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GUILHERME PEREZ PINHEIRO

STUDY OF THE CHEMICAL PROFILE OF VOLATILES AND SECRETORY STRUCTURES OF *Plectranthus amboinicus* (Lour.) Spreng. - LAMIACEAE

ESTUDO DO PERFIL QUÍMICO DE VOLÁTEIS E ESTRUTURAS SECRETORAS DE *Plectranthus amboinicus* (Lour.) Spreng. - LAMIACEAE

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Dissertation presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Master, in the area of Plant Biology.

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Orientador: ALEXANDRA CHRISTINE HELENA FRANKLAND SAWAYA

Co-Orientador: JULIANA LISCHKA SAMPAIO MAYER

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RESUMO

Plectranthus amboinicus é uma espécie frequentemente utilizada na medicina popular brasileira e possui diversos usos etnobotânicos, como tratamento de resfriados e gripes, asma e inflamações. A revisão da literatura revela variações nos perfis químicos de *P. amboinicus*, recentemente discutidas como consequência das condições experimentais. O presente estudo avaliou a composição e atividade antioxidante do óleo essencial, bem como o perfil de voláteis durante um ano e a distribuição dos tricomas glandulares nas folhas da espécie. O principal componente do óleo essencial foi carvacrol, seguido pelo *p*-cimeno, *(E)*-cariofileno e *γ*-terpineno. Dois ensaios com diferentes mecanismos de ação foram utilizados para avaliar a atividade antioxidante do óleo essencial, a qual foi baixa a moderada no ensaio DPPH, corroborado pela técnica CCD, ao passo que o ensaio $ORAC_{FL}$ resultou em atividade antioxidante moderada. O perfil de voláteis demonstrou que folhas em diferentes estágios de desenvolvimento são quimicamente distintas enquanto os diferentes indivíduos apresentaram perfis de voláteis similares, portanto não foram observadas interações entre genótipo e ambiente. Terpinen-4-ol e outro componente minoritário não identificado (*mass feature* H) foram estatisticamente diferentes entre manhã e tarde e a maior parte dos componentes voláteis variaram entre os meses de coleta, com exceção do *γ*-terpineno, carvacrol e um componente minoritário não identificado (*mass feature* O). Nenhuma correlação entre precipitação e variação na composição foi observada e somente *p*-cimeno variou de acordo com a temperatura. Quatro tipos de tricomas capitados foram identificados em *P. amboinicus*, três tipos estavam presentes em todas as folhas enquanto o quarto tipo era predominante em folhas jovens, sugerindo que estas estruturas secretoras podem estar relacionadas com a variação na composição dos voláteis em folhas em diferentes estágios de desenvolvimento. Estes resultados fornecem novas informações acerca da química de *P. amboinicus* de acordo com fatores ambientais, atividade antioxidante do óleo essencial e anatomia da espécie.

Palavras-chave: Plantas medicinais; Metabolômica; Essências e óleos essenciais; Cromatografia gasosa; Tricomas; Compostos voláteis; Atividade antioxidante.

ABSTRACT

Plectranthus amboinicus is a species commonly used in Brazilian folk medicine and has several ethnobotanical uses, such as treatment of the common cold and the flu, asthma, inflammation, constipation, headache, cough, fever and skin diseases. A review of the literature reveals variations in the chemical profile of *P. amboinicus*, possibly as a consequence of the experimental conditions. The present study evaluated the essential oil composition and antioxidant activity, as well as the volatile profile during a year and the distribution of leaf glandular trichomes of the species. The main essential oil component was carvacrol, the most abundant component in area percentage, followed by *p*-cymene, *(E)*-caryophyllene and *γ*-terpinene. Two assays with different mechanisms of action were used to evaluate the essential oil's antioxidant activity, which was low to moderate in the DPPH assay, corroborated via the TLC technique, while the $ORAC_{FL}$ assay resulted in a moderate antioxidant activity. The leaf volatile profile showed that leaves in different stages of development are chemically distinct while the different individuals showed similar volatile profile, thus no underlying interaction between genotype and environment was observed. Terpinen-4-ol and the unidentified mass feature H, two minor components, were statistically different between morning and afternoon and most volatile components varied significantly among months of collection except for *γ*-terpinene, carvacrol and the unidentified mass feature O, a minor component. No correlation was observed between rainfall and the variation in composition and only *p*-cymene varied with the temperature. Four types of capitate trichomes were identified in *P. amboinicus*, three of them were present on every leaf while the fourth type was predominant on younger leaves, suggesting that these secretory structures may correlate to the variation in volatile composition of leaves in different stages of development. These results provided new insights about the chemistry of *P. amboinicus* according to environmental factors, the antioxidant activity of the essential oil and anatomy of this species.

Keywords: Medicinal plants; Metabolomics; Essences and essential oils; Gas chromatography; Trichomes; Volatile compounds; Antioxidant activity.

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SUMMARY

GENERAL INTRODUCTION

Since the beginning of mankind, people search for herbs that can cure diseases and relieve symptoms[1]. Pollen clusters discovered in a Neanderthal grave dating from 60,000 years ago are thought to be the oldest evidence of the use of medicinal plants[2,3], although the first written record describing this use in prescriptions dates from approximately 5,000 years ago[4]. It was only in the eleventh century that the first written compendium was created, the codex entitled Herbarium of Apuleius Platonicus which describes medicinal plants with illustrations and their respective uses[5]. However, the search for plants with therapeutic potential is not limited to remote times. In recent years, the increase in related scientific publication and the launch of new herbal medicines are a reflection of the growing demand for these products[6].

Natural products, such as plant secondary metabolites, were the sole source of therapeutic agents for centuries and have been the inspiration for drug development throughout history[7]. The cardiotonic glycosides, digoxin and digitoxin, extracted from *Digitalis purpurea* for the treatment of cardiac insufficiency[5], and quinine, extracted from *Cinchona* spp. for treatment of malaria[8], are examples of the discovery of bioactive compounds that follow a sequence of steps closely related to ethnobotany. At first, there is an accumulation of popular knowledge about the therapeutic activities of the plant, followed by traditional use by the local population and reporting of biological potentials to scientists. The specialists collect and identify the plant species, perform preliminary biological tests and later isolate and characterize the compounds of interest[5]. Thus, both the use of certain plant species for therapeutic purposes and the popular report of their activities are indicative of the medicinal potential of these plants, guiding future scientific research about them.

Numerous plants are popularly used for medicinal purposes around the globe. In Brazil, there is a great diversity of both native and exotic medicinal plant species, the Lamiaceae family stands out in number of representatives[9]. *Salvia* L. (*e.g.*

"sálvia"), *Ocimum* L. (*e.g.* "manjericão"), *Mentha* L. (*e.g.* "menta") and *Plectranthus* L'Hér. (*e.g.* "boldo") are examples of frequently used genera in Brazil, both in folk medicine and in cooking[10,11]. Of these, the *Plectranthus* genus has a remarkable diversity of ethnobotanical uses[11,12], which is the reason why it is considered of great therapeutic and economic potential[13], of interest for both researchers and agricultural producers. *Plectranthus amboinicus* (Lour.) Spreng. ("hortelã-graúda"), *Plectranthus barbatus* Andrews ("falso-boldo"), *Plectranthus neochilus* Schltr. ("boldo-gambá") e *Plectranthus ornatus* Codd ("boldo-miúdo") are the best known species in Brazil[10], *P. amboinicus* has different ethnobotanical uses from the other species[12].

Plectranthus amboinicus (Fig. 1) is popularly known as "malvarisco", "malvariço", "hortelã-graúda", "hortelã-pimenta" and "hortelã-gorda" in Brazil, and it is used both as a medicinal plant and as an unconventional edible plant[10,14]. The species is a perennial, succulent and pleasantly aromatic herb, up to 1.5 m in height, with ovate-deltoid leaves rich in glandular trichomes and inflorescence in long racemes of purple, lilac or whitish flowers[15,16].

Figure 1 - *Plectranthus amboinicus* (Lour.) Spreng. growing in the Experimental Field of the Institute of Biology, University of Campinas (São Paulo, Brazil).

The volatile compounds of *P. amboinicus* are responsible for its strong and pleasant odor, which is appreciated in cooking, as well as promoting the species' bioactivities[17]. In folk medicine, *P. amboinicus* is mainly used in infusions or syrups for

the treatment of the common cold and the flu, asthma, inflammation, constipation, headache, cough, fever and skin diseases[10,12]. These therapeutic properties are believed to be related to the antimicrobial and antioxidant activities of the essential oil, and to the anti-inflammatory activity of the extracts[17]. In addition to traditional uses, the potential of the species' essential oil as a mosquito repellent to *Aedes aegypti* has recently been described[18].

The use of *P. amboinicus* is not restricted to Brazil, the species is widely consumed around the world in countries like India, Cambodia and the USA, mainly in tropical and subtropical countries, being one of the best documented species of Lamiaceae[17]. Arumugam *et al.* (2016)[17] reviewed *P. amboinicus* literature and indicate that the chemical composition of the essential oil differs according to the region of the globe where the plant material was collected, differences which may explain divergent results regarding the bioactivities of this medicinal plant. Despite the large number of publications exploring the chemical composition and biological activities of *P. amboinicus*, the efforts to understand the variations found in the published results are scarce. Therefore, the present work evaluated the chemical composition of *P. amboinicus* volatiles of individuals obtained in different regions of São Paulo, the bioactivity of the essential oil and the leaf secretory structures containing this oil.

CHAPTER 1

CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF *Plectranthus amboinicus* (Lour.) Spreng. ESSENTIAL OIL FROM BRAZIL

Plants are capable of producing various organic compounds that apparently have no function in growth and development, and have restricted distribution in the plant kingdom[19]. These compounds, called secondary metabolites, which mediate plant defenses and ecological interactions, are the constituents of the essential oils produced by various taxonomic groups[20]. In addition to their physiological and ecological role for plants, these volatile essential oils are valued for their aromatic characteristics and therapeutic properties, being widely used as flavoring and medicine[21]. From the plants that produce essential oils, the Lamiaceae family stands out in relation to the number of studied species, which generally have anatomical structures capable of secreting and storing these oils[20,22].

As one of the best chemically documented Lamiaceae species, the volatile profile of *P. amboinicus* essential oil is characterized by the abundance of terpenoids, particularly the phenolic monoterpenes thymol and carvacrol[17]. Arumugam *et al.* (2016)[17] compiled a total of 76 volatiles in a review of publications from different places around the globe, however large variations are observed regarding the presence and abundance of compounds. Only 28 volatiles were identified in published studies with *P. amboinicus* individuals collected in northeast and southeast of Brazil[23–26]. Gobbo-Neto & Lopes (2007)[27] indicate that the variation in chemical composition of secondary

metabolites is a consequence of the interaction of intrinsic plant factors (*e.g.* genetics) with environmental factors such as seasonality, circadian cycle and nutrient availability.

P. amboinicus is used in Brazilian folk medicine to treat cough, sore throat, bronchitis, skin wounds, ovarian and uterine problems[10]. The essential oil from this species has therapeutic potential in the treatment of respiratory and skin diseases[17] due to its antimicrobial[23,25,28–31] and antioxidant[26,32] properties, which are a consequence of the abundance of thymol and carvacrol[33,34] and other components in lower concentration such as *p*-cymene, *γ*-terpinene, *β*-caryophyllene and *α*-humulene[23]. Both antimicrobial and antioxidant activities of this natural product are of great interest in the medical field in regard to the growing antimicrobial resistance and reactive oxygen species generated in the human organism, respectively[35,36].

Oxidative stress has been associated with degenerative and inflammatory diseases, becoming a global health issue[37]. Our bodies naturally produce a disproportional amount of reactive oxygen species (ROS) when compared to endogenous enzymatic and non-enzymatic antioxidants, thus the dietary intake of antioxidants is necessary and inversely proportional to the incidence of these diseases[38]. In this scenario, there has been an increased interest in natural antioxidants, such as plant extracts, due to their pharmacological potential and low or no side effects[39].

There are many available *in vitro* methods for measurement of antioxidant capacity with different reaction mechanisms, such as Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET)[40]. Prior *et al.* (2005)[40] indicate that HAT mechanisms are quite fast, but solvent and pH dependent, while SET mechanisms are frequently slower and trace components or contaminants can interfere, accounting for unreliable results. Thus, antioxidants may respond differently to assays with different reaction mechanisms, making it necessary to apply more than one reaction mechanism in order to evaluate the antioxidant potential of a natural product[41].

The present chapter aimed to assess the chemical profile of *P. amboinicus* essential oil from individuals growing in Brazil and evaluate its antioxidant potential. Comparisons with the literature were established for a better understanding of the influence of environmental variables and the interaction between genotype and

environment on the essential oil composition. In addition, antioxidant assays with different mechanisms of action and a chromatographic technique associated with the antioxidant assay were performed, providing new perspectives on the antioxidant potential of this medicinal plant.

1.1 Material and Methods

1.1.1 Plant Material

Plectranthus amboinicus individuals were collected at different sites in São Paulo state, propagated by cutting and planted in the experimental field of the Institute of Biology, University of Campinas (UNICAMP, Campinas-SP) in early 2018. Vouchers were deposited in the Unicamp Herbarium of individuals collected in: Mandala Space at UNICAMP, Campinas-SP (UEC 203557); Coordination of Integral Technical Assistance (CATI), Campinas-SP (UEC 203558); and Valinhos-SP (UEC 201900). A pool sample of fresh leaves from the three individuals was collected in September 2019, from which essential oil was extracted by hydrodistillation using Clevenger-type apparatus for 3 hours and the oil was subsequently stored in amber vials in a freezer. The essential oil yield was calculated at the end of extraction based on total weight of fresh leaves.

1.1.2 Essential Oil Analysis

The essential oil sample was diluted in ethyl acetate 1:10 (v / v) and analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Analysis was performed on a Thermo Scientific™ Model TRACE 1300 Gas Chromatograph, fitted with a HP-5ms fused silica capillary column (Agilent J&W, USA - 30 m x 0.25 mm x 0.25 µm film thickness) and coupled to a Thermo Scientific™ mass spectrometer model ISQ QD. The following conditions were used for essential oil analysis: injector temperature at 220°C, transfer line temperature at 250°C, oven temperature programmed from 60°C to 246°C at 3°C/min, carrier gas He at 1.00 mL/min, injection volume of 0.50 µL, split 1:400, solvent delay 2.00 min and acquisition mode full scan (50 - 600 *m/z*). An external standard of n-alkanes (C8-C20, Sigma-Aldrich) was analyzed in the same conditions for

calculation of the linear retention index. Chemical compounds were identified through mass spectra comparison with NIST Library and retention index comparison with Adams (2017)[42].

1.1.3 Rapid Screening of Components with Radical Scavenging Capacity

Thin-layer chromatography (TLC) was performed for rapid screening of essential oil components that have antioxidant activity through radical scavenging capacity. The essential oil was diluted in hexane 1:10 (v/v) and 3 µL of this solution was applied to silica gel 60 F254 plates (Merck, USA). The elution was realized with hexane - ethyl acetate 93:7 (v/v) as mobile phase and the plates were revealed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) ethanolic solution (45 µM), acid solution of *p*-anisaldehyde 0.97% and UV-light (254 nm and 365 nm).

1.1.4 Radical Scavenging Capacity

Antioxidant activity was evaluated by radical scavenging capacity using the DPPH radical. The DPPH assay, a SET based method, was performed in triplicate according to Mimica-Dukic *et al.* (2004)[43], with the following modifications: 96-well plate assay with ethanol as solvent; sample concentrations ranging from 2 µg/mL to 1000 µg/mL, obtained by serial dilution, quercetin as positive control and the DPPH solution as blank. The absorbance decay was measured in microplate reader Molecular Devices SpectraMax M3 at 515 nm, with readings every 10 minutes until reaction stabilization. Radical scavenging capacity was calculated according to the following equation:

$$RSC_{\%} = 100 \times (Absorbance_{blank} - Absorbance_{sample}) / Absorbance_{blank}$$

Linear regression was used for estimation of the inhibitory concentration of 50% of the DPPH radical (IC_{50}).

1.1.5 Oxygen Radical Absorbance Capacity

Antioxidant activity was evaluated by oxygen radical absorbance capacity ($ORAC_{FL}$), a HAT based method, in an assay using Fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one) as a fluorescent probe, AAPH (2,2′-azobis(2- amidinopropane) dihydrochloride) as radical source, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as reference substance. The assays were performed in 96-well microplates according to Ou *et al.* (2001)[44] and Prior *et al.* (2003)[45], with sample dilution (v/v) ranging from 1:1200 up to 1:9600.

1.2 Results and Discussion

The essential oil yield was approximately 0.06 % in terms of leaf fresh weight and the identified components are described in Table 1, as well as the respective chromatogram (Figure 2). The most abundant component is carvacrol representing 57.67 % of total peak area, followed by *p*-cymene (13.08 %), *(E)*-caryophyllene (8.42 %) and *γ*-terpinene (6.76 %). Components eluted after 30 minutes were not identified since both sample and n-alkane standard chromatograms showed similar profile (Figure 2), although quantitatively different, due to a possible column contamination.

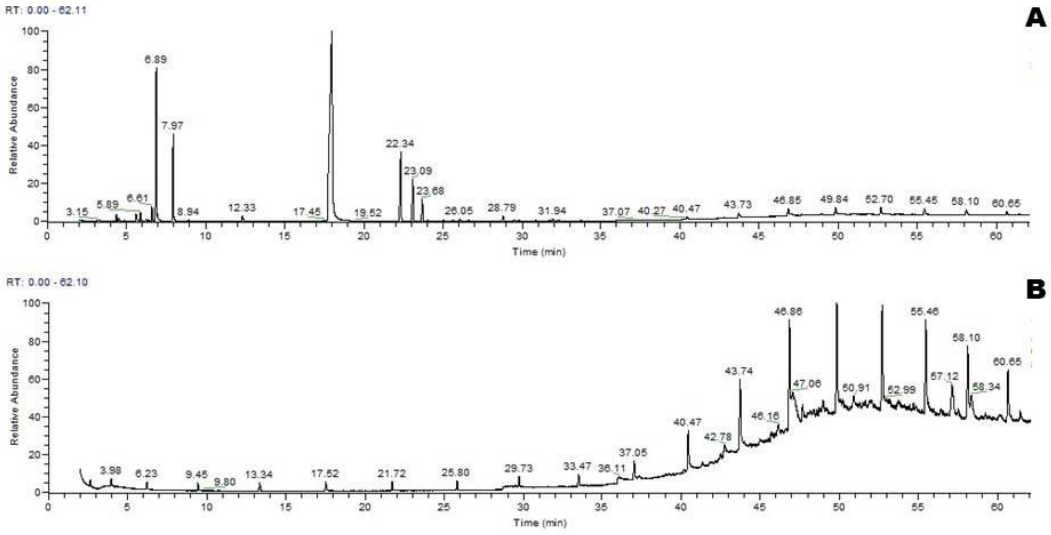

Figure 2 - Chromatograms of *Plectranthus amboinicus* essential oil (A) and n-alkane standard (B) obtained via GC-MS analysis.

Table 1. Composition of *Plectranthus amboinicus* essential oil analyzed by GC-MS.

RT (min)	RI*	Name	Area (%)
4.37	924	*α*-Thujene	0.53
4.52	932	*α*-Pinene	0.32
5.61	974	1-Octen-3-ol	0.89
5.89	974	*β*-Pinene	0.81
6.61	1014	*α*-Terpinene	1.26
6.89	1020	*p*-Cymene	13.08
7.97	1054	*γ*-Terpinene	6.76
12.33	1174	Terpinen-4-ol	0.77
17.99	1298	Carvacrol	57.67
22.34	1417	*(E)*-Caryophyllene	8.42
23.09	1432	*α-trans*-Bergamotene	4.63
23.68	1452	*α*-Humulene	2.26
25.64	1500	*α*-Muurolene	0.15
26.05	1505	*β*-Bisabolene	0.25
28.79	1582	Caryophyllene oxide	0.57
		Monoterpenes	81.21
		Sesquiterpenes	16.28
		Oxygenated Compounds	59.90
		Total Identified Compounds	98.38

*Retention Indexes from Adams (2017)[42].

The essential oil composition described in this chapter is similar to a previous publication of our research group[46], as well as in other investigations of *P. amboinicus* plants growing in Brazil[25,26], indicating that carvacrol, *p*-cymene, *γ*-terpinene and *(E)*-caryophyllene represent the majority of total peak area (Table 1). Although Pinheiro *et al.* (2019)[46] described a total of 25 identified compounds, in the present study a higher

split ratio and a lower injection volume were used for better chromatographic resolution. Generally, *P. amboinicus* essential oil is rich in phenolic monoterpenes carvacrol and/or thymol, depending on the assessed literature[17]. These terpenes are isomers and present close retention indexes[42], making it difficult to separate them along the chromatographic process. As examples, Bezerra *et al.* (2010)[26] reported that carvacrol was the main peak in area percentage using individuals collected in Brazil, while Senthilkumar *et al.* (2010)[47] indicate both thymol and carvacrol with similar area percentage using individuals collected in India. In this study, only carvacrol was identified as a single chromatographic peak and thymol, if present, was below detection limit.

A preliminary screening of essential oil components that have antioxidant activity was performed using TLC (Figure 3). The acid solution of *p*-anisaldehyde as well as the 254 nm UV light were efficient chemical and physical visualization methods, respectively, whereas the 365 nm UV light resulted in no visible bands. Although several components were observed (Figure 3B and 3C), only one spot presented antioxidant activity (Figure 3A). A quercetin solution was used as positive control for the DPPH solution. This compound was observed as a single spot in all visualization methods.

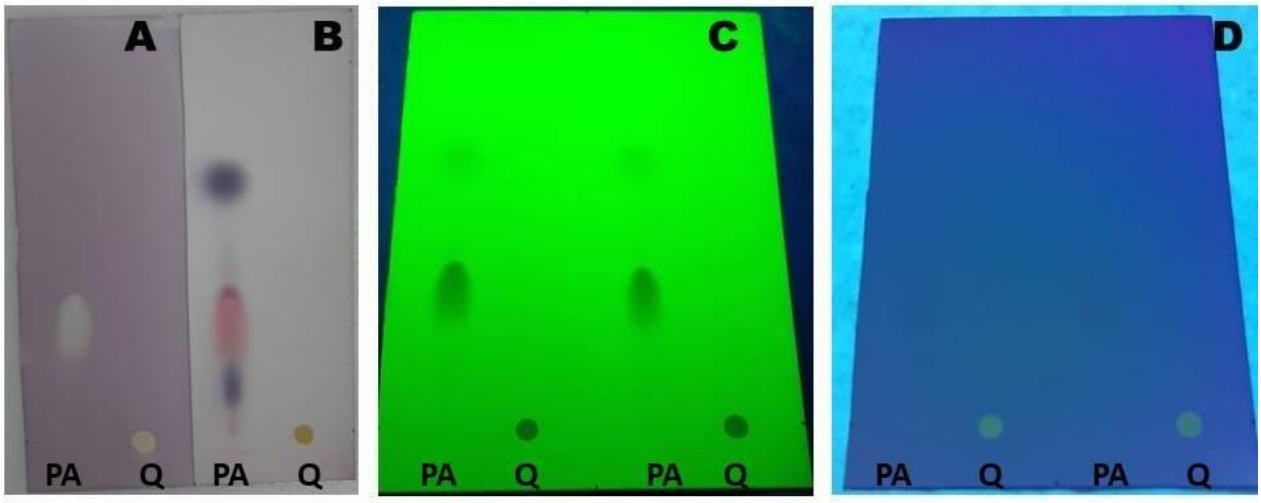

Figure 3 - TLC analysis of *Plectranthus amboinicus* essential oil (PA) and Quercetin (Q) visualized using: DPPH solution (A), acid solution of p-anisaldehyde (B), 254 nm UV light (C) and 365 nm UV light (D).

The antioxidant activity of *P. amboinicus* essential oil was evaluated with two assays with different mechanisms of action. The DPPH assays were performed twice, an exploratory assay utilizing essential oil concentrations ranging from 2 µg/mL to 1000 µg/mL, and a second assay ranging from 100 µg/mL to 1000 µg/mL (Figure 4). The IC_{50} of the essential oil was approximately 396.7 µg/mL whilst the IC_{50} of the quercetin control was approximately 1.4 µg/mL. An exploratory assay for $ORAC_{FL}$ was also performed, the final assay was done using the essential oil dilutions (v/v) 1:1200, 1:2400, 1:4800 and 1:9600 while the dilution 1:4800 was used for antioxidant activity estimation because of curve fitting of the protocol. The essential oil presented an antioxidant activity of approximately 1736.8±2.1 µmol Trolox equivalent (TE)/mL.

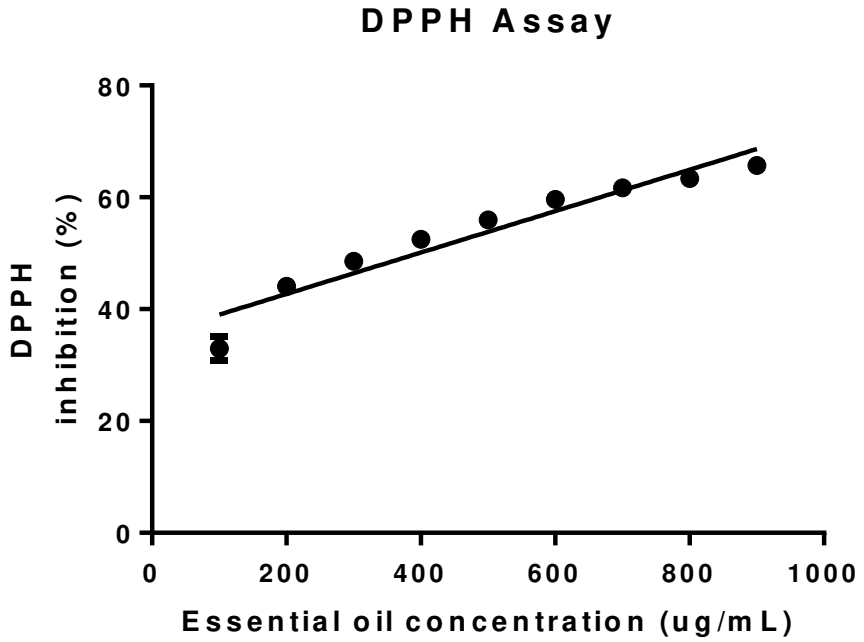

Figure 4 - Linear regression of the DPPH assay of *Plectranthus amboinicus* essential oil (linear equation: Y = 0,03703*X + 35,31; R^2 = 0,9225).

TLC was performed to screen for essential oil components with antioxidant potential. This technique has been used as a fast method to determine the chemical composition and biological activity of essential oil components[48]. A single spot showed antioxidant activity against the DPPH radical, indicating that a single compound or a

group of a few components are responsible for the antioxidant activity of the whole essential oil. Arumugam et al. (2017)[17] speculate that the biological activities of *P. amboinicus* essential oil is due to its abundance of bioactive components such as carvacrol, thymol and β-caryophyllene. However, no standards for the components was evaluated in the experiments.

The DPPH assay of the essential oil resulted in a IC_{50} of 396.7 µg/mL. Similarly, Bezerra *et al.* (2017)[26] reported values between 125.0 and 325.1 ppm (equivalent to µg/mL), depending on the month of collection of the leaves, while León Méndez (2015)[49] reported approximately 327.5 µg/mL. These results indicate that *P. amboinicus* presents a low to moderate antioxidant activity evaluated by the DPPH method. This result is corroborated by the previously discussed TLC result that only a fraction of the whole essential oil presents antioxidant activity. As the DPPH method evaluates SET, generally phenolic components, such as carvacrol, are responsible for this activity. However, Mimica-Dukic et al. (2004)[43], from which the DPPH assay protocol was adapted in this study, evaluated the essential oil of *Melissa officinalis* L. (Lamiaceae) and indicated a IC_{50} of approximately 7.6 µg/mL. The most powerful scavenging compounds identified were monoterpene aldehydes, ketones and sesquiterpene hydrocarbons such as (E)-caryophyllene, which is also present in the *P. amboinicus* essential oil analyzed herein.

The $ORAC_{FL}$ assay resulted in an antioxidant activity of approximately 1736.8 µmol TE/mL. This assay is usually applied to food products, such as juices and plant extracts[45]. Dudonné *et al.* (2009)[50] evaluated 30 aqueous plant extracts and reported values between 183 µmol TE/mL and 8515 µmol TE/mL. The results found in this study indicate that the *P. amboinicus* essential oil presents a moderate antioxidant activity evaluated by the $ORAC_{FL}$ assay, a HAT based method. Sharifi-Rad *et al.* (2018)[34] described that carvacrol, the main component of *P. amboinicus* essential oil evaluated in this study, presents higher *in vitro* antioxidant activity than most common volatile components of essential oils. Hence, carvacrol may be responsible for most of the antioxidant activity of *P. amboinicus* essential oil.

Therefore, the essential oil of *P. amboinicus* contains components that present antioxidant activity by both HAT and SET mechanisms. This corroborates its

popular use to treat cough, sore throat, bronchitis and other inflammatory diseases, as this antioxidant activity helps the patient to overcome the excess of ROS liberated under these conditions.

1.3 Conclusion

Plectranthus amboinicus essential oil presented a similar composition to those reported in other studies of plants growing in Brazil, with carvacrol being the most abundant component in area percentage and containing other major components such as *p*-cymene, *(E)*-caryophyllene and *γ*-terpinene. The essential oil presented a low to moderate antioxidant activity in the DPPH assay which was corroborated via the TLC technique, whilst the $ORAC_{FL}$ assay resulted in a moderate antioxidant activity. These results suggest that the antioxidant activity of *P. amboinicus* essential oil presents more than one mechanism of action, corroborating its popular use to treat colds and other inflammatory diseases.

CHAPTER 2

INFLUENCE OF ENVIRONMENTAL FACTORS ON THE VOLATILE PROFILE OF *Plectranthus amboinicus* (Lour.) Spreng.: AN UNTARGETED METABOLOMICS APPROACH

Norms for collecting medicinal and toxic plants have been reported since the 4th century B.C., like the Greek executioners who collected hemlock (*Conium maculatum* L.) in the morning, probably due to the greater potency of the poison. In the 1960s, it was proven that in the early hours of the day there is an accumulation of the alkaloid conine, responsible for the toxicity of the species[51]. Currently, it is known that the concentration of secondary metabolites in plants, compounds responsible for medicinal or toxic properties, are influenced by environmental and genetic factors[27], although the relationship is still unclear for most species used by man.

In the literature on medicinal plants, authors often report conflicting results regarding the composition and concentration of secondary metabolites. As previously seen in the Chapter 1, the chemical profile of *Plectranthus amboinicus* varies widely according to the sampling place[17]. Official documents such as the Brazilian Pharmacopoeia[52], seek to standardize the concentration of key compounds of medicinal species as a way of guaranteeing the bioactivity of the medicinal plant, however *P. amboinicus* is not included in these references. Seasonal variations are observed in the composition and biological activities of essential oil of the species[26,53,54], nonetheless more extensive studies, with a metabolomics approach, are necessary to understand the

influence of environmental and intrinsic factors of individuals on the chemical composition of *P. amboinicus* volatiles.

Metabolomics is one of the areas of systems biology that focuses on the holistic study of low molecular mass metabolites, starting from limited biological knowledge, through the analysis of a large number of samples in order to quickly observe disturbances in the metabolome[55]. The metabolomic analyses are divided into targeted metabolomics, in which metabolites or classes of target metabolites are selected, and untargeted metabolomics, which is based on the analysis of the largest possible number of metabolites[56]. The untargeted approach associated with chemometric data analysis has been used in investigations about the influence of environmental factors on the chemical composition of medicinal plants and is presented as an indispensable tool in the study of *P. amboinicus*.

The phytochemistry of *P. amboinicus* is extensively investigated in the literature with a focus on the chemical composition of essential oil. The low yield of the extraction of this product makes more extensive approaches such as metabolomics difficult, given the large amount of plant material required to obtain a few µL of oil[26]. In recent years, there has been an analytical trend towards simplification in sample preparation. The headspace solid phase microextraction (HS-SPME) technique stands out for the analysis of volatiles and metabolomics studies of several plant species[57]. In addition to requiring very little sample material, the technique integrates the extraction process with the concentration and transfer of the sample to the GC injector[58]; however, some aspects of optimization such as choosing the appropriate microextraction fiber must be taken into account.

The present chapter describes, through an untargeted metabolomic approach, the evaluation of the influence of environmental factors (*i.e.* rainfall and temperature) on the volatile profile of different individuals of *P. amboinicus*. HS-SPME combined with GC-MS were used to determine the volatile profile of *P. amboinicus* of (i) leaves in different stages of development, (ii) individuals collected at different sites in the Metropolitan Region of Campinas (São Paulo, Brazil), (iii) leaves sampled at different hours of the day and (iv) leaves sampled in different months of the year. Untargeted

metabolomic analyses and chemometrics were applied to compare the volatile profiles and identify the chemical variability related to these factors.

2.1 Material and Methods

2.1.1 Plant Material and Sampling

Monthly collections of *P. amboinicus* leaves, pool sampling of both expanding and expanded leaves, were performed twice on the same day (early morning and early afternoon), from each of the following individuals obtained from Mandala Space at UNICAMP, Campinas-SP (UEC 203557); Coordination of Integral Technical Assistance (CATI), Campinas-SP (UEC 203558); and Valinhos-SP (UEC 201900). The procedure was repeated over a year (June 2018 to May 2019). Samples of leaves in seven different stages of development, from the first exposed leaf up to the seventh leaf, were collected separately at the end of the experiment, pooling the leaves of the four individuals. The collected leaves were immediately frozen in liquid nitrogen and later stored in a biofreezer (-80 °C). All samples were analyzed in sequence at the end of the collections so that any variations in the equipment did not interfere with the results. A total of 79 samples were obtained and subsequently analyzed in duplicate, in addition to Quality control (QC) samples that were made with equal parts of each sample as a way to guarantee the quality of the metabolomic analyses.

2.1.2 HS-SPME/GC-MS Analysis

Frozen *P. amboinicus* leaves were ground with liquid nitrogen and 0.5 g was transferred to 20 mL SPME vials for each sample, which were immediately closed and stored at -80°C until the extraction. Each vial was incubated at 50°C for 5.00 min and the HS-SPME was conducted at the same temperature for 10.00 min using divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS - 50/30 µm) fiber assembly obtained from Supelco, Bellefonte, PA, USA. The choice of fiber was based on a previous study by Pinheiro *et al.* (2019)[46].

The GC-MS analyses were performed on a Thermo Scientific™ TRACE 1300 gas chromatographer fitted with a HP-5ms fused silica capillary column (Agilent J&W, USA - 30 m × 0.25 mm × 0.25 µm film thickness) and coupled to a Thermo Scientific™ ISQ QD mass spectrometer. The following GC-MS operating conditions were used: injector temperature at 220°C, transfer line at 240°C, oven program from 60°C up to 246°C at 3°C/min, carrier gas used was hydrogen at 1.00 mL/min, split ratio of 1:20 and the mass spectrometer acquisition mode was full scan (40-500 m/z).

2.1.3 Data Processing and Feature Identification

The raw GC-MS data were preprocessed through XCMS package[59–61] in R software (Version 3.6.2) using the parameters optimized via IPO package[62] (Table 2). The detected mass features were compared to the mass spectra from the original chromatographic peaks and only those representing the base peak were used. If no mass feature from a chromatographic peak met this condition, the mass feature with the highest abundance was selected. The selected mass features were identified by mass spectra comparison with NIST Library version 11 and with the previous study by Pinheiro *et al.* (2019)[46].

2.1.4 Chemometrics and Statistical Analysis

The filtered mass features obtained via XCMS processing were normalized by sum and auto-scaled, then the Principal Component Analysis (PCA), heatmaps and statistical analysis (*i.e.* t-test and ANOVA) were performed using the MetaboAnalyst online software[63]. The analysis of leaves in different stages of development was performed in duplicate, the mean of the duplicate was used as a third replicate for each sample in order to statistically compare the groups via ANOVA. GraphPad software (version 6) was used in order to establish a comparison of climate variables between months and period of the day via t-test or ANOVA with Tukey's multiple comparison test, as well as the correlation between mass features and these climate variables via Pearson correlation coefficient with the raw (not normalized) data. The climate data were provided by the Centro de Pesquisas Meteorológicas e Climáticas Aplicadas à Agricultura (CEPAGRI/UNICAMP).

2.2 Results and Discussion

The XCMS parameters optimized via IPO package are described in Table 2. The data was subsequently preprocessed via XCMS package. The analyses of leaves in different stages of development generated a total of 2,162 mass features, which resulted in 30 valid mass features (Table 3). A total of 2,768 mass features were detected in the monthly collection analyses, resulting in 17 valid mass features after comparison to the mass spectra and chromatograms (Table 4).

Table 2 - Optimized XCMS parameters via IPO package in R software for analysis of *Plectranthus amboinicus* volatiles obtained via HS-SPME/GC-MS.

Untargeted Metabolomics		Leaves in Different Stages of Development	Monthly Collection Analyses
Feature Detection	Method	matchedFilter	matchedFilter
	FWHM	1.0	1.0
	step	0.21	0.285
Retention Time Correction	Method	obiwarp	obiwarp
	profStep	0.99	0.99
Alignment	bw	0.25	0.25
	minfrac	0.3	0.5
	mzwid	0.0194	1.0

The valid mass features from both experiments were analyzed via MetaboAnalyst applying normalization by sum and auto-scaling parameters. PCAs were performed in order to compare the three individuals obtained in different sites, period of the day and months within each experiment and heatmaps were created to complement and facilitate visualization of the results.

Table 3 - Detected valid mass features via XCMS package (R software) from *Plectranthus amboinicus* leaf volatile analyses of leaves in different stages of development (mass features are identified as fragment_retention time).

Leaves in Different Stages of Development		
Number	**Feature**	**Identification**
1	102_1.82	Butanoic acid, 2-methyl, ethyl ester*
2	93_2.71	*β*-Thujene*
3	93_2.81	*α*-Pinene**
4	57_3.60	1-Octen-3-ol**
5	93_3.82	Myrcene**
6	59_3.94	3-Octanol*
7	91_4.62	*p*-Cymene*
8	91_4.70	**NI**
9	77_4.73	**NI**
10	118_4.86	**NI**
11	93_5.42	*γ*-Terpinene**
12	93_5.66	**NI**
13	71_6.51	**NI**
14	73_8.72	**NI**
15	71_9.06	Terpinen-4-ol**
16	151_11.81	Thymoquinone**
17	135_14.17	Carvacrol**
18	73_15.50	**NI**
19	206_17.53	**NI**
20	96_18.04	**NI**
21	93_18.38	*(E)*-Caryophyllene**
22	109_18.63	**NI**
23	109_18.73	**NI**
24	119_19.22	*α*-trans-Bergamotene**
25	123_19.40	**NI**
26	93_19.68	*α*-Humulene**
27	191_20.74	**NI**
28	161_21.62	*α*-Muurolene**
29	151_24.37	**NI**
30	131_24.59	Caryophyllene oxide**

* Identified by comparison with NIST library.

** Identified by comparison with NIST library and Pinheiro *et al.* (2019)[46].

NI Unidentified mass features.

Almost every mass feature varied significantly between the leaves in different stages of development, with the exception of mass features number 12 (93_5.66) and 25 (123_19.40). The PCA (Figure 5A) reflects the modification in the volatile composition going from the left to the right along PC1, with L1 on the far left and L7 on the far right. The biplot (Figure 5B) shows which mass features promote this grouping. However, the heatmap (Figure 6) shows more clearly which compounds are more intense in each stage of leaf development. Hierarchical grouping of L2 and L3, which alongside L1 represent the young leaves, while the mature leaves are placed in two sub-groups: L4 - L5 and L6 - L7.

These results show that volatiles are present in *P. amboinicus* leaves in all stages of development, but their composition varies significantly between stages. Both mass features number 12 and 25, which are statistically similar in all stages, are minor components of the chemical profile. As indicated in Chapter 1 and in the reviewed bibliography[17,23,25,26,46], carvacrol is the main component of the leaf volatile profile and gives the essential oil most of its biological activity. This compound differed between stages of leaf development and is more intense in older leaves (Figure 6). As the essential oil contained in leaves in different stages of development varied in composition, its biological activities may not be the same. The variation in volatile profile may be partially due to differences in the glandular trichomes of the leaves, which will be discussed in Chapter 3. Although the samples for this analysis were collected at the end of the experiment, in the monthly collection it was previously established that several leaves in different stages of development would be collected from each individual, avoiding this type of variation between samples.

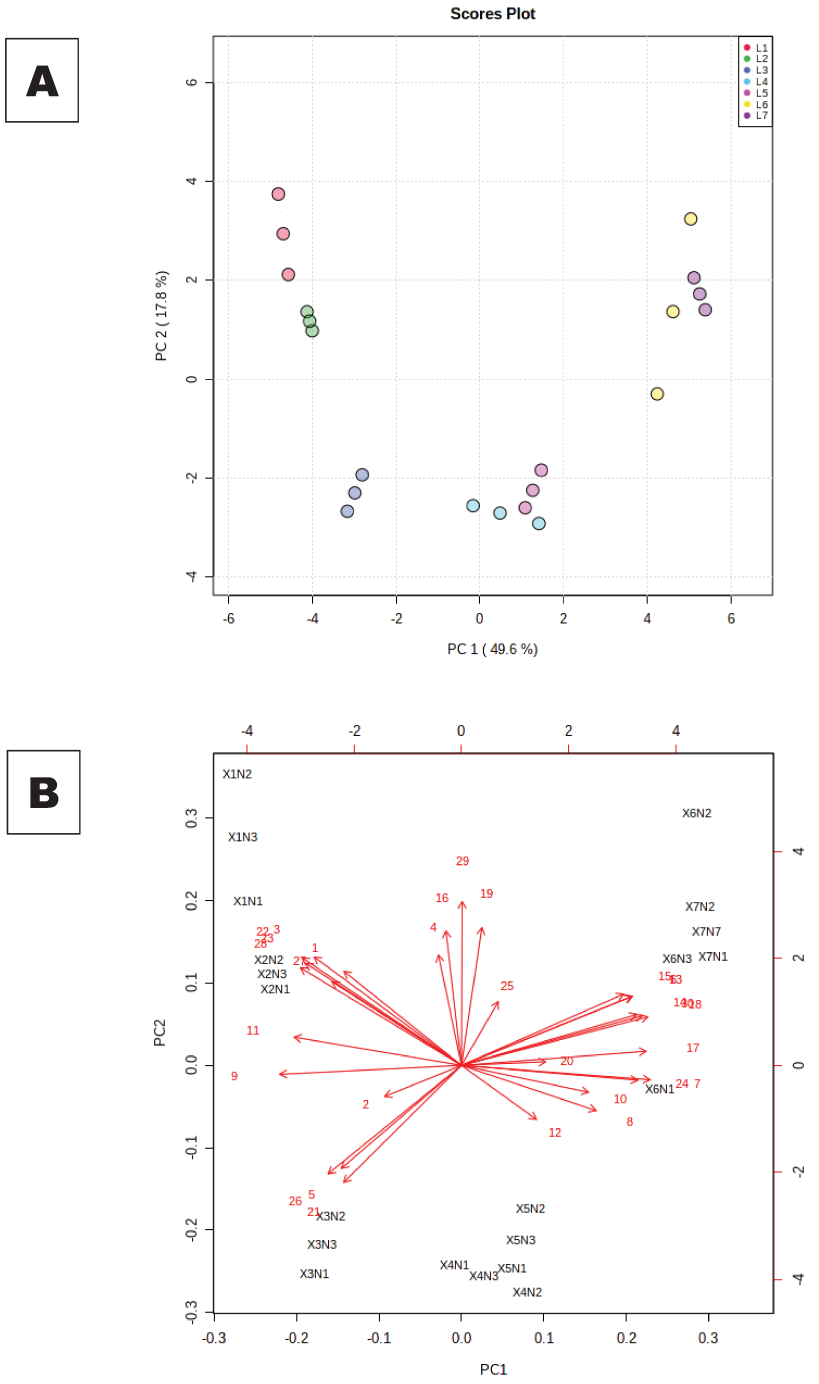

Figure 5 - Principal Component Analysis (PCA) 2D scores plot (A) and biplot (B) of *Plectranthus amboinicus* volatiles from leaves in different stages of development (L1-7, ranging from emerging to expanded leaves, respectively). Numbers (red) indicate each feature while letters and numbers (black) indicate each sample (B).

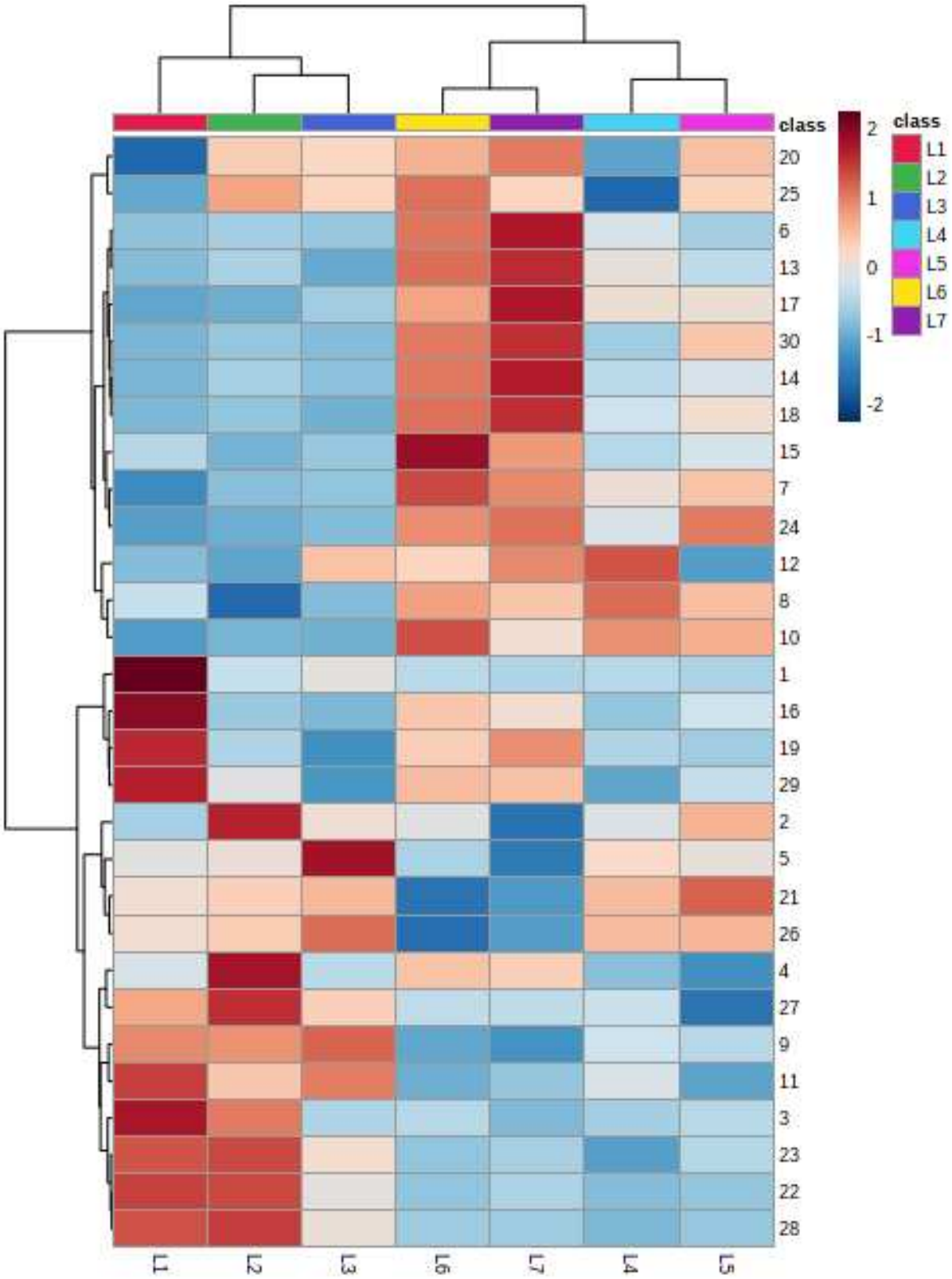

Figure 6 - Heatmap of *Plectranthus amboinicus* volatiles from leaves of different stages of development using all the valid mass features (1 - 30 as in Table 3).

Table 4 - Detected valid mass features via XCMS package (R software) from *Plectranthus amboinicus* leaf volatile analyses of monthly collection of leaves (mass features are identified as fragment_retention time).

Monthly Collection Analyses		
Letter	**Feature**	**Identification**
A	93_2.71	*β*-Thujene*
B	93_2.81	*α*-Pinene**
C	57_3.61	1-Octen-3-ol**
D	93_3.82	Myrcene**
E	59_3.93	3-Octanol*
F	119_4.58	*p*-Cymene*
G	93_5.39	*γ*-Terpinene**
H	121_5.61	**NI**
I	93_9.06	Terpinen-4-ol**
J	135_14.16	Carvacrol**
K	124_17.56	**NI**
L	93_18.27	*(E)*-Caryophyllene**
M	109_18.64	**NI**
N	93_19.13	*α*-trans-Bergamotene**
O	123_19.36	**NI**
P	93_19.60	*α*-Humulene**
Q	131_24.59	Caryophyllene oxide**

* Identified by comparison with NIST library.

** Identified by comparison with NIST library and Pinheiro *et al.* (2019)[46].

NI Unidentified mass features.

For the monthly collection analyses, using a pool of expanding and expanded leaves, the same mass features were analyzed by MetaboAnalyst using different grouping parameters. Initially the analyses were executed labeling the individuals. The comparison between individuals showed no statistical difference (ANOVA) and no grouping tendency in the PCA (Figure 7). This result indicated that the analyzed individuals did not present very different chemical phenotypes (chemotypes), eliminating possible interaction between genotype and environment that may explain differences in the volatile profile.

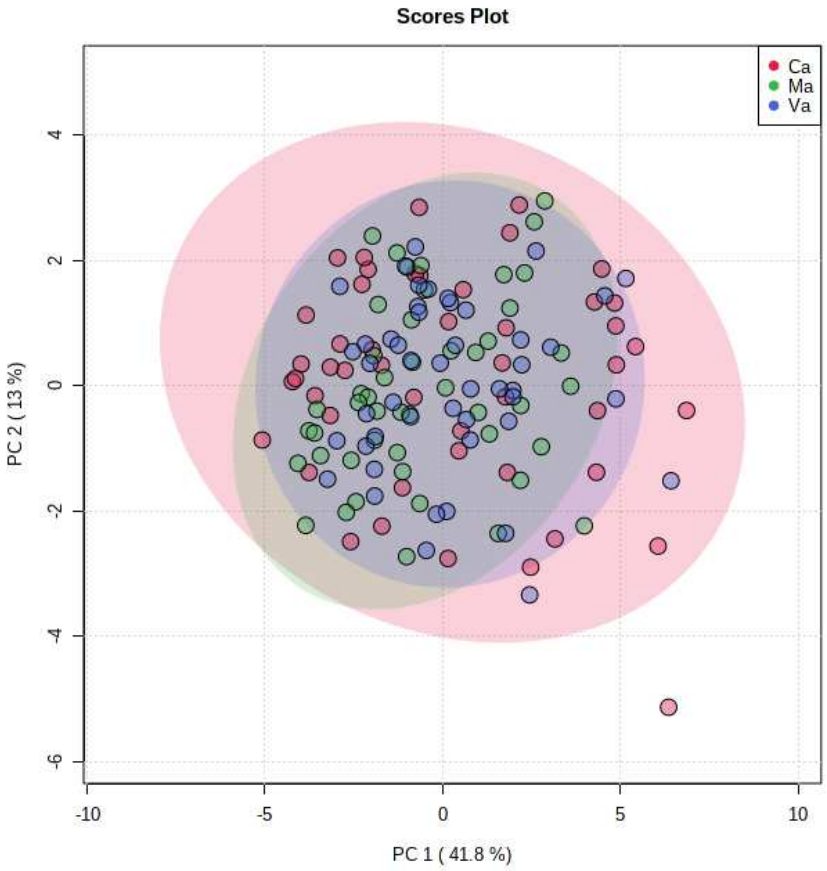

Figure 7 - Principal Component Analysis (PCA) 2D scores plot of *Plectranthus amboinicus* volatiles from leaves of individuals originally from: Ma - Mandala Space at UNICAMP, Ca - CATI- Campinas-SP and Va - Valinhos-SP).

The same results were then labeled as morning or afternoon, irrespective of which individual plant they derived from. Although no clear groups were observed in the PCA (Figure 8A), the t-test indicated a significant difference between morning and afternoon (Figure 8B) for the mass features H (Unidentified mass feature - NI) and I (terpinen-4-ol), which are minor components of the volatile profile.

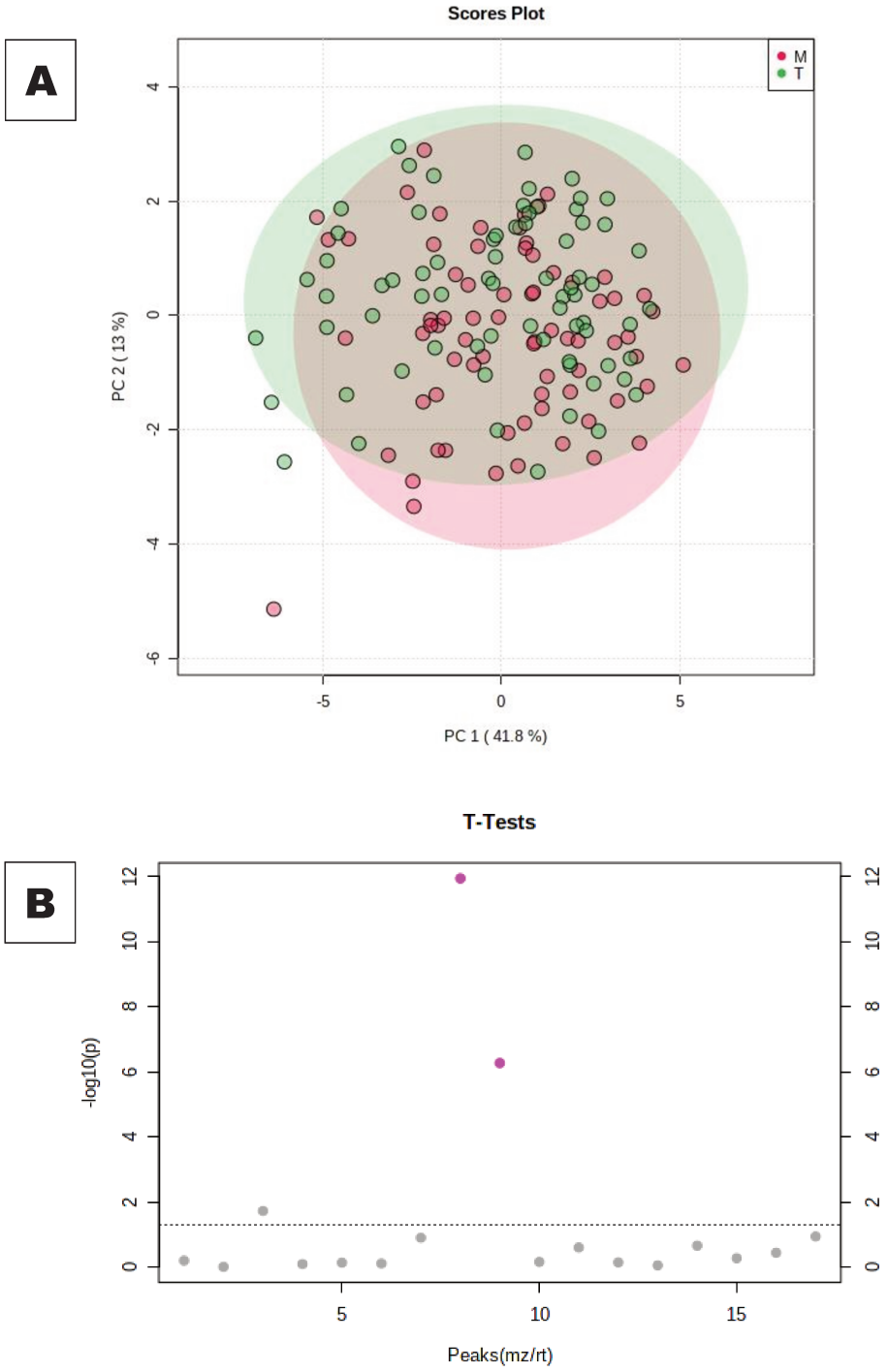

Figure 8 - Principal Component Analysis (PCA) 2D scores plot (A) and T-test (B) of *Plectranthus amboinicus* volatiles from leaves collected in the M - morning and T - afternoon.

As no significant difference was observed between individuals, these were grouped to observe if there was a monthly variation. In this way almost every mass feature varied significantly between months, with exception of the mass features G (γ-terpinene), J (carvacrol) and O (NI). The heatmap shown in Figure 9 indicates two main groups; one comprising samples collected from January (Jan) to June (Jun) and another from July (Jul) to December (Dez). In general, mass features were generally more intense in the samples collected from January to June.

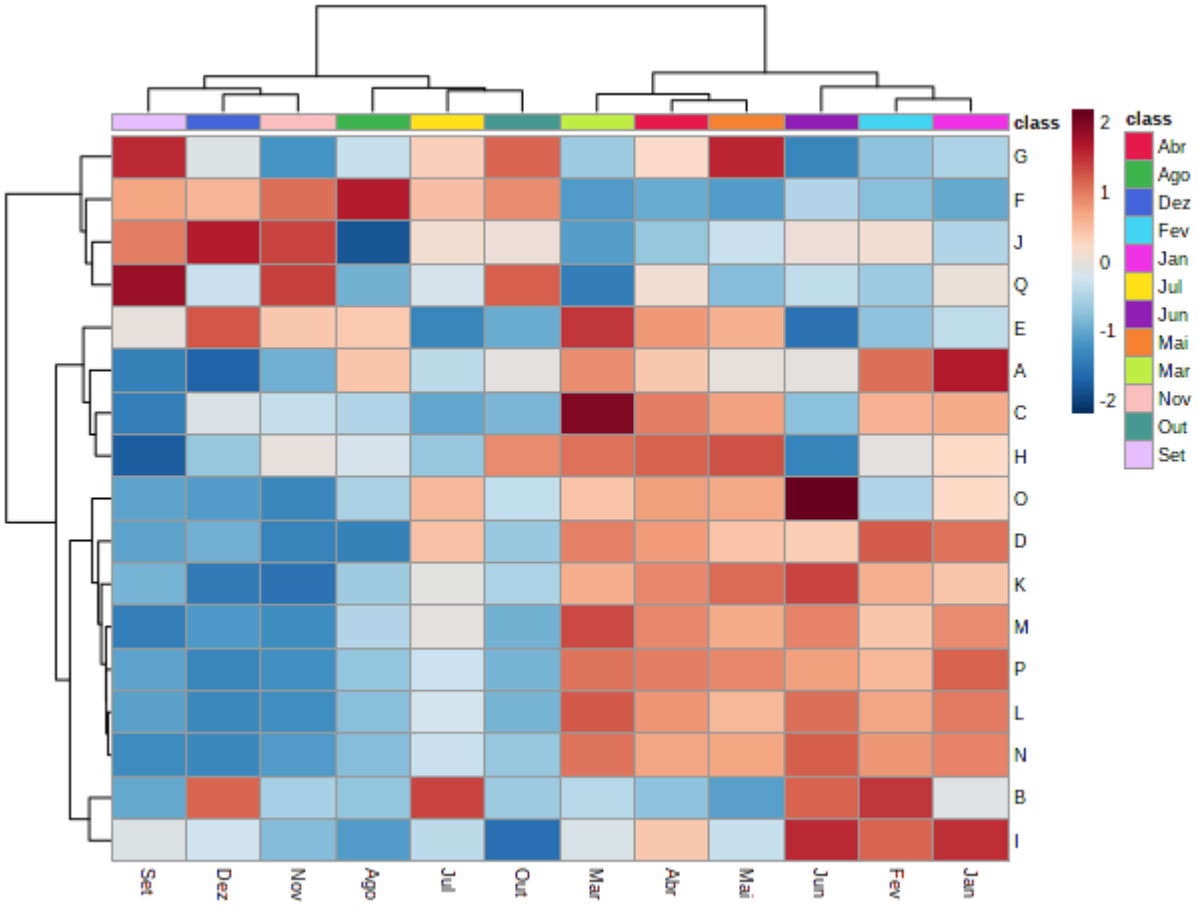

Figure 9 - Heatmap of *Plectranthus amboinicus* volatiles from leaves collected every month over one year using all the valid mass features (A - Q as in Table 4).

With regard to the mass features that did not differ between months, γ-terpinene and mass feature O (NI) are minor components of the volatile profile, whereas carvacrol is the main volatile component. Carvacrol, as a targeted compound for *P.*

amboinicus, which may be used for standardization of the natural product, would indicate that essential oil was similar in all evaluated months, although the untargeted approach of the volatile profile shows that there was variation along a year. The use of sum normalization and autoscaling reduces the influence of the intensity of peaks on the result, that is, the variation of major and minor components is equally considered. Therefore, it is clear that in spite of the monthly variation in composition, carvacrol, an important volatile and bioactive component, is present throughout the year and can be considered a marker for this species. Bezerra *et al.* (2017)[26] published one of the few seasonal studies of *P. amboinicus* essential oil from individuals collected in northeast Brazil, which showed that most chemical components varied between seasons, although no statistical analyses nor correlations were established, and the variation culminated in difference in antioxidant activity. Besides carvacrol, other essential components may account for its biological activity either by their individual biological potential or acting synergistically.

The month with the highest mean daily precipitation was November 2018 (9.99±18.66 mm), although the standard deviation was also the highest amongst the other months (Figure 10A), while July 2018 presented the lowest mean daily precipitation (0.33±1.80 mm). The total precipitation per month followed the same pattern with the months of November 2018 and July 2018 with 299.73 mm and 10.16 mm, respectively, however the precipitation in June 2018 was numerically similar to July 2018 (Figure 10B). As the mean precipitation per day presented high standard deviations, Pearson correlations were calculated between total precipitation and the mean of every mass feature per month (Table 5). The r values ranged from -0.4060 to 0.3522, thus no significant correlation was observed indicating that rainfall did not affect the volatile composition.

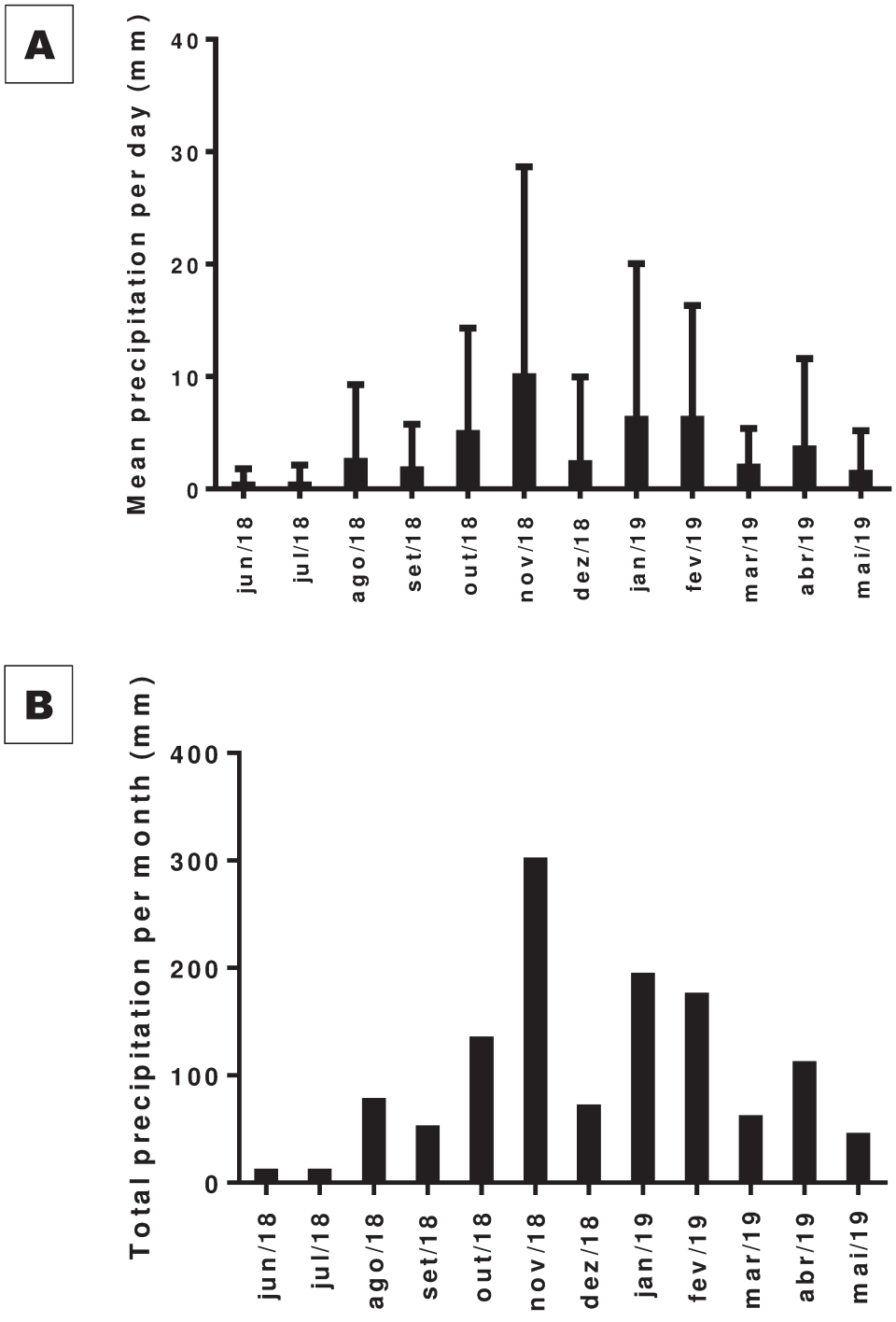

Figure 10 - Mean precipitation per day (mm) (A - whiskers above bars represent standard deviation) and total precipitation per month (mm) (B).

Table 5 - Pearson correlation coefficient (r) between total precipitation (TP) from the months (mm) and the mean of every mass feature per month (1 - 17) (ns - non-significant).

Pearson correlation	r	95% confidence interval	R square	P (two-tailed)	P value summary
TP vs A	0.0671	-0,5272 to 0,6173	0.0045	0.8359	ns
TPvs B	-0.2140	-0,7015 to 0,4108	0.0456	0.5052	ns
TP vs C	0.0779	-0,5194 to 0,6240	0.0061	0.8099	ns
TP vs D	-0.1270	-0,6531 to 0,4826	0.016	0.6953	ns
TP vs E	-0.0370	-0,5983 to 0,5486	0.0014	0.909	ns
TP vs F	-0.2600	-0,7255 to 0,3695	0.0674	0.4152	ns
TP vs G	-0.3750	-0,7808 to 0,2538	0.1405	0.23	ns
TP vs H	0.1515	-0,4627 to 0,6675	0.023	0.6383	ns
TP vs I	-0.0610	-0,6133 to 0,5319	0.0037	0.8516	ns
TP vs J	-0.1570	-0,6705 to 0,4584	0.0246	0.6263	ns
TP vs K	-0.2830	-0,7373 to 0,3474	0.0801	0.3727	ns
TP vs L	-0.1780	-0,6823 to 0,4410	0.0317	0.5798	ns
TP vs M	-0.1880	-0,6878 to 0,4326	0.0354	0.5582	ns
TP vs N	-0.1510	-0,6672 to 0,4631	0.0228	0.6392	ns
TP vs O	-0.4060	-0,7949 to 0,2188	0.165	0.1901	ns
TP vs P	-0.1490	-0,6659 to 0,4650	0.0221	0.6447	ns
TP vs Q	0.3522	-0,2780 to 0,7705	0.124	0.2615	ns

The maximum and minimum daily air temperatures ranged from 25.3 to 32.9 °C and 13.3 to 20.3 °C, respectively, with the highest mean value recorded in January 2019 (32.9 °C) and lowest in July 2018 (13.3 °C) (Figure 11). The mean of maximum and minimum temperature of the day per month was used to calculate Pearson's correlation coefficient. The r values ranged from -0.6630 to 0.5428, only one significant correlation (p=0.0189) between mean temperature and mass feature F (*p*-Cymene) was determined, although it was not a strong correlation (r=-0.6630) (Table 6). The mean recorded air temperature at the time of sample collection was used in order to compare the results between morning and afternoon (Figure 12). Multiple t-tests indicated that both periods of the day were statistically different in every month of the year ($p<0.0001$), being mornings colder than afternoons, as expected.

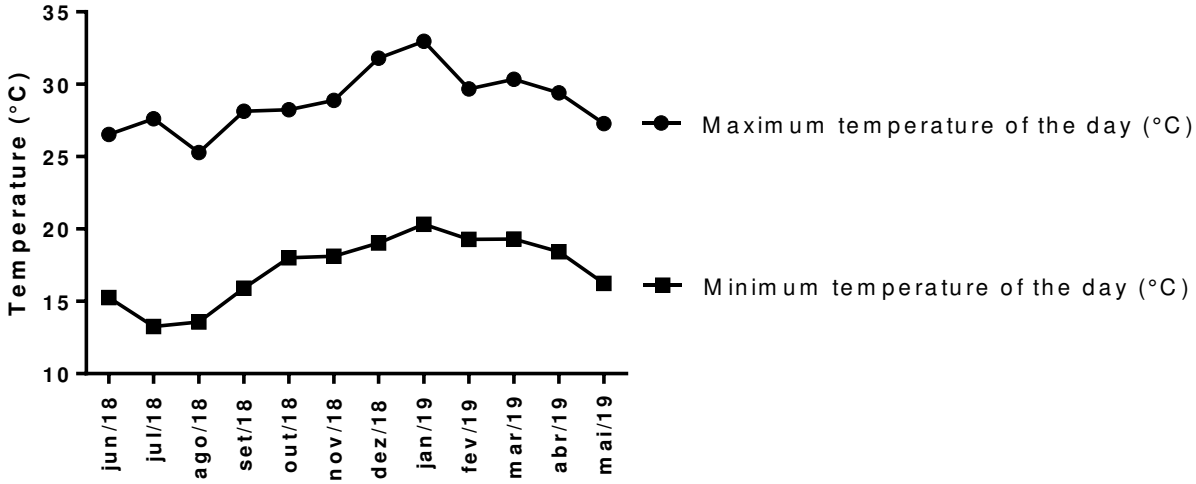

Figure 11- Mean of the monthly maximum and minimum temperature of the day (°C).

Table 6 - Pearson correlation coefficient (r) between mean temperature (MT) of the day during every month of a year (°C) and the mean of every mass feature per month (A - Q) (ns - non-significant).

Pearson correlation	r	95% confidence interval	R square	P (two-tailed)	P value summary
MT vs A	0.3581	-0,2718 to 0,7732	0.1282	0.2531	ns
MT vs B	0.2005	-0,4221 to 0,6946	0.0402	0.5321	ns
MT vs C	0.5428	-0,04539 to 0,8515	0.2946	0.0682	ns
MT vs D	0.3738	-0,2549 to 0,7804	0.1397	0.2313	ns
MT vs E	0.4437	-0,1748 to 0,8111	0.1969	0.1485	ns
MT vs F	-0.6630	-0,8959 to -0,1428	0.439	0.0189	*
MT vs G	-0.1230	-0,6513 to 0,4850	0.0152	0.7024	ns
MT vs H	0.3373	-0,2935 to 0,7635	0.1138	0.2836	ns
MT vs I	0.3464	-0,2841 to 0,7677	0.12	0.2701	ns
MT vs J	0.3596	-0,2702 to 0,7739	0.1293	0.2509	ns
MT vs K	0.0725	-0,5233 to 0,6207	0.0053	0.8228	ns
MT vs L	0.2470	-0,3810 to 0,7191	0.061	0.439	ns
MT vs M	0.2297	-0,3966 to 0,7101	0.0527	0.4727	ns
MT vs N	0.2311	-0,3954 to 0,7108	0.0534	0.4699	ns
MT vs O	-0.0570	-0,6109 to 0,5346	0.0032	0.8607	ns
MT vs P	0.2592	-0,3699 to 0,7253	0.0672	0.416	ns
MT vs Q	0.0828	-0,5157 to 0,6270	0.0069	0.7981	ns

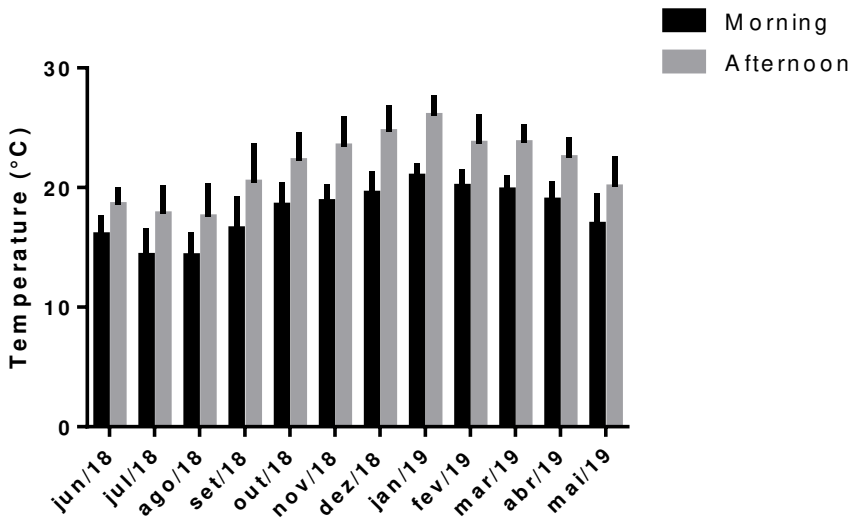

Figure 12 - Mean air temperature (°C) at the time of sample collection during a year (whiskers above bars represent standard deviation).

In the monthly collection analyses, the only established correlation was between mean temperature and *p*-cymene, which is one of the largest peak areas in the essential oil, as seen in Chapter 1, although in Pinheiro *et al.* (2019)[46] *o*-cymene, an isomer of the compound, is described as one of the volatiles constituents. Collin *et al.* (2010)[64] indicate that it is difficult to unambiguously identify the cymene isomers and that there are discrepancies in various data banks in terms of order of elution and retention indexes. This study identified the volatile components using the retention indexes described in Adams (2017)[42] and mass spectra comparison with NIST library, resulting in *p*-cymene as one of the major chromatographic peaks in terms of area percentage. This component is a precursor of carvacrol via hydroxylation of *p*-cymene molecule[65,66] and the inversely proportional correlation between mean temperature and *p*-cymene suggests that this environmental factor may influence carvacrol biosynthesis pathway, although no strong correlations were observed and carvacrol did not vary significantly among months.

No strong correlation between the evaluated environmental factors and mass features were detected. Unlike the analyses using MetaboAnalyst online software, the raw data were not pre-processed (i.e. normalized and auto-scaled) in order to establish

these correlations, thus the variation of major and minor component is unequally considered. Although data pre-processing is efficient in indicating subtle changes in the chemical profile (Figures 5 - 9), the raw data processing reduces the influence of the intensity of peaks on the result. Moreover, other environmental factors not evaluated in this study may influence *P. amboinicus* volatile composition.

Secondary metabolites mediate the interaction between plants and the environment, thus the synthesis of these compounds is not only affected by the plant life cycle but also by the environmental conditions[67]. Gobbo-Neto and Lopes (2007)[27] pointed out that the main factors that coordinate or alter the secondary metabolism are seasonality, circadian rhythm, development, temperature, water availability, ultraviolet radiation, nutrients, altitude, atmospheric pollution and induction by mechanical stimuli or attack by pathogens. Thus, there is a complex interaction between these environmental factors and the essential oil synthesis, which may indicate why there were no strong correlations between rainfall and air temperature, individually, with the obtained mass features.

2.3 Conclusion

The leaf volatile profile of *P. amboinicus* individuals collected at different sites, leaves in different stages of development and leaves sampled at different hours of the day and in different months of the year varied in different ways. It was shown that the volatile profile of leaves in different stages of development was both qualitatively and quantitatively different, suggesting that the essential oils produced in different leaves may differ as well as their biological activities. The three individuals, collected from different regions in the Metropolitan Region of Campinas (São Paulo, Brazil), presented similar volatile composition indicating that there were no underlying interactions between genotype and environment. The period of collection, both within a day and along a year, influenced the volatile composition of *P. amboinicus*. Comparing the results of leaves collected in the morning and afternoon, only terpinen-4-ol and the unidentified mass feature H, two minor components, varied significantly, although the temperature at these times was significantly different throughout the year. The chemometric analysis

(heatmap) indicates that most mass features varied significantly among months of collection except for *γ*-terpinene, carvacrol and the unidentified mass feature O. In spite of the monthly variation in composition, carvacrol, an important volatile and bioactive component, is present throughout the year. No correlation was observed between rainfall and the variation in composition and only *p*-cymene varied with the temperature. These results indicate that carvacrol may be considered as a marker for the species and that further investigations are required in order to understand how each environmental factor influences the volatile composition of the species, and the impact on the essential oil biological activities, as a way to standardize *P. amboinicus* leaf collection for medicinal purposes.

CHAPTER 3

GLANDULAR TRICHOMES OF *Plectranthus amboinicus* (Lour.) Spreng. AND THEIR DISTRIBUTION ON LEAVES IN DIFFERENT STAGES OF DEVELOPMENT

In principle, all plants are capable of producing volatile compounds, but only in two situations are they of commercial interest for essential oils: the production of a mixture of volatiles such as flower aromas, and the secretion and accumulation of volatiles in specialized anatomical structures[20]. An example of the second case, essential oils in the Lamiaceae family are secreted by structures called glandular trichomes[22]. These trichomes are classified into different types, which are distinguished by the morphology, onset and duration of secretory activity, mode of secretion and type of secreted material[68]. Evert (2006)[22] points out that two main types of trichomes are found in Lamiaceae, the peltate trichomes, which consist of a basal cell, a short peduncle and a secretory head with 4 to 18 cells; and capitate trichomes, which have a basal cell, a peduncle of variable length and a spherical head with 1 to 4 cells. In addition to the chemical characteristic attributed to species by the secretion of glandular trichomes, the morphology of these structures is of great taxonomic importance[69,70].

The distribution of glandular trichomes, onset of secretion and type of secreted material, influence the chemical composition throughout the plant. Werker *et al.* (1985)[68] described the essential oil composition of *Majorana syriaca* (L.) Raf. (synonymous of *Origanum syriacum* L. - Lamiaceae) using leaves at different stages of

development and associated the increase in Thymol and Carvacrol content with the transition of types of glandular trichomes prevalent in each stage. Similarly, as seen in Chapter 2 for *P. amboinicus*, the transition from leaves in expansion to expanded leaves is characterized by an increase in Carvacrol content, suggesting that the chemical variation may be due to glandular trichome distribution.

The glandular trichomes of several species of the genus *Plectranthus* have been extensively described[69–74]. As example, Kalicharan *et al.* (2015)[69] described the glandular trichomes of *Plectranthus zuluensis* and indicated that the capitate trichomes of *Plectranthus zuluensis* in the secretory phase are present in emerging and young leaves, whereas peltate trichomes are active in all stages of leaf development. The few anatomical studies of *P. amboinicus* indicated the presence of both non-glandular and glandular trichomes[75], which were classified as short-stalked and long-stalked capitate trichomes and capitate trichomes with a large spherical head[76,77]. Further investigation of glandular trichome distribution in *P. amboinicus* was required in order to determine correlations between volatile composition and these secretory structures.

This final chapter is focused on the morphology and distribution of glandular trichomes on *P. amboinicus* leaves, evaluated via optical microscopy and scanning electron microscopy. Moreover, correlations between trichome distribution and chemical variation of leaves in different stages of development were established. In addition to a better understanding of the production and secretion of the species' essential oil, the characterization of glandular trichomes may promote systematic implications within the genus.

3.1 Material and Methods

3.1.1 Plant Material

Plectranthus amboinicus individuals planted in the experimental field of the Institute of Biology, University of Campinas (Unicamp, Campinas-SP) were used in the experiments according to demand. The individuals were deposited in the Unicamp Herbarium as described in the previous chapters: Mandala Space at UNICAMP,

Campinas-SP (UEC 203557); Coordination of Integral Technical Assistance (CATI), Campinas-SP (UEC 203558); and Valinhos-SP (UEC 201900).

3.1.2 Scanning Electron Microscopy (SEM)

Leaf samples at different stages of development (*i.e.* emerging, expanding and expanded leaves) were collected from individuals of *P. amboinicus*. The emerging leaves were collected near the apical meristem, the first and second evident leaves on the branch apex were collected as expanding whereas the first fully expanded leaf was collected as expanded. The samples were fixed in neutral-buffered formalin (TNF)[78] for 48 h and subsequently subjected to the dehydration series of Ethanol in various concentrations, ranging from 10 % to 100 % (v/v), respectively. After dehydration, the samples were dried via critical point drying and sputter coated with gold particles. The samples were analyzed via SEM using an electron microscope model JSM - 5800LV - JEOL, available in the Electron Microscopy Laboratory Institute of Biology (UNICAMP).

3.1.3 Optical Microscopy (OM)

Leaf samples at different stages of development (*i.e.* expanding and expanded leaves) were collected from individuals of *P. amboinicus* and freehand sections were cut. These sections were placed onto a microscope slide together with a drop of water, then covered with a glass coverslip. The samples were analyzed via OM using an optical microscope model Leica DM 750 coupled to a digital microscope color camera model Leica DFC/295.

3.2 Results and Discussion

The glandular trichomes of *P. amboinicus* leaves were evaluated by OM and SEM, and four trichome types were observed (Figure 13). The first type (Type I) was characterized by a tiny peduncle and a secretory head with two cells (Figure 13A, 14A); the second type (Type II) had a single or bicellular peduncle and an elongated unicellular secretory head, which often presented lipophilic droplets on its surface

(Figure 13B, 14B). The third and fourth types had uni- or bicellular peduncles, however the secretory head of third type (Type III) was characterized by cells located at the base of the head and a large subcuticular space (Figure 13C, 14C), while the fourth type (Type IV) was characterized by an unicellular bulb-shaped head (Figure 13D, 14D).

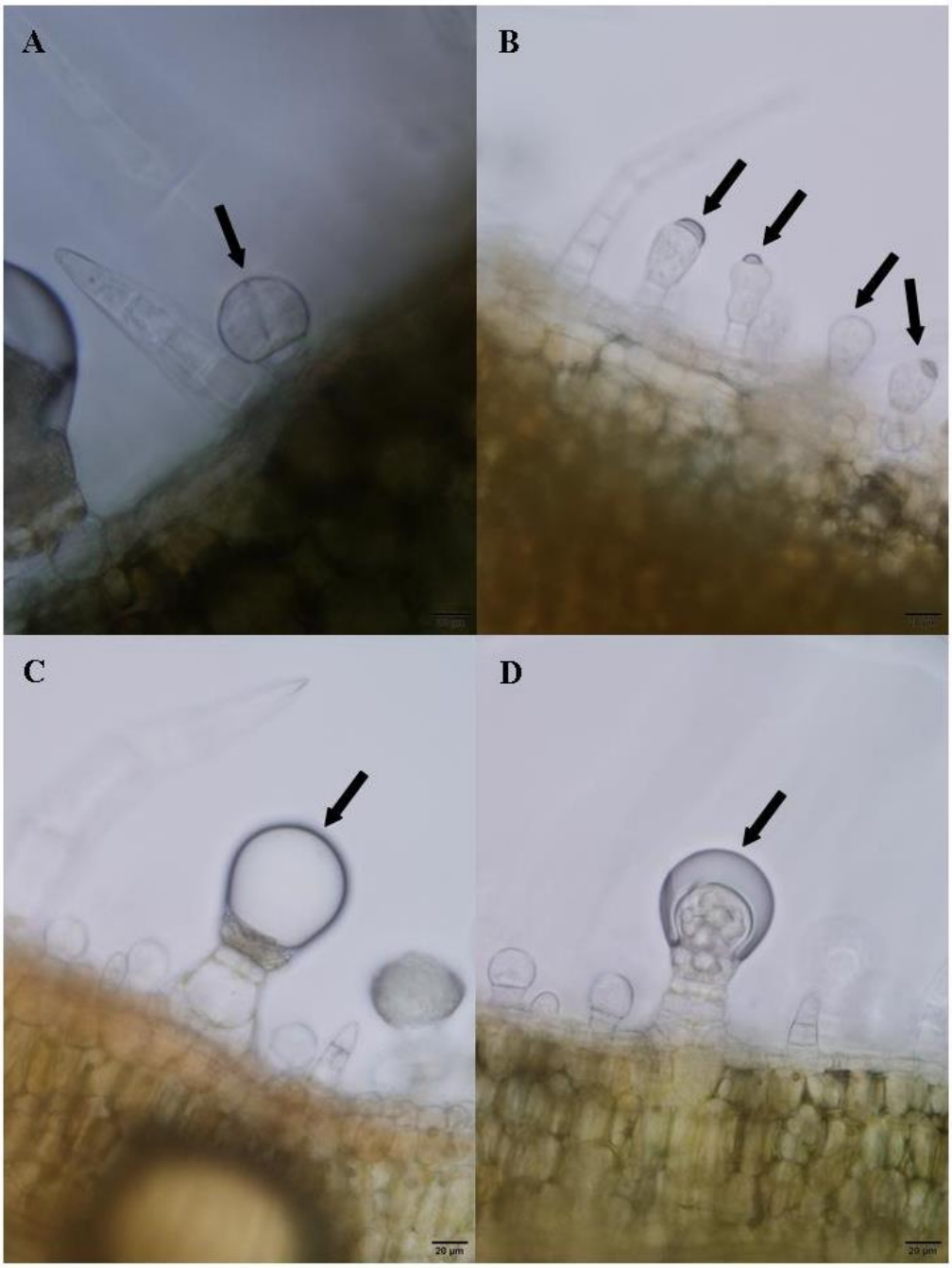

Figure 13 - Optical microscopy of *Plectranthus amboinicus* (Lour.) Spreng. leaf glandular trichomes (A - D, arrows point the different types of trichomes).

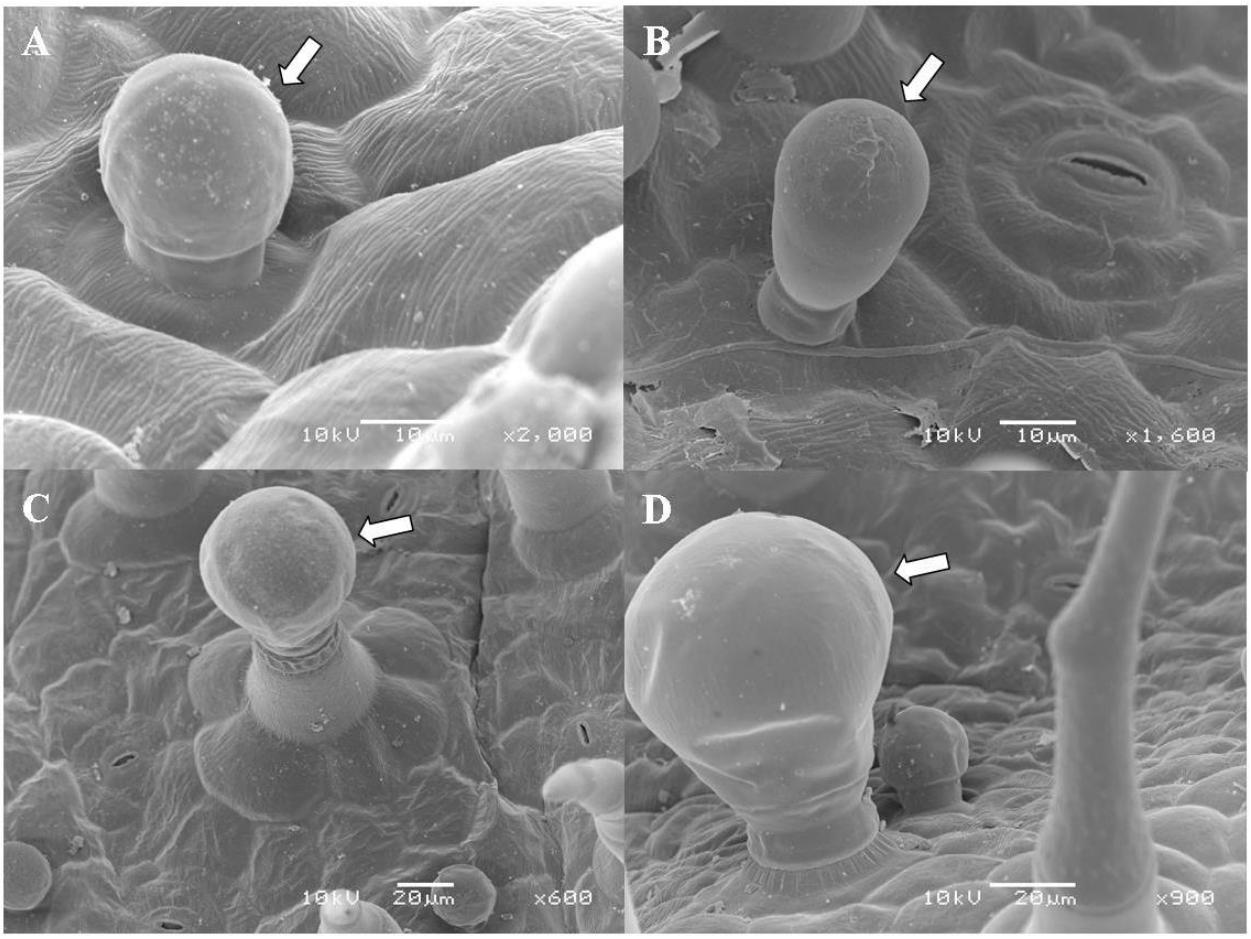

Figure 14 - Scanning electron microscopy of *Plectranthus amboinicus* (Lour.) Spreng. leaf glandular trichomes (A - D, arrows point the different types of trichomes).

The emerging leaves were characterized by an abundance of glandular trichomes, whilst these structures became scarce as the leaves expanded (Figure 15). The Figure 15A shows the emerging leaves near de apical meristem, detailed in Figure 15B, leaves which are also characterized by an abundance of non-glandular multicellular trichomes. On the surface of expanded leaves, many glandular trichomes with broken heads can be observed, particularly the glandular trichomes Type III (Figure 15C), probably due to their length.

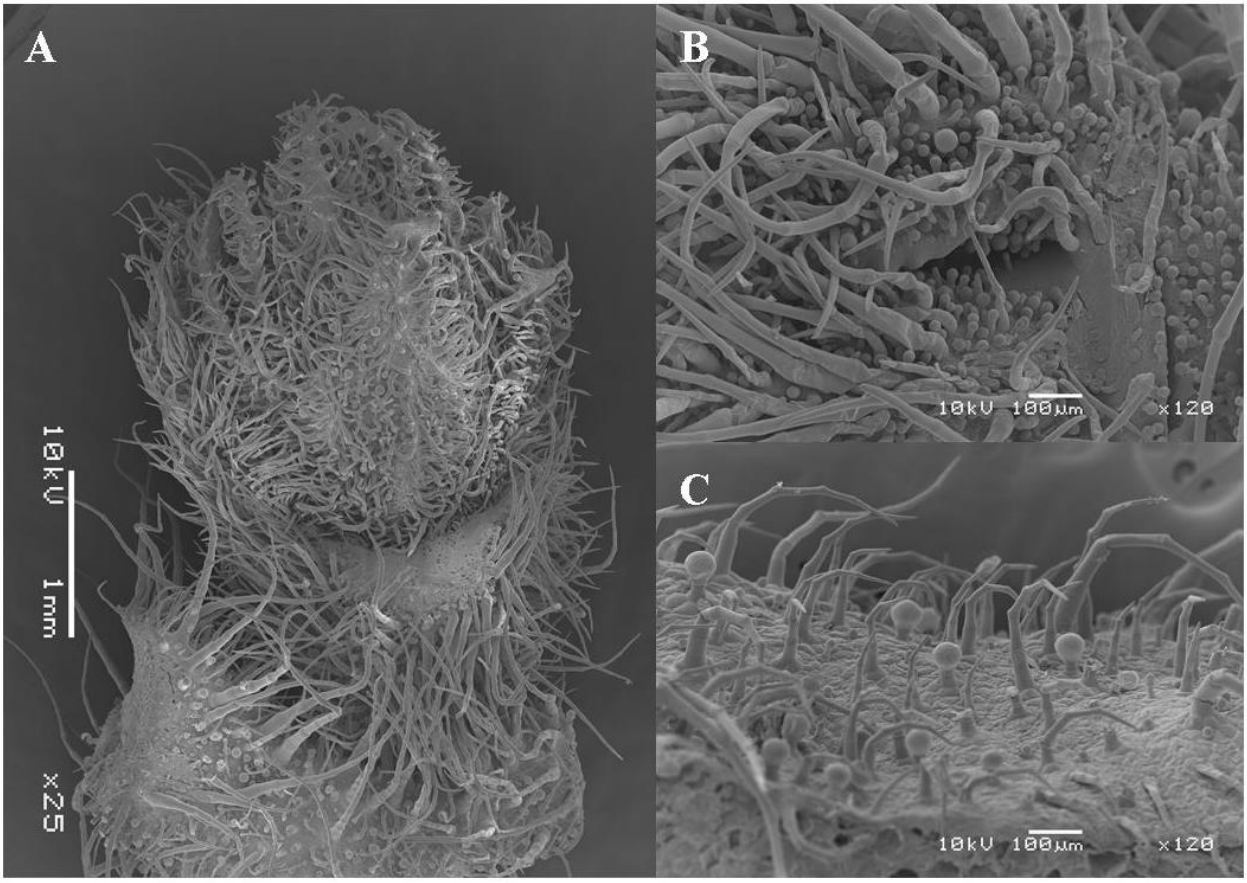

Figure 15 - Scanning electron microscopy of *Plectranthus amboinicus* (Lour.) Spreng. leaf trichomes (A - B - emerging leaves; C - expanded leaf).

The glandular trichomes presented a differential distribution on leaves in different stages of development. The glandular trichomes Types I, II and III, were observed in all stages of leaf development, while the Type IV was predominant on younger leaves (Figure 16).

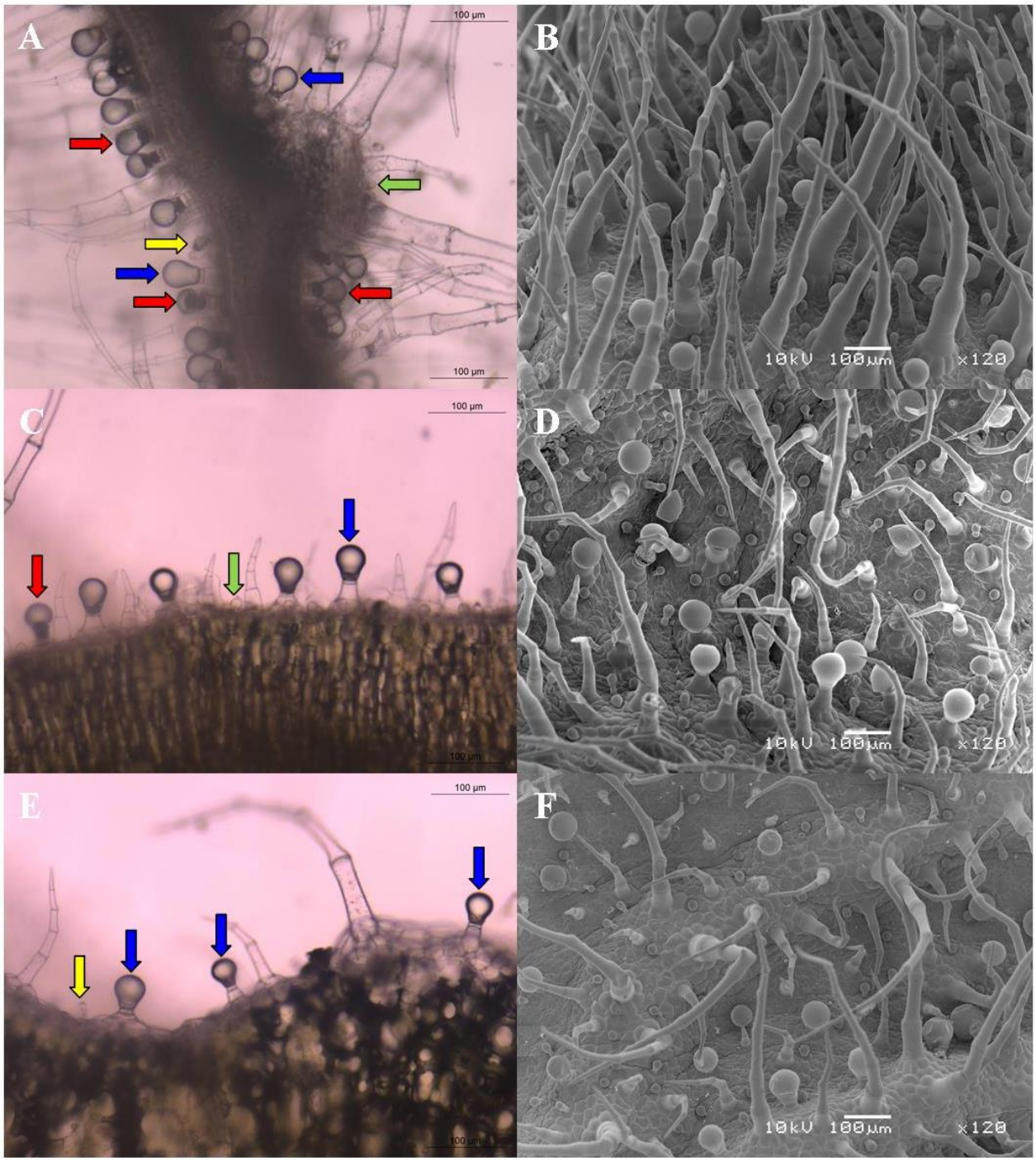

Figure 16 - Optical microscopy (A, C and E) and scanning electron microscopy (B, D and F) of *Plectranthus amboinicus* (Lour.) Spreng. leaves in different stages of development: expanding leaves (A-B and C-D) and expanded leaves (E-F). Arrows point the different types of glandular trichomes (green - Type I; yellow - Type II; blue - Type III; red - Type IV).

All the glandular trichomes observed in this study were identified as capitate trichomes, whereas no peltate trichome, a fairly common type in *Plectranthus*, was identified. These results are in agreement with Mauro et al. (2008)[76] and Muthukumarana *et al.* (2014)[77] that described three types of capitate trichomes (*i.e.* short-stalked, long-stalked and capitate trichomes with a large spherical head) as the glandular trichomes of *P. amboinicus*. Peltate trichomes are usually characterized by a short basal cell and a round head with 4-14 secretory cells[69]. The length of these trichomes can hinder identification, once short-stalked capitate trichomes are often mistakenly identified as peltate trichomes. Every anatomical characteristic was considered in this investigation, ensuring that no peltate trichome was observed. Besides, the absence of this type of glandular trichomes may be of taxonomic importance and a systematic criteria for species delimitation in *Plectranthus*[69,70].

As seen in chapter 2, almost every volatile component varied significantly between the leaves in different stages of development. The increase in carvacrol content, as the leaves expanded, marked the transition of the stages. Moreover, the increase of this component, in terms of area percentage, was due to the decrease of several minor components that were abundant in younger leaves. Coincidently, Type IV trichomes were abundant on these leaves and decreased in number as the leaves expanded. These results suggest that the capitate trichomes Type IV secrete minor components of the volatile profile, while the other three types may be responsible for secreting the other components, such as carvacrol. Few papers correlated secretory structures and chemical composition in Lamiaceae, Werker *et al.* (1985)[68] associated the increase in thymol and carvacrol content with the transition of the main secreting glandular trichome types, from capitate to peltate trichomes, as the leaves expanded. This correlation for the genus *Plectranthus* is still unclear, a promissory area for future research.

2.3 Conclusion

Plectranthus amboinicus leaves showed four different types of capitate glandular trichomes with singular characteristics: Type I - tiny peduncle and a secretory

head with two cells; Type II - single or bicellular peduncle and an elongated unicellular secretory head; Type III - uni- or bicellular peduncle with a secretory head with cells located at the base of the head and a large subcuticular space; Type IV - uni- or bicellular peduncle with an unicellular bulb-shaped head. The trichomes Type I-III were observed in all leaves, whilst the Type IV was predominant on younger leaves. This distribution correlates to the volatile profile, younger leaves showed an abundance of minor components possibly due to the Type IV trichome secretion, while the other trichomes may be responsible for secreting other components such as carvacrol, the main essential oil component. Further research on the glandular trichome secretion in *P. amboinicus* is required for better understand of how these secretory structures influence the volatile profile of the species.

FINAL CONCLUSION

Plectranthus amboinicus is a medicinal plant used worldwide for its therapeutic potential, such as treating common cold, asthma, inflammation and the flu. Many studies described the volatile composition of *P. amboinicus*, mainly focusing on essential oil composition, but few explored the chemical variation within the species. In this study, not only the composition of the essential oil and its bioactivity were evaluated, but the volatile profiles during a year and during leaf expansion, as well as the secretory structures of the species are reported herein.

With regard to the essential oil, the main components were: carvacrol, the most abundant component in area percentage, followed by p-cymene, (E)-caryophyllene and γ-terpinene. The essential oil showed moderate antioxidant activity via two tests with different mechanisms of action (*i.e.* SET and HAT), corroborating its popular use. The leaf volatile profile showed that: (i) leaves in different stages of development are chemically distinct, suggesting that the essential oils produced in different leaves may differ as well as their biological activities; (ii) the evaluated individuals presented similar volatile profile, thus no underlying interaction between genotype and environment was observed; (iii) although terpinen-4-ol and an unidentified mass feature were statistically different between morning and afternoon, they were minor components and this difference might not alter essential oil bioactivity; and (iv) most volatile components varied significantly among months of collection except for γ-terpinene, carvacrol and an unidentified mass feature, but no correlation was observed between rainfall and the variation in composition and only p-cymene varied with the temperature. These results indicate that carvacrol, the major component, was present throughout a year and may be considered as a marker for the species.

The secretory structures in *P. amboinicus* leaves found in this study were glandular trichomes, identified as four types of capitate trichomes: Type I - tiny peduncle and a secretory head with two cells; Type II - single or bicellular peduncle and an elongated unicellular secretory head; Type III - uni- or bicellular peduncle with a

secretory head with cells located at the basis of the head and a large subcuticular space; Type IV - uni- or bicellular peduncle with an unicellular bulb-shaped head. These trichomes had a differential distribution on the leaves, Types I-III were present in all stages of development, whereas Type IV trichomes were mainly found in younger leaves. This result correlates with the volatile composition of leaves in different stages of development, as younger leaves showed an abundance of minor components possibly due the Type IV trichome secretion, while the other trichomes may be responsible for secreting other components.

Although *P. amboinicus* is one of the best documented species of Lamiaceae, there are variations regarding its composition in the published results which have not been fully explained. This study aimed to explore the gaps in knowledge of the chemical composition of this medicinal plant, which is widely used in Brazil and in other countries around the world. As seen herein, the essential oil presented a similar profile to the ones evaluated by other Brazilian studies and presented moderate antioxidant activity, which was evaluated by two antioxidant assays with different mechanisms of action. Furthermore, this is the only investigation about the volatile profile with a metabolomics approach, as well as the study of glandular trichome distribution. Further correlation between environmental factors and the volatile composition of the species are required as a way to standardize *P. amboinicus* leaf collection for medicinal purposes, along with the impact of chemical variation on the essential oil's biological activities and the secretion process of the different types of glandular trichomes.

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Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "***Study of the chemical profile of volatiles and secretory structures of Plectranthus amboinicus (Lour.) Spreng. - Lamiaceae***", desenvolvida no Programa de Pós-Graduação em Biologia Vegetal do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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