium AZB−Ruy Telles Palhinha of the same University.

B. Essential Oil (EO) extraction (duration 6 months)

The CJF-EO will be obtained by HD through a Clevenger-type apparatus, according to the European Pharmacopoeia. In detail, the sample to water ratio is 1:10 g/mL, and the distillation time is approximately 3 h. The isolated EOs will be dehydrated and stored in sealed amber vials at 4ºC until further analysis. Each HD process will be performed at least in triplicate. EO yield (%) will be calculated as the EO mass (g) per 100 g of fresh weight (fw) of CJF.

C. Essential Oil composition analysis (duration 1 month)

The CJF-EO will be analyzed by gas chromatography-mass spectroscopy (GC–MS) and by gas chromatography with flame ionization detection (GC–FID) for component identification and quantification, respectively, as detailed in Lima et al., 2023a.

D. Preparation of CJF-EO-loaded chitosan (or cellulose acetate/maltodextrin) nanoparticles (NPs) (duration 4 months)

The NPs will be prepared through emulsification, by dissolving the material in an appropriate solvent, using a surfactant. The EO would then be added into the solution, which would be stirring. After evaporating the solvent, the NPs will be washed with distilled water.

E. Determination of retained CJF-EO in chitosan (or cellulose acetate/maltodextrin) NPs (duration 4 months)

The amount of loaded EO in the NPs would be determined by UV-VIS spectrophotometry. NPs without EO would serve as a blank. The amount of EO inside the NPs would then be calculated through a calibration curve.

F. Instrumental analysis of CJF-EO-loaded NPs (duration 4 months)

A variety of different tests would be performed to evaluate the properties of the NPs. Starting with dynamic light scattering (DLS) to determine the particle size, zeta potential and polydispersity index. The chemical structure analysis would be performed through Fourier-transform infrared spectroscopy (FTIR). In addition, the surface morphology and the shape of the NPs would be determined through scanning electronic microscopy (SEM), and thermogravimetric analysis (TGA), to evaluate the thermal properties of the NPs.

G. Determination of antibacterial properties (duration 12 months)

The efficacy of Azorean CJF-EO encapsulation will be evaluated in terms of its biological activity using the *in vitro* disc diffusion and microdilution methods against important pathogenic gram-positive and gram-negative bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, Methicillin-resistant

Staphylococcus aureus, *S. epidermidis*, *Escherichia coli*, *Enterobacter cloacae*, and *Serratia marcescens*). The synergistic potential of CJF-EO encapsulated with common antibiotics will be evaluated using checkerboard assay. All microbial strains will be obtained from the Department of Biology, University of the Azores. Kanamycin and ampicillin will be used as common antibiotics. Additionally, the inhibitory activity of chitosan (or CA/maltodextrin) NPs, CJF-EO-loaded chitosan (or CA/maltodextrin) NPs and pure CFJ-EO against the growth of mentioned bacterial strains will be examined to evaluate synergistic, additive, and antagonistic interactions. All experiments will be performed in at least triplicate.

H. Determination of antifungal properties (duration 9 months)

Phythopatogenic fungal strains, *Penicillium digitatum* (Person) Saccardo, *P. italicum* Wehmer, *Thielaviopsis paradoxa* (Dade) C. Moreau (1952), and *Botrytis cinerea* (De Bary) Whetzel, will be isolated from infected fruits, and maintained on potato dextrose agar (PDA) medium. The antifungal activity will be evaluated by agar dilution method. Clotrimazole will be used as positive control. All experiments will be performed in at least triplicate.

I. *In vitro* assay of COX-1/COX-2 inhibitory activity (duration 3 months)

The *in vitro* ability of the test compounds (CJF-EO loaded NPs, blank NPs and the CJF-EO) to inhibit COX-1 and COX-2 isoenzymes will be determined using a Cayman colorimetric COX (ovine and human) inhibitor screening assay kit (catalog number 701050, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Samples will be dissolved in DMSO, and aspirin will be used as a standard.

J. Antioxidant activity (duration 1 month)

Spectrophotometric method based on the inhibitory effects of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) will be carried out in flat-bottom 96-well plates, using a microplate reader, based on the methodology described by Ellman et al. (1961). Donepezil will be used as the standard. The IC_{50} values, which are the concentrations of the sample that inhibited 50% of

the enzyme activity will be determined. A similar method would be used to determine antioxidant activity, by measuring the ability of the EOs to quench the 2,2-Diphenyl-1-picrylhydrazyl stable free radical, also to be carried out in 96-well plates, using a microplate reader. The results would also be expressed in the form of IC_{50}.

K. *Artemia salina* toxicity assay (duration 6 months)

The toxicity of the NPs would also be tested, through a toxicity test in *Artemia salina*. *A. salina* cysts would be hatched in artificial seawater for 48 h, at 25 ºC. These nauplii would then be brought into contact with the CJF-EO loaded NPs, blank NPs and the CJF-EO, in a 96-well microplate. The microplate would then be incubated at 25 ºC, for 24 h. After incubation, the dead and total nauplii would be counted in each well, adjusting the mortality rate with the Abbott formula.

L. Statistical analysis and publication of data (duration 7 months)

All data obtained will be inspected for statistical distribution and individual outlying values. One-way analysis of variance (ANOVA) followed by multiple comparison test will be applied at a significance level of $p < 0.05$ to determine the significant differences among groups (chitosan NPs, CJF-EO-loaded chitosan NPs and pure CFJ-EO). All data analyses will be performed using the Microsoft excel or SPSS software. Subsequently, results will be published in high impact factor peer-reviewed journals. Also, should be done oral and poster presentations in international and national meetings.

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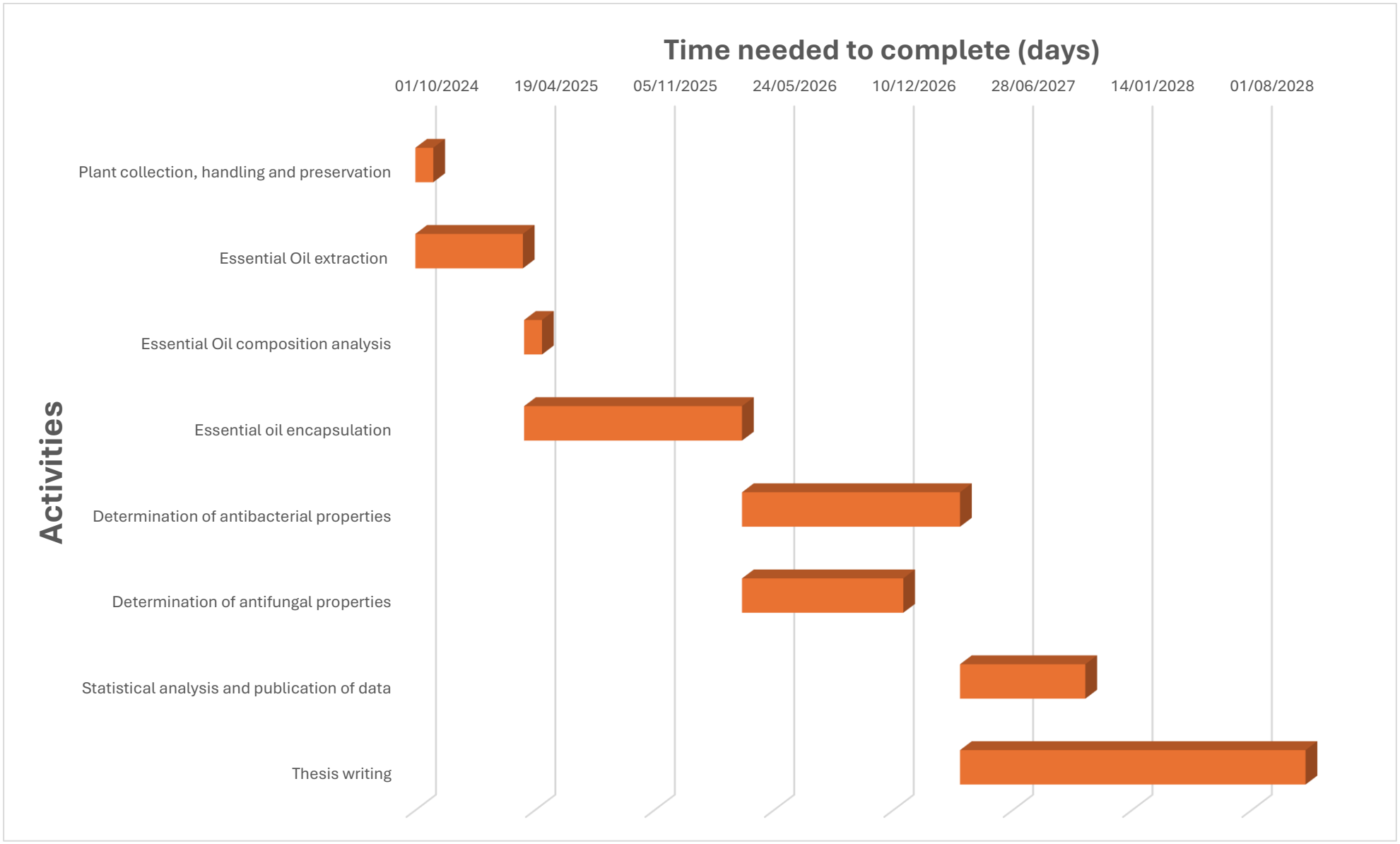
Time needed to complete (days)
01/10/2024
19/04/2025
05/11/2025
24/05/2026
10/12/2026
28/06/2027
14/01/2028
01/08/2028
Activities
Plant collection, handling and preservation
Essential Oil extraction
Essential Oil composition analysis
Essential oil encapsulation
Determination of antibacterial properties
Determination of antifungal properties
Statistical analysis and publication of data
Thesis writing