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EXTRACTION AND FRACTIONATION OF CROWBERRY AND TAMARILLO LEAVES FOR BIOLOGICAL ACTIVITY SCREENING

Dissertation submitted to the University of Coimbra for the fulfillment of the necessary requirements for the obtention of the degree of Master in Biodiversity and Vegetal Biotechnology, with scientific supervision of Dr. Jorge Manuel Pataca Leal Canhoto (CEF, DCV) and Dr. Ricardo Manuel Fernandes da Costa (MF-Q, CEF)

October 2021

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Extração e fracionamento de folhas de camarinha e tamarilho para *screening* de atividade biológica

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Endorsements:

Apoios:

"Dripping water hollows out stone, not through force but through persistence."

Ovid

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ABSTRACT

The urge for new environmentally friendly solutions to the pesticides has led to a recent increasing interest in the research and production of plant-based biopesticides and the compounds they produce. In the present study, leaves of the species *Solanum betaceum* Cav. and *Corema album* (L.) D. Don were used to create crude ethanol extracts which were subsequently fractioned using organic solvents. The species leaf fractions were tested *in vitro* for their anti-oomycete activity on the growth of *Phytophthora cinnamomi* Rands. The initial extract of the leaf material was prepared with ethanol and the fractionation was carried out with n-hexane, chloroform, ethyl acetate and butanol, in that order, through a biphasic separation system. *P. cinnamomi* shows sensitivity to the butanolic fraction of *S. betaceum* and to all fractions of *C. album*, particularly to the initial *C. album* ethanolic extract. The butanolic fraction of *S. betaceum* proves to be rich in polyphenols, polysaccharides and esters. The various fractions of *C. album* contain pectins, esters, triterpenoids and aromatic compounds such as phenols.

Keywords: Anti-oomycete; *Corema album*; Pesticide; *Phytophthora cinnamomi*; *Solanum betaceum*.

RESUMO

A necessidade de novas soluções biologicamente sustentáveis ao uso de pesticidas levou a um recente crescimento no interesse à investigação e produção de biopesticidas à base de plantas e os compostos por elas produzidos. No presente estudo foram realizados e fracionados à base de solventes orgânicos frações vegetais de folhas das espécies *Solanum betaceum* Cav. e *Corema album* (L.) D. Don, e a atividade antioomicética dos mesmos foi testada *in vitro* no crescimento de *Phytophthora cinnamomi* Rands. O extrato inicial do material foliar foi preparado com etanol e o fracionamento do mesmo foi realizado com n-hexano, clorofórmio, acetato de etilo e butanol, por esta ordem, através de um sistema de separação bifásica. *P. cinnamomi* demonstra sensibilidade à fração butanólica de *S. betaceum* e a todas as frações de *C. album*, particularmente ao extrato inicial etanólico. A fração butanólica de *S. betaceum* demonstra ser rica em polifenóis, polissacarídeos e ésteres. As várias frações de *C. album* possuem pectinas, ésteres, triterpenóides e compostos aromáticos como fenóis.

Palavras-chave: Anti-oomycete; *Corema album*; Pesticida; *Phytophthora cinnamomi*; *Solanum betaceum.*

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
0-EtOH	Ethanol extract
1-HEX	n-Hexane extract fraction
2-CHL	Chloroform extract fraction
3-ETA	Ethyl acetate extract fraction
4-BUT	n-Butanol extract fraction
5-AQU	Aqueous extract fraction
ATR	Attenuated total reflectance attachment
BE	β-carotene equivalent
C-3-GE	Cyanidin-3-glucoside equivalent
CAM	Portuguese-crowberry (*Corema album*)
DMSO	Dimethyl sulfoxide
DW	Dry weight
ECHA	European Chemicals Agency
EFAA	Extract activity assay(s)
EFSA	European Food Safety Authority
EW	Edible weight
FA	Fatty acids
FAO	Food and Agriculture Organization of the United Nations
FTIR	Fourier-Transform Infrared Spectroscopy
FW	Fresh weight
GAE	Gallic acid equivalent
GR	Growth reduction
HGA	Hyphal growth area
ISSG	Invasive Species Specialist Group
IU	International unit (1 mg β-carotene = 1667 IU Vitamin A activity)
LBS	Liquid biphasic system
LE	Lutein equivalent
MAA	Multiwell activity assay(s)
MIC	Minimum inhibitory concentration
MLC	Minimum lethal concentration
MUFA	Monounsaturated fatty acids
NAP	National Action Plan
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PES	Polyethersulfone
PTG	Post treatment growth
PUFA	Polyunsaturated fatty acids
QE	Quercetin equivalent
RE	Rutin equivalent
SC-CO_2	Supercritical CO_2 extraction
SE	Soxhlet extraction
SEM	Scanning electron microscopy
SFA	Saturated fatty acids
TAM	Tamarillo (*Solanum betaceum*)
WHO	World Health Organization

1. INTRODUCTION

1.1. Pesticides

Pesticides - Definition and their applications

A pesticide is a chemical substance, or a biological agent intentionally released into the environment, capable of deterring, preventing or controlling populations of harmful pests such as animals, weeds, fungi, bacteria or viruses. These pests are defined as organisms that can be hazardous to our food and health (Mahmood et al., 2016) and are liable for yearly expenses of billions of dollars through the production of costly synthetic chemicals (Chattopadhyay et al., 2017). The term "pesticide" is a general term that refers to a wide range of compounds such as insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, among others. Insecticides are generally the most acutely toxic class of pesticides (Aktar et al., 2009).

The world population is largely dependent of agrochemicals use, being the only reliable way of protecting large scale food production (Andréa et al., 2000) necessary for the subsistence of an ever-growing world population. That being said, the use of pesticides seems to be a necessary evil to mankind food necessities as a whole (Carvalho, 2006; Peshin & Zhang, 2014). This assessment is supported by the increasing production and use of these chemicals around the world **(Fig. 1)**.

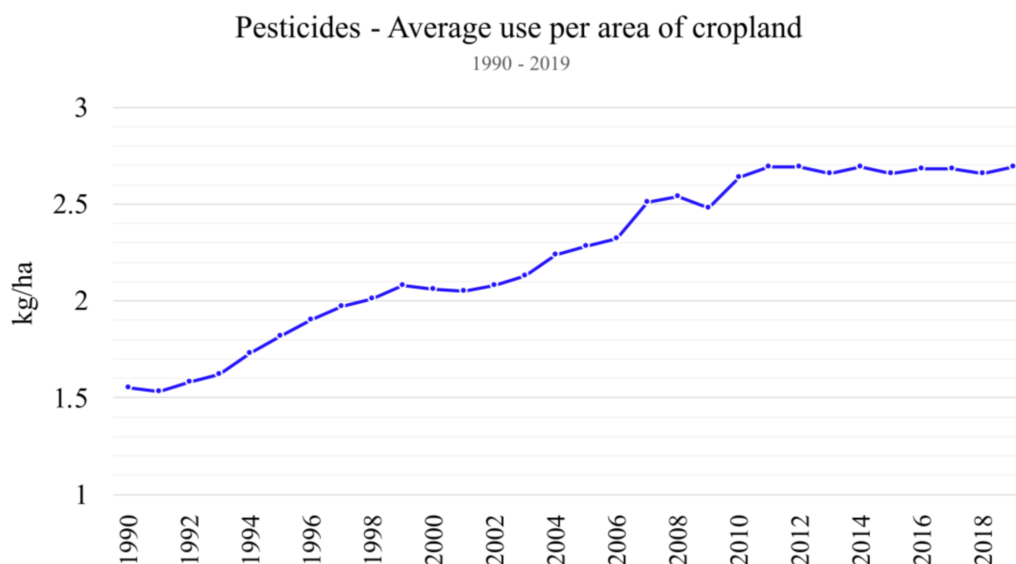

Figure 1. Average use of pesticides per area of cropland worldwide from 1990-2019. *(source: fao.org/faostat. Accessed: 12/08/2021)*

Pesticides polution and risks

Pesticides overuse and pollution is increasing around the globe, especially in countries outside Europe, such as India, China and Brazil (Zhang & Liu, 2017). The frequent use of pesticides negatively affects soils and water bodies quality, and contaminate surface waters by drift, run-off, drainage and leeching (Houtman, 2010). Studies conducted in Italy reveal dangerously high concentrations of pesticides in surface and groundwaters in most part associated with agricultural activities taken place at local watersheds (Meffe & de Bustamante, 2014). Other monitoring studies conducted in water bodies around the world have shown similar concerning results (Gao et al., 2009; Fadaei et al., 2012; De Gerónimo et al., 2014). Being designed to be biologically active (Schuster & Schröder, 1990a), pesticides can affect other species causing unintended side effects and accumulate on crops that enter our food chain and are directly ingested by humans along foodstuffs and water (Taylor et al., 2002; FAO/WHO, 2006). Besides ingestion, two other main routes of human body exposure to pesticides are inhalation and dermal exposure (Tomer et al., 2015). The exposure to these agrochemicals is responsible for numerous cases of related illnesses and injuries, especially among agricultural industry workers, who are directly exposed to these pesticides. Data reports by Calvert et al. (2016) in the USA reveals that the rates of pesticide-related ailments are staggeringly greater among agricultural workers that in nonagricultural workers.

The general population is also directly exposed to agrochemicals, mainly in public places such as office buildings, restaurants, schools, parks, and along roads and walkways (Bolognesi & Merlo, 2019). Pesticide exposure may result in biochemical alterations in the body long before more glaring and adverse symptoms are manifested (Tomer et al., 2015). The chronic exposure to pesticides significantly increase the risk of developing various pathophysiological, respiratory and neurological conditions (Agrawal & Sharma, 2010). For example, the natural occurring rotenone (derived from plant roots), widely used as an active agent in insecticides commonly applied in gardens, lakes and reservoirs, is proven to induce behavioral and neuropathological features of Parkinson's disease (Panov et al., 2005; Agrawal & Sharma, 2010). Given the hazardous nature of synthetic pesticides, rigid regulations are imposed to their use and many known substances are regulated and prohibited, especially at an European Union (EU) level and its Member States.

Pesticide use and legislation in the EU

There are strict legislations concerning the use of pesticides in EU, which aim to regulate all types of plant protection products and biocidal usage across all EU Member States. Pesticide regulations are undertaken at an EU level by Parliament directives alongside Member States national laws, or National Action Plans (NAPs). Although NAPs have the freedom to implement their particular laws towards the use of pesticides within the given country borders, these regulations must comply and work in tandem with the collective EU. NAPs should advocate a proper training amongst producers, distributors, advisors and professional users of pesticides, the implementation of certification systems explicitly disclaiming potential health and environmental risks, and the public sensibilization and education towards the use of agrochemicals (Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009). Pesticide placement in the market is also thoroughly revised. These regulations are mediated the Regulation on Plant Protection Products (Regulation No 1107/2009 of the European Parliament and of the Council, 2009) and the Regulation on Biocidal Products (Regulation No 524/2013 of the European Parliament and of the Council, 2012). Those regulations were implemented with the objective of protecting human, animal and environment health, as well as to standardize the rules to which the plant protection products and biocides must adhere to be approved for marketing. Both types of agrochemicals are subject to a dual approval process, where the products active substances are revised at an EU level and the products themselves are at a Member State level. The approval of active substances by the EU is mediated by the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA) by a set number of "exemption criteria" **(Table 1)**. As of June 2021, EU regulation entities assesses the state of 1518 active substances, among them 456 are approved, 921 are not approved, 61 are under review and 17 are yet to be assessed at an EU level.

Table 1. Principal exemption criteria for substance approval in the EU.
(Adapted from European Parliamentary Research Service 2017)

<table>
<tr><th></th><th>Plant protection substances</th><th>Biocidal substances</th></tr>
<tr><td rowspan="2">Effects on human health</td><td colspan="2">Cannot be classified as carcinogenic, mutagenic or toxic to reproduction</td></tr>
<tr><td colspan="2">Cannot be considered an endocrine disruptor</td></tr>
<tr><td rowspan="2">Effects on the environment</td><td>Cannot be considered a persistent organic pollutant (POP)</td><td></td></tr>
<tr><td colspan="2">Cannot be considered as a persistent, bio-accumulative and toxic (PBT) substance, or a very persistent, very bio-accumulative substance (vPvB)</td></tr>
</table>

Biopesticides and alternatives

Given the potentially hazardous consequences of pesticide overuse and the strict regulations imposed in the pesticides active substances and products, it is of utmost importance to search for environmentally friendly options in our immediate future. The use of organic-based compounds seems to be a potential solution to develop effective new products to tackle pest management. This biopesticides, as they are named, have several crucial differences that distinguishes them from chemical pesticides. Their active compounds come from animals, plants and bacteria (EPA, n.d.) and usually target specific pests and leave the remaining organisms unscathed (Dar et al., 2021). They are also biodegradable with little to no residual effects and are less prone to be rendered useless by pest resistances (Gupta & Dikshit, 2010). Currently there are many plant-based products in use in organic agriculture, with effective results in the control of many economical important pests, such as biopesticide products based of neem (*Azadirachta indica*) (Khater, 2012) and butterfly-pea (*Clitoria ternatea*) (Damalas & Koutroubas, 2018).

Pesticides and Phytophthora cinnamomi

Currently, only a single class of pesticides has proven to be effective in the control of the pathogen *P. cinnamomi* and readily available in the global market, the phosphites. This class of compounds are anionic forms of phosphonic acid (H_3PO_3) and their application in the infected plant decrease (but not prevent) the production of the oomycete sporangia and zoospores, and trigger a defense response in the targeted plant which inhibit the oomycete growth (Guest & Grant, 1991; Wilkinson et al., 2001). Despite these positive effects, zoospores produced by *P. cinnamomi* previously sprayed with phosphites are still viable and capable of infecting new plants (Wilkinson et al., 2001). Phosphites are also phytotoxic, inducing foliar necrosis in many studied species. Their intake and retention is highly variable from species to species and is directly correlated with the appearance and severity of the symptoms shown in treated plants, making these compounds application potentially harmful to some species (Pilbeam et al., 2000; Barrett et al., 2004). The practical application of these phosphites in large areas is conducted via aerial spraying, covering the entirety of the treated areas that usually comprises communities with rare and threatened plant species (Hardy et al., 2001). These detrimental consequences that come from the use of pesticides is unfortunately very common and does not only extend to the destruction of the surrounding flora, but also the contamination of the environment soils and waters.

1.2. *Phytophthora cinnamomi* Rands

P. cinnamomi is a diploid pathogenic oomycete (Kroon et al., 2004) capable of infecting a large number of plant species (likely close to 5000 species), among them important crops such as the avocado, chestnut, peach and pineapple (Hardham, 2005). The pathogen was first described by Rands in the west coast of Sumatra, causing bark canker in infected cinnamon trees *(Cinnamomum burmanni)*, hence its name (Rands, 1922). *P. cinnamomi* exhibits mycelial growth and produces motile flagellated asexual zoospores (Hardham, 2005; Beakes et al., 2012). The pathogen has both asexual and sexual phases during its life cycle, being the former favored over the latter (Hardham, 2005) **(Fig. 2)**.

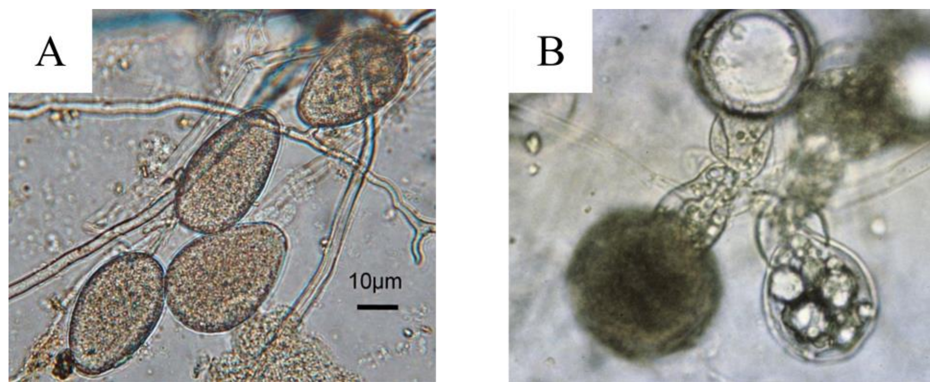

Figure 2. *Phytophthora cinnamomi*. **(A)** Asexual spore, sporangia; **(B)** Sexual spore, oogonia and antheridia.
(source: bugwood.org. photos: ***A*** *- Elizabeth Bush;* ***B*** *- Mary Ann Hansen)*

This oomycete has a cosmopolitan global distribution **(Fig. 3)**, reproducing rapidly in moist soils and in temperatures of 16-30 ºC, ideally around 22-28 ºC, at 4.0-7.0 pH (Chee & Newhook, 1965) and can survive up to six years at favorable conditions (Zentmyer & Mircetich, 1966). Thriving in waterlogged soils and after events of heavy rain, moisture is crucial to the development of the sporangia and the production and movement of the motile zoospores (Hardham, 2005). The oomycete is a major threat to natural ecosystems and biodiversity worldwide, causing heavy economic losses in agriculture and forestry (Hardham & Blackman, 2018). The pathogen causes the rotting of fibrous roots and stem cankers, as well as the dieback of young shoots. The oomycete usually invades its host via the finer roots, where the zoospores form cysts that germinate and grow a tube that penetrates the outer cell layer (Cahill et al., 2008). The infected roots have a significantly reduced water intake, which results in foliage chlorosis and the rapid wilting of the plant (Hardham & Blackman, 2018).

Having a large number of possible hosts, *P. cinnamomi* is responsible for many types of plant diseases worldwide, such as the Jarrah Dieback *(Eucalyptus marginata)* in south-western Australia (Shearer & Tippett, 1989; Hardham, 2005), root rot in *Quercus* species in the Iberian Peninsula (Brasier et al., 1993; Brasier, 1996), stem canker in peach tress *(Prunus persica)* in several USA states (Haygood et al., 1986; Mircetich & Keil, 1970) and dieback or "ink disease" in chestnut trees *(Castanea sativa)* (Crandall et al., 1945; Vettraino et al., 2005) across Europe, among others. The oomycete also indirectly affects the fauna of the habitats it infects, disrupting food chains and reducing birds nesting sites, as the infection results in the substantial loss of plant canopy area (Cahill et al., 2008; Garkaklis et al., 2004). Given its high contagion rate and global dispersion, the pathogen was placed in the top 100 most dangerous species in the world by the Invasive Species Specialist Group (ISSG) (van der Weijden et al., 2017).

No thoroughly effective solutions currently exist to eradicate *P. cinnamomi* once the infection is established. Human activity management is the main approach to *P. cinnamomi* control, as humans are the fastest and widest vector of this oomycete propagation, which by itself spreads rather slowly (O'Gara et al., 2005). This management consists in the adoption of preventive practices and operations in risk-prone areas and field personnel sensibilization and training (Hardy et al., 2001).

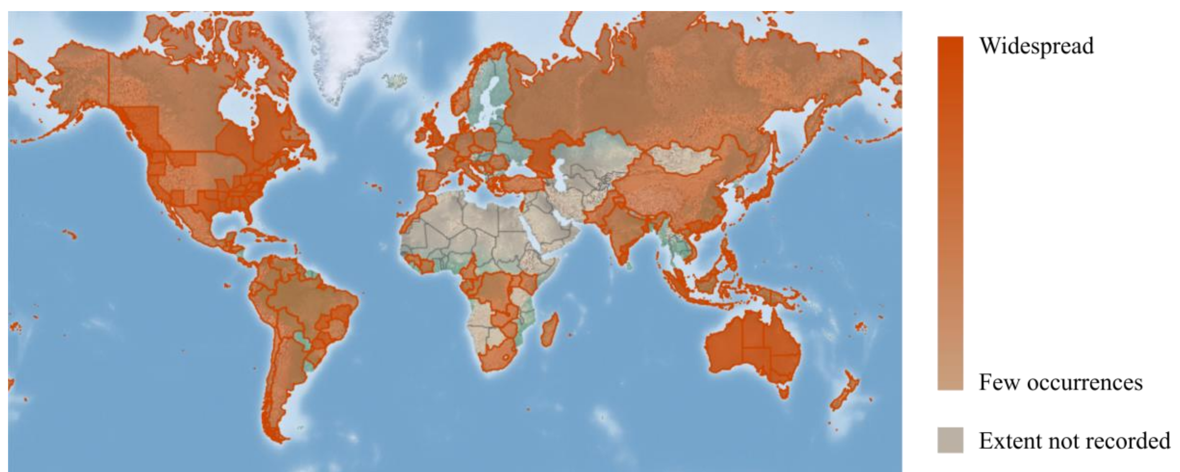

Figure 3. Global distribution of *Phytophthora cinnamomi*.
(source: cabi.org. Accessed: 17/06/2021)

1.3. *Solanum betaceum* Cav.

Taxonomy, ecology and distribution

Solanum betaceum (Cav.), tamarillo or "*tree-tomato*" (Wiersema & León, 2016) belongs to the *Solanum* genus and Solanaceae family. This genus accounts for half this family diversity

and is considered the most economically important genus within Solanaceae (Frodin, 2004; Bohs, 2007). The species is a subtropical plant native to the Andes of Peru, Chile, Ecuador, Colombia and Bolivia **(Fig. 4A)**, where wild populations of tamarillo occur along with the other closely related species of the same genus (Bohs, 1989b; Prohens & Nuez, 2001). The tamarillo tree is commonly found growing in mountainous regions in medium to high altitudes, especially in its native regions of South America. (Morton, 1982; Duke & DuCellier, 1993). Ideally, tamarillo naturally grows in regions with an average annual temperature of 15 to 25 ºC, and rainfall between 1000-2000 mm (minimum 800 mm). Tamarillo flowers and fruits throughout the entire year, suggesting no photoperiodic response (Duarte & Paull, 2015). The species was probably spread by the Spanish throughout Latin America, and later was dispersed to the tropics and subtropics of Africa, South Asia, China, Australia, New Zealand, USA and Europe **(Fig. 4B)**. Studies report that the tamarillo shows effective establishment conditions in the Mediterranean region (Prohens et al., 1997). To this day the species is cultivated in all these regions around the world, such as Jamaica, Puerto Rico, Costa Rica, Haiti, Guatemala, Sri Lanka, New Guinea, China, South Africa and many others (Bohs, 1989b; Duke & duCellier, 1993).

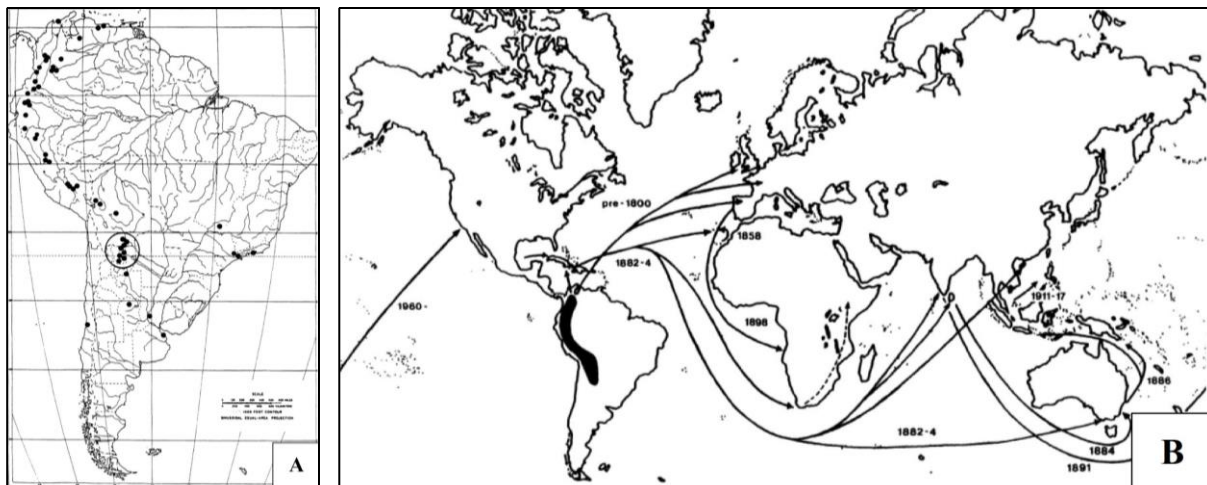

Figure 4. Distribution of *S. betaceum* in the native region of South America. **(A)** Native distribution in South America; **(B)** Global dispersal. (Adapted from Bohs, 1989b)

Botanical characterization

S. betaceum is a tree which normally grows 2-4 m tall. Its branches are narrow and brittle, branching out of the main stem to form a wide dichasial crown (Bohs, 1989b; Bohs, 1994; Lewis & Considine, 1999b) **(Fig. 5A)**. The tree starts to produce fruits after 1-2 years (Prohens

& Nuez, 2001) and can produce fruit for 8-12 years (Bakshi et al., 2016). The tamarillo tree has large simple leaves with long petioles, are softly pubescent on both sides and have a characteristic musky smell. (Bohs, 1989b; Bohs, 1994; Prohens & Nuez, 2001; Duarte & Paull, 2015). *S. betaceum* flowers grow in buds of 10-50 flowers (Lewis & Considine, 1999b). They grow 1.5-2 cm wide, have a stellate shape with a pinkish-white pentamerous corolla, green-purple calyx and five thick stamens. Tamarillo flower petals are fleshy, glabrous and slightly curved at the tip (Bohs, 1994; Lewis & Considine, 1999b; Duarte & Paull, 2015) **(Fig. 5B)**.

The fruit has a ellipsoid to ovoid shape and grow singly or in clusters of 3-12 fruit sets (Bohs, 1994; Duarte and Paull, 2015). They are glabrous with a yellow to orange, red or purple color, often presenting long longitudinal stripes depending on the fruit variety **(Fig. 5C)**. The mesocarp is meaty and firm, with a bland to bitter flavor (Bohs, 1989b; Bohs, 1994; Duarte & Paull, 2015). The fruit grows rapidly, reaching full size in about 16 weeks and maturity in around 27 weeks after anthesis (Heatherbell et al., 1982). The fruit is acidic with a pH=4.0 ± 0.17 (Romero-Rodriguez et al., 1994) and is highly nutritious (Morton, 1982).

Figure 5. *Solanum betaceum*. **(A)** Young tamarillo tree supported by a stake; **(B)** Tamarillo flower bud with flowers in different stages of maturation; **(C)** Tamarillo fruit (red-type).
*(photos: **A** - Bruno Costa; **B,C** – Gonçalo Pereira)*

1.4. *Solanum betaceum* chemical characterization

Phenolic compounds

Having many varieties and cultivars, tamarillo bioactive compounds concentration differ between varieties and cultivars (Acosta-Quezada et al., 2015). Nevertheless, all tamarillo fruit varieties present high concentrations of similar phenolic compounds, conferring the fruit

substantial antioxidant properties (Acosta-Quezada et al., 2015; Espin et al., 2016). Ghosal et al. (2013) reports that the fruit accumulates phenols as it matures, reaching an high concentration of these compounds in later maturity stages. This increasingly higher reducing power throughout fruit ripening was also observed by Vasco et al. (2009). Studies in cultivars from Spain (Espin et al., 2016) and Ecuador (Vasco et al., 2009) report hydroxycinnamoyl derivatives (yellow-giant: 60.25-110.23 mg/100 g DW; purple-giant: 132.57-421.55 mg/100 g DW) and hydroxycinnamic acid derivatives (golden-yellow: 39 mg/100 g FW; purple-red: 61 mg/100 g FW) as the most abundant phenolic compounds present in the fruits, respectively. Mertz et al. (2009) also reports an abundant presence of hydroxycinnamic acids in *S. betaceum* fruit extracts, such as caffeoylquinic acid (red: 54.8 mg/100 g DW; yellow: 32.8 mg/100 g DW). DW stands for “dry weight”. Tamarillo phenolic content is found in the **Table 2**.

Anthocyanins and flavonoids

Tamarillo fruits presents a variable concentration of anthocyanins according to cultivar location and fruit color (Hurtado et al., 2009; Espin et al., 2016). These compounds accumulate as the fruit ripens (Heatherbell et al., 1982) and are more concentrated in the fruit pulp (4.15 mg C-3-GE/100 g DW) than in the peel (1.36 mg C-3-GE/100 g DW) (Hassan & Bakar, 2013). The yellow fruit varieties tend to have negligeable amounts of anthocyanins, as shown in Vasco et al. (2009), Mertz et al. (2009) and Espin et al. (2016). The major anthocyanins found in tamarillo fruit are delphinidin-3-rutinoside, cyanidin-3-rutinoside and pelargonidin-3-rutinoside (Wrolstad & Heatherbell, 1974; Mertz et al., 2009; Hassan & Bakar, 2013). Delphinidin-3-rutinoside is the main anthocyanin in the fruit pulp and cyanidin-3-rutinoside is the main anthocyanin in the fruit peel, in most of the worldwide studied varieties. Pelargonidin-3-rutinoside is the main anthocyanin present in studied Ecuadorian cultivars (Wrolstad & Heatherbell, 1974). The total flavonoid content was higher in the fruit peel (3.36 mg RE/g) than in the fruit pulp (2.41 mg RE/g) (Hassan & Bakar, 2013). Vasco et al. (2009) found the flavonols quercetin (golden-yellow: 6 mg/100 g FW; purple-red: 4 mg/100 g FW) and myricetin (golden-yellow: 1.2 mg/100 g FW; purple-red: 1.4 mg/100 g FW) in tamarillo fruits. FW and RE stands for “fresh weight” and “rutin equivalent”, respectively. Tamarillo anthocyanin and flavonoid content is found in the **Table 2**.

Table 2. Phenolic compounds content in *S. betaceum* fruits.

Reference	[A]		[B]		[C]		[D]		
Region	Ecuador		Penampang, Malaysia		New Zealand		Loja, Ecuador		
Obs.	Golden-yellow	Purple-red	Striped-red		N.D.		Orange + orange-pointed	Red + red-pointed	Purple
			Pulp	*Peel*	*Pulp*	*Peel*			
Total phenolic compounds	125 ± 6.2[1]	187 ± 3.7[1]	261 ± 12.0[3]	489 ± 4.0[3]	-	-	2.43 – 6.18[6]	2.94 – 4.39[6]	2.58 – 6.12[6]
Anthocyanins	undetected	38 ± 0.2[2]	2.15 ± 0.14[4]	1.36 ± 0.1[4]	0.98[7]	0.32[7]	-	-	-
Flavonoids	-	-	241 ± 2.0[5]	336 ± 1.0[5]	-	-	-	-	-

[A] Vasco et al., 2009; **[B]** Hassan & Bakar, 2013; **[C]** Wrolstad & Heatherbell, 1974; **[D]** Acosta-Quezada et al., 2015.

[1] Gallic acid equivalent (mg GAE/100 g FW)
[2] Cyanidin-3-glucoside equivalent (mg C-3-GE/100 g FW)
[3] Gallic acid equivalent (mg GAE/100 g DW); Results converted from mg GAE/g to mg GAE/100 g
[4] Cyanidin-3-glucoside equivalent (mg C-3-GE/100g DW)
[5] Rutin equivalent (mg RE/100 g DW); Results converted from mg RE/g to mg RE/100 g
[6] g/100 g DW
[7] µmol/g FW

Fatty acids

S. betaceum fruits are extremely poor in lipids (Wang & Zhu, 2020). Previous studies by Castro-Vargas et al. (2013) report low yields of nonpolar lipidic compounds in the pericarp of tamarillo fruits, corroborating this assessment to a certain degree. Ramakrishnan et al. (2013) and Achicanoy et al. (2018) conducted studies assessing the fatty acid composition of tamarillo seeds using supercritical CO_2 extraction (SC-CO_2) and Soxhlet extraction (SE). Both studies revealed that tamarillo seeds seem to be much richer in fatty acids than the rest of the fruit, as proven by lipid extraction yields: 17.4%, optimal SC-CO_2 yield; 21.13 and -24.05%, SE yield. The major type of fatty acids (FA) present in the fruit seeds are polyunsaturated fatty acids (PUFAs) (72.20% and 72.05%), followed by monounsaturated fatty acids (MUFAs) (15.47% and 16.18%), and saturated fatty acids (SFAs) (12.32% and 11.80%). The most abundant fatty acids in tamarillo seeds are linoleic (SC-CO_2: 66.67%; SE: 70.47% and 71.30%), oleic (SC-CO_2: 17.94%; SE: 14.93% and 17.3%) and palmitic (SC-CO_2: 10.41%; SE: 9.41% and 8.0%) (Ramakrishnan et al., 2013; Achicanoy et al., 2018). Tamarillo fatty acids content is found in the **Table 3**.

Table 3. Fatty acid percentage content in *S. betaceum* seed oil.

Reference	[A]	[B]
Region	Cameron Highlands, Malaysia	Colombia
Obs.	Seed oil (%)	Seed oil (%)
Polyunsaturated fatty acids	72.20	72.05
Monounsaturated fatty acids	15.47	16.18
Saturated fatty acids	12.32	11.80

[A] Ramakrishnan et al., 2013; **[B]** Achicanoy et al., 2018.

Carotenoids

Tamarillo has a high content in terpenoids (Wang & Zhu, 2020), mainly in carotenoids. Studied cultivars from Ecuador (Mertz et al., 2009), Colombia (Giuffrida et al., 2018), Brazil (Rodriguez-Amaya et al., 1983) and China (Yang & Zhao, 2013) present a rich diversity of carotenoids, such as β-carotene, cryptoxanthin, lutein and zeaxanthin. The commonest carotenoids found in the tamarillo fruit are precursors of provitamin A (Bauernfeind, 1972), making the fruit a valuable source of vitamin A (Rodriguez-Amaya, 1999). Rodriguez-Amaya et al. (1983) reports a vitamin A value of 2475 IU/100 g EW, and Mertz et al. (2009) 2000 RE/kg FW. EW stands for "edible weight". Hassan & Bakar (2013) determined that the total carotenoid content in the pulp is 25.13 mg BE/100g DW, and 19.13 mg BE/100 g DW in the peel. Rodriguez-Amaya et al. (1983) report similar carotenoid concentration results, with 24.3 μg/g FW in the pulp and 22.0 μg/g FW in the peel. "BE" stands for "β-carotene equivalent". Studies in extracts of tamarillo fruit indicate that the major free carotenoids in tamarillo fruits are β-carotene and β-cryptoxanthin. (Rodriguez-Amaya et al., 1983; Mertz et al., 2009; Giuffrida et al., 2018) Carotenoid esters account for 78% of the total carotenoid composition (Mertz et al., 2009). Tamarillo carotenoids content is found in the **Table 4**.

Table 4. Total carotenoid content and main carotenoids in *S. betaceum* fruits.

Reference	[A]		[B]		[C]	
Region	Ecuador		Brazil		Penampang, Malaysia	
Obs.	Yellow (μg LE/g FW)	Red (μg LE/g FW)	Fruit pulp (μg/g FW)	Fruit peel (μg/g FW)	Fruit pulp (mg BE/100 g DW)	Fruit peel (mg BE/100 g DW)
β-Carotene	4.6 ± 0.3[1]	5.1 ± 0.3[1]	7.9 ± 3.6	8.8 ± 3.5	-	-
Cryptoxanthin	1.1 ± 0.1[1]	1.5 ± 0.08[1]	13.9 ± 4.2	10.0 ± 2.8	-	-
Zeaxanthin	0.1 ± 0.02[1]	0.3 ± 0.06[1]	0.6 ± 0.6	1.1 ± 0.6	-	-
Lutein	0.98 ± 0.05[2]	1.25 ± 0.05[2]	1.7 ± 1.1	1.5 ± 0.3	-	-
Total carotenoids	-	-	24.3	22.0	25.13 ± 0.35	19.13 ± 1.93

[A] Metz et al., 2009; **[B]** Rodriguez-Amaya et al., 1982; **[C]** Hassan & Bakar, 2013.

[1] Before saponification; [2] After saponification (Lutein concentration is exceptionally noted in β-carotene equivalents (μg BE/g FW).

Alkaloids

The presence of alkaloids and other N-containing compounds in *S. betaceum* is well documented. Nevertheless, the specific plants organs where these compounds exist and their quantification is not clearly reported (Wang & Zhu, 2020). Eich (2008) compiled and described various alkaloids found in *S. betaceum*, alongside the studies in which these compounds were found. Pyrrolidines like solamine, solacaproine, tropinone, cuscohygrine and tomatidenol have

been found in unspecified parts of the tamarillo plant (Wang & Zhu, 2020). Evans et al. (1972) also reports the presence of the aforementioned compounds (with the exception of tomatidenol) in the tamarillo roots, alongside other compounds like amines. Schröter & Neumann (1964) also describe the presence of tropane and steroid alkaloids in *S. betaceum* roots. Trace amounts of tropane-derived calystegins were found in tamarillo fruits by Asano et al. (1997b), but more recent tests seem to indicate the absence or an undetectable concentration of alkaloids in *S. betaceum* fruits (Vasco et al., 2009).

Sugars and other acids

Tamarillo fruits have a moderate amount of sugar compounds and other carbohydrates such as organics acids and pectins. In comparison with other tropical fruits, *S. betaceum* holds a relatively low sugar content (Wills et al., 1986; Vasco et al., 2009), which make the fruit an healthier alternative to its counterparts. The sugar concentration increases as the fruit matures, and it slightly varies between different tamarillo varieties (Heatherbell et al., 1982; Acosta-Quezada et al., 2015). The three main sugars found in the tamarillo fruit are glucose, sucrose and fructose (Heatherbell et al., 1982). *S. betaceum* fruits present considerable amounts of citric acid and ascorbic acid (vitamin C), with comparatively low concentrations of malic acid and quinic acid (Romero-Rodriguez et al., 1994; Vasco et al., 2009; Acosta-Quezada et al., 2015). Both citric acid and malic acid are produced during fruit maturation, and their concentration decreases substantially after fruit ripening (Heatherbell et al., 1982). Various monosaccharides were isolated from tamarillo pulp and seed mucilage, the main ones being arabinose, xylose, galactose and uronic acids. The detection of these compounds indicate the presence of long chain pectins in the tamarillo fruit and mucilage (do Nascimento et al., 2013; do Nascimento et al., 2016). Cacioppo (1984) and Romero-Rodriguez et al. (1994) report a high content of ascorbic acid in yellow (30.0-35.0 mg/100g FW and 19.7 mg/100g FW, respectively) and red (35.0-45.0 mg/100g FW and 21.9 mg/100g FW, respectively) tamarillo varieties, making its fruits a great source of vitamin C. Tamarillo sugar and acid content is found in the **Table 5**.

Table 5. Sugar and organic acids compounds in *S. betaceum* fruits.

Reference	[A]		[B]		[C]			[D]		
Region	Galicia, Spain		Ecuador		Loja, Ecuador			New Zealand		
Obs.	Yellow (%)	Red (%)	Golden-yellow (%)	Purple-red (%)	Orange + orange-pointed (g/100 g DW)	Red + red-conical (g/100 g DW)	Purple (g/100 g DW)	ASR (g/100 g FW)	SR (g/100 g FW)	GM (g/100 g FW)
Glucose	0.5 ± 0.10	1.0 ± 0.23	1.7 ± 0.02	1.4 ± 0.10	6.3 – 12.4	8.0 – 10.2	7.4 – 9.6	0.774	0.707	0.923
Fructose	0.7 ± 0.14	1.2 ± 0.14	1.6 ± 0.10	1.4 ± 0.10	6.3 – 13.2	8.4 – 9.9	7.4 – 10.1	1.079	0.910	1.139
Sucrose	1.6 ± 0.26	2.5 ± 0.27	1.9 ± 0.10	1.7 ± 0.10	15.5 - 27.1	20.8 – 24.1	18.3 – 25.8	1.813	1.100	1.891
Citric acid	1.8 ± 0.11	1.7 ± 0.03	2.5 ± 0.10	2.7 ± 0.04	5.42 – 7.54	4.01 – 6.39	5.50 – 6.91	1.27	1.35	1.71
Malic acid	0.07 ± 0.01	0.05 ± 0.01	0.07 ± 0.03	0.53 ± 0.02	0.34 – 0.78	0.46 – 0.99	0.53 – 0.88	0.14	0.13	0.15
Quinic acid	0.8 ± 0.06	0.4 ± 0.03	-	-	-	-	-	-	-	-

[A] Romero-Rodriguez et al., 1994; **[B]** Vasco et al., 2019; **[C]** Acosta-Quezada et al., 2015; **[D]** Boyes & Strübi, 2010.

ASR – Andys Sweet Red variety; **SR** – Secombes Red variety; **GM** – Goldmine variety.

Minerals

The mineral content in tamarillo fruits is highly variable between different cultivars and regions, but all studied cultivars are particularly rich in potassium and phosphorus, minerals which are usually present at low concentrations in fruits (Dawes & Callaghan, 1970). Romero-Rodriguez et al. (1994) reports concentrations of potassium as high as 404 ± 47.1 mg/100 g FW in yellow tamarillo variant in Galicia cultivars and Vasco et al. (2009) reports concentrations reaching 398 ± 11.3 mg/100 g FW in Ecuador cultivars. The species fruits also hold a relatively high concentration of calcium and magnesium (Vasco et al., 2009; Acosta-Quezada et al., 2015). Tamarillo mineral content is found in the **Table 6**.

Table 6. Mineral content in *S. betaceum* fruits.

Reference	[A]		[B]		[C]		[D]		
Region	Galicia, Spain		Ecuador		New Zealand		Loja, Ecuador		
Obs.	Yellow (mg/100 g FW)	Red (mg/100 g FW)	Golden-yellow (mg/100 g FW)	Purple-red (mg/100 g FW)	Yellow (mg/100 g FW)	Red (mg/100 g FW)	Orange + orange-pointed (mg/100 g DW)	Red + red-conical (mg/100 g DW)	Purple (mg/100 g DW)
Sodium (Na)	4.9 ± 0.66	8.9 ± 2.9	0.06 ± 0.001	0.20 ± 0.001	-	-	-	-	-
Potassium (K)	404 ± 47.1	347 ± 14.7	398 ± 11.3	379 ± 3.4	292.0	321.0	-	-	-
Calcium (Ca)	10.6 ± 0.05	9.3 ± 1.09	25 ± 0.8	22 ± 0.2	11.0	11.0	34 – 80	42 – 70	16 – 78
Magnesium (Mg)	22.3 ± 2.09	19.7 ± 1.8	16 ± 0.5	14 ± 0.4	20.0	21.0	58 – 230	48 – 221	54 – 214
Iron (Fe)	0.4 ± 0.03	0.4 ± 0.05	0.22 ± 0.02	0.46 ± 0.01	0.44	0.57	0.68 – 2.32	1.12 – 1.64	1.00 – 2.43
Copper (Cu)	0.2 ± 0.06	0.2 ± 0.05	0.08 ± 0.01	0.12 ± 0.01	0.06	0.05	0.07 – 3.25	0.06 – 0.82	0.07 – 0.70
Zinc (Zn)	0.2	0.2 ± 0.03	0.20 ± 0.02	0.17 ± 0.01	0.17	0.15	0.21 – 1.44	0.46 – 1.83	0.35 – 1.13
Manganese (Mn)	0.1	0.1	-	-	0.185	0.114	-	-	-
Phosphorus (P)	-	-	-	-	40.0	39.0	-	-	-

[A] Romero-Rodriguez et al., 1994; **[B]** Vasco et al., 2009; **[C]** Diep et al., 2020; **[D]** Acosta-Quezada et al., 2015.

1.5. *Corema album* (L.) D. Don

Taxonomy, ecology and distribution

Corema album (L.) D. Don, Portuguese-crowberry or "*camarinha*" is a member of the Ericaceae family. *Corema* is a small genus with only two species, *C. album* and *C. conradii*, both being small coastal dioecious shrubs with drupaceous fruits. *C. album* has two subspecies, *C. album* subsp. *album* and *C. album* subsp. *azoricum* P. Silva, the latter being endemic to the Azores islands of Faial, Graciosa, Pico, São Jorge and São Miguel (DRAAC, 2010), being a protected species in the region (Decreto Legislativo Regional 15/2012/A of April 2 of Regime Jurídico Da Conservação Da Natureza e Da Proteção Da Biodiversidade, 2012).

C. album occurs in the Atlantic coast of the Iberian peninsula (León-González et al., 2013). The crowberry habits sand dunes and rocky-coast sites (Guitián et al., 1997). Its distribution spans the entire west Iberian peninsula coastal region, from the north of Galicia to the south of Gibraltar (Álvarez-Cansino et al., 2010; Zunzunegui et al., 2006), except for the coastal region of Douro in Portugal, where the species does not seem to be established (Blanca et al., 2000) **(Fig. 6)**. Crowberry is commonly found forming associations alongside species like *Pinus pinea*, *Juniperus phoenicea*, *Pistacia lentiscus*, *Calluna vulgaris*, *Cistus crispus*, among several other species (Ferreira, 2018; López-Dóriga, 2018). Crowberry is found from seaside up to 50 meters above sea level (López-Dóriga, 2018), growing in regions with an annual temperature of roughly 15 ºC (13.4-16.8 ºC) and high variations in annual rainfall, ranging from 540 mm to 1355 mm, according to reports from studied population sites in Álvarez-Cansino et al. (2013). *C. album* can withstand harsh summer drought events, which are progressively common in the Mediterranean basin (Christensen & Christensen, 2007). The species is especially vulnerable to habitat changes, as it inhabits coastal regions prone to touristic recreation, infrastructure expansion and sand gathering for construction materials (Blanca et al., 2000; Clavijo et al., 2002; Gil-López, 2011).

Figure 6. Distribution of *C. album* in the Iberian Peninsula coastline, with several studied populations marked. (Adapted from Álvarez-Cansino et al., 2013)

NOTE: Although the name "crowberry" is more commonly used to identify the *Empetrum nigrum* species (black-crowberry), throughout the entire thesis *Corema album* (Portuguese-crowberry) is commonly referred as "crowberry" for readability sake.

Currently in Spain there are only two populations of *C. album* that have male and female plants in all age ranges and therefore relatively safe from extinction, while all the other isolated populations of crowberry consist mainly of old individuals and are doomed to disappear, unless protective conservation measures are urgently implemented (Aguilella & Laguna, 2009).

Botanical characterization

C. album is a perennial evergreen shrub that grows between 30–75 cm tall (Guitián et al., 1997; Oliveira & Dale, 2012), is densely branched from the base and can reach up to 3 m in diameter (Clavijo et al., 2002) **(Fig. 7A)**. Although the crowberry is mostly a dioecious species, hermaphrodite crowberries may occur in low proportions, only occurring in bigger ratios in specific regions and populations (Zunzunegui et al., 2006). The species deep root system can be complemented by adventitious roots sprouting from branches that may become buried in the sediment, such as sand blown by the wind (Álvarez-Cansino et al., 2010). *C. album* has small linear ericoid dark-green leaves with a prominent groove along the abaxial axis. This groove is a result of the leaf blade folding into itself (Tutin et al., 1972; Villar, 1993; Oliveira & Dale, 2012). Crowberry leaves have a short petiole and grow laid against the stem alternated in whorls of 3-4 leaves (Villar, 1993; Oliveira & Dale, 2012). The leaves are covered in sessile glands when young, becoming glabrous when mature (Tutin et al., 1972).

C. album flowers are tightly packed in terminal racemose inflorescences of variable morphology, depending on the plant sex (Guitián et al., 1997; Oliveira & Dale, 2012). Female plants have 1-2 flowers per inflorescence and male plants have 4-14 flowers per inflorescence with ovate to acuminate bracts (Villar, 1993; Zunzunegui et al., 2006). Both the male and female flowers are actinomorphic with 3 pinkish-red obovate petals and 3 green pubescent sepals **(Fig. 7B, 7D)**. Male flowers have 3 exert stamens with conspicuous red-purple anthers (Villar, 1993; Oliveira & Dale, 2012). Female flowers are smaller than their male counterparts, and in some cases may completely lack their petals (Tutin et al., 1972). Crowberries usually flowers from early March to mid-April, but can flower as early as February (Blanca et al., 2000). The fruit is a small pinkish-white spherical berry-like drupe (Tutin et al., 1972; Guitián et al., 1997; Oliveira & Dale, 2012) **(Fig. 7C)**, highly acidic with a pH of around 3, a moisture content of 81.7% (Brito et al., 2021) and a characteristic lemony flavor. The fruit usually contains three seeds enclosed by a thick woody endocarp (pyrenes), but the number can change between 2-9 (Guitián et al., 1997; Calviño-Cancela, 2002; Zunzunegui et al., 2006). Fruiting occurs during early summer through late autumn, peaking during August and early September (Oliveira & Dale, 2012).

Figure 7. *Corema album*. **(A)** Crowberry shrub **(B)** Male flower; **(C)** Crowberry fruit; **(D)** Female flower.
(source: jb.utad.pt; floradegalicia.wordpress.com. photos: ***A*** *– José Guimarães;* ***B*** *– João Moleiro;* ***C*** *– Martin Sirovs;* ***D*** *- Henry David Thoreau)*

1.6. *Corema album* chemical characterization

Phenolic compounds

C. album is rich in phenolic compounds, especially in the plant leaves and fruits, conferring the plant a high antioxidative power (Brito et al., 2021). Crowberry fruits have a high concentration of phenolic acids (2268.1 mg/kg DW), flavonoids (1437 mg GAE/100 g pulp), tannins (871 mg GAE/100 g pulp), and in lower concentrations, ortho-diphenols (21.8 mg GAE/100 g pulp) and anthocyanins (39.2 mg/kg DW) (León-González et al., 2013; Andrade et al., 2017). GAE stands for "gallic acid equivalents". Having a white to colorless fruit, a low concentration of anthocyanins is to be expected in *C. album*, as opposed to other colored berries which have high concentrations of these phenols (Jakobek et al., 2007). The seeds alone have almost as much phenolic compounds as the entirety of the crowberry fruit. Phenolic compounds are abundant in *C. album* leaves (247 mg GAE/100 g leaf weight) and can be found in trace amounts on the plant flowers (32 mg GAE/100g flower weight) (Brito et al., 2021).The main phenolic compounds found in *C. album* fruit are chlorogenic acid, caffeic acid and flavonol derivatives, mainly myricetin and quercetin (León-González et al., 2013). In the leaves, the phenols consist mostly of flavonols, such as myricetin and epicatechin (Macedo et al., 2015). Crowberry phenolics content is found in the **Table 7**.

Table 7. Phenolic contents in *C. album* fruits, leaves and flowers.

Reference	[A]				[B]	[C]			
Region	Mira, Portugal				Huelva, Spain	Leiria, Portugal			
Obs.	White fruit ($\bar{X}$*)		Translucent fruit ($\bar{X}$*)		Fruit (mg/100 g DW)	Fruit (mg GAE/100 g DW)		Leaves (mg GAE/100g FW)	Flowers (mg GAE/100g FW)
	Pulp	*Seed*	*Pulp*	*Seed*		*Pulp*	*Seed*		
Total phenolic compounds	1517.7[1]	737.3[1]	1342.3[1]	777.3[1]	226.81 ± 22.99	3 ± 1.0	29 ± 1.0	247 ± 21.0	32 ± 2.0
Anthocyanins	-	-	-	-	3.92 ± 0.48	-	-	-	-
Flavonoids	1204.0[2]	546.0[2]	1055.0[2]	531.3[2]	-	-	-	-	-
Flavonols	-	-	-	-	63.83 ± 8.01	-	-	-	-
Tannins	818.3[3]	614.0[3]	732.0[3]	577.0[3]	-	-	-	-	-
Ortho-diphenols	18.8[1]	15.7[1]	17.9[1]	14.8[1]	-	-	-	-	-

[A] Andrade et al., 2017; **[B]** León-González et al., 2013; **[C]** Brito et al., 2021.
* Average of three different extraction methods: ethanol:water (50:50, v:v); acetone:water (60:40, v:v); methanol (100%).

[1] Gallic acid equivalent (mg GAE/100 g)
[2] Quercetin equivalent (mg QE/100 g)
[3] mg/100 g

Fatty acids

C. album is an interesting source of healthy lipids. The fatty acids (FA) present in *C. album* leaves and fruits are predominantly polyunsaturated (PUFAs), especially in the fruit seeds, where PUFAs constitute 81.9% of their total fatty acid content (Brito et al., 2021). These results go in accordance with prior studies by Martin et al. (2019), which revealed that *C. album* fruit seeds are rich in unsaturated lipids. Saturated fatty acids (SFAs) are the second most common fatty acids present in the leaves (31.6%) and fruit pulp (40.4%), and the least common in the fruit seeds (5.8%). Monounsaturated fatty acids (MUFAs) appear in low quantities in the leaves (7.77%) and fruit pulp (11.9%) in comparison with the PUFAs and SFAs. The most common fatty acid in crowberry's leaves and seeds is alpha-linolenic (leaves: 13.87%; seeds: 50.8%), and in the fruit pulp is eicosapentanoic (30.7%) (Brito et al., 2021). Crowberry fatty acids content is found in the **Table 8**.

Table 8. Fatty acid percentage content in *C. album* fruit and leaves.

Fatty acid (%)	Fruit pulp	Fruit seeds	Leaves
Polyunsaturated FA	47.0 ± 2.0	81.9 ± 1.1	55.4 ± 1.1
Monounsaturated FA	11.9 ± 1.4	12.3 ± 0.3	7.8 ± 0.3
Saturated FA	40.4 ± 1.3	5.8 ± 0.9	31.6 ± 1.1

Terpenoids and alkaloids

Martin et al. (2019) reports the presence of various undetermined terpenoid compounds in *C. album* berries using FTIR and Raman analysis. These terpenoids are prevalent in the cuticular wax of the external fruit skin. Limited research has been published regarding the description of alkaloids and other nitrogen-containing compounds in *C. album*. Ferreira (2018) reports the presence of trigonelline in crowberry leaves (10.2 µg/100 mg) and fruits (2.5 µg/100 mg). This alkaloid is effective in the treatment of diabetes-associated neuropathy (Zhou et al., 2012) and various types of cancer (Hirakawa et al., 2005; Liao et al., 2015).

Minerals

The mineral content of the crowberry berry is generally higher than more typical berries, such as blackberries, raspberries and strawberries (Baby et al., 2018). The fruit is a good source of various minerals per edible weight (EW) such as potassium (230 mg/100 g EW), calcium (91.2 mg/100 g EW), iron (10.0 mg/100 g EW) and zinc (1.8 mg/100 g EW) (Brito et al., 2021). *C. album* fruit also has an high content in copper (2.3 mg/100 g of EW), which is a concerning factor to human consumption as copper's negative effects in human cells are well documented (Agarwal et al., 1989; Pablo Rodriguez et al., 2002; Ahamed et al., 2010). Crowberry mineral content is found in the **Table 9**.

Table 9. Mineral content in *C. album* fruits.

	Edible weight (mg/100 g)	**Dry weight (mg/100 g)**
Calcium (Ca)	91.2 ± 36.1	580 ± 230
Iron (Fe)	10.0 ± 4.8	63.5 ± 30.6
Copper (Cu)	2.3 ± 0.8	14.9 ± 5.0
Zinc (Zn)	1.8 ± 0.6	11.5 ± 3.8
Manganese (Mn)	0.9 ± 0.6	5.8 ± 4.1
Nickel (Ni)	0.4 ± 0.1	2.2 ± 0.3
Strontium (Sr)	1.0 ± 0.4	6.0 ± 2.8
Rubidium (Rb)	0.5 ± 0.3	3.2 ± 1.9
Potassium (K)	230 ± 10	2700 ± 100

1.7. Trichomes

Definition and functions

Trichomes are defined as unicellular or multicellular appendages which extend outwards from the surface of plant organs and originate exclusively from the epidermal cells (Johnson, 1975; Werker, 2000). Although to different extents, all major terrestrial plants possess

trichomes (Johnson, 1975) and the layer of trichomes as a whole in a given plant organ is referred as the indumentum (Karabourniotis et al., 2020). Trichomes can be found anywhere on the plant, be it in the stems and leaves or the flowers and fruits. They can also grow on the subterranean parts of the plant, consisting predominantly of roots hairs, formed by specialized cells called trichoblasts (Bibikova & Gilroy, 2002).

Trichomes are vital to the plant development, being a primary barrier against hazards such as herbivores, pathogens and UV irradiation (Xiao et al., 2016) by conferring the plant epidermis robust structural features and chemical protection (LoPresti, 2016). Various studies notice that plants produce more trichomes in newer leaves after herbivory damage (Agrawal, 1999; Björkman et al., 2008; Dalin et al., 2008), and trichome secretions compounds may help to deter pathogen infections (Karamanoli et al., 2005; Kliebenstein et al., 2005; Shepherd & Wagner, 2007). In contrast, the density of trichomes seems to be related to higher fungal infection success, as a more dense coat of trichomes provides easier adhesion sites to fungal cysts and higher humidity levels (Calo et al., 2006; Łaźniewska et al., 2012; Wang et al., 2020). The production of resinous secretions have been proven to regulate temperature and transpiration in evergreen xerophytes (Dell & McComb, 1979), and indirectly affect photosynthesis by eliminating potentially toxic waste in halophytes (Paulino et al., 2020). Trichomes can also guide and attract pollinators through their secretions (Wagner, 1991). Given the extensive diversity of trichomes their objective classification is not always easy (Werker, 2000). The major distinction made among trichomes is whether they are glandular or non-glandular (Wang et al., 2021; Werker, 2000).

Glandular trichomes

Glandular trichomes can be unicellular or multicellular (predominantly the latter) and are defined as secretory structures that can synthetize and store exudates in specialized secretory cells, rich in secondary metabolites such as terpenoids, phenols (Karabourniotis et al., 2020; Wagner et al., 2004; Wagner, 1991) and, to a lesser extent, alkaloids (Laue et al., 2000; Dai et al., 2010). Being capable of producing secretions rich in secondary metabolites, these glandular trichomes play a direct role in the protection against herbivory and pathogen infections, water loss regulation and pollinators attraction, based on the compounds present in the trichomes secretions (Peter & Shanower, 1998; Dai et al., 2010).

Non-glandular trichomes

Non-glandular trichomes are the trichomes whose main function is not the synthesis and secretion of exudates (Karabourniotis et al., 2020), rather acting as protective physical barriers (Hanley et al., 2007; Liu et al., 2017a). Non-glandular trichomes are more varied in terms of their morphology, size and density compared to glandular trichomes, being highly variable among plant species and within an individual plant itself (Dalin et al., 2008; Werker, 2000). Non-glandular trichomes can be unicellular and multicellular, branched or unbranched, and can range from small, soft hairs to stiff thorns and hooks. They can be described based on their general morphology as needle-like, hook-like, or antenna-like, and based on their shape and appendage number as simple, peltate, or stellate, among other morphological distinctions (Dalin et al., 2008; Liu et al., 2017a). Usually more than one type of non-glandular trichomes are present at the surface of the leaves (Werker, 2000). Although non-glandular trichomes lack a secretory mechanism, they are reportedly able to store phenolic compounds in vast quantities, especially during their early stages of development (El-Negoumy et al., 1986; Tattini et al., 2007; Karabourniotis et al., 2020).

1.8. Objective

The present study objective is to test and assess the anti-oomycete activity of two different plant species leaf ethanolic extracts fractions against *P. cinnamomi*. The species studied were the tree *Solanum betaceum*, native to the Andes region in South America, and the shrub *Corema album,* endemic to the Atlantic coast of the Iberian Peninsula.

2. MATERIALS AND METHODS

2.1. Plant material

Material collection locations

The plant material used in this study were the leaves of tamarillo trees and crowberry bushes. Both leaf types were collected by hand. *S. betaceum* leaves were collected from twelve different trees growing at the Botanic Garden of the University of Coimbra (40º12’22”N, 8º25’31”W, altitude 68m) whereas *C. album* leaves were collected from several bushes at Quiaios beach (40º13’27”N, 8º53’22”W, altitude 8m). Both collection locations can be seen in **Fig. 8**. After collection, both the tamarillo and crowberry leaves were stored in airtight plastic bags at -40 ºC and later freeze-dried.

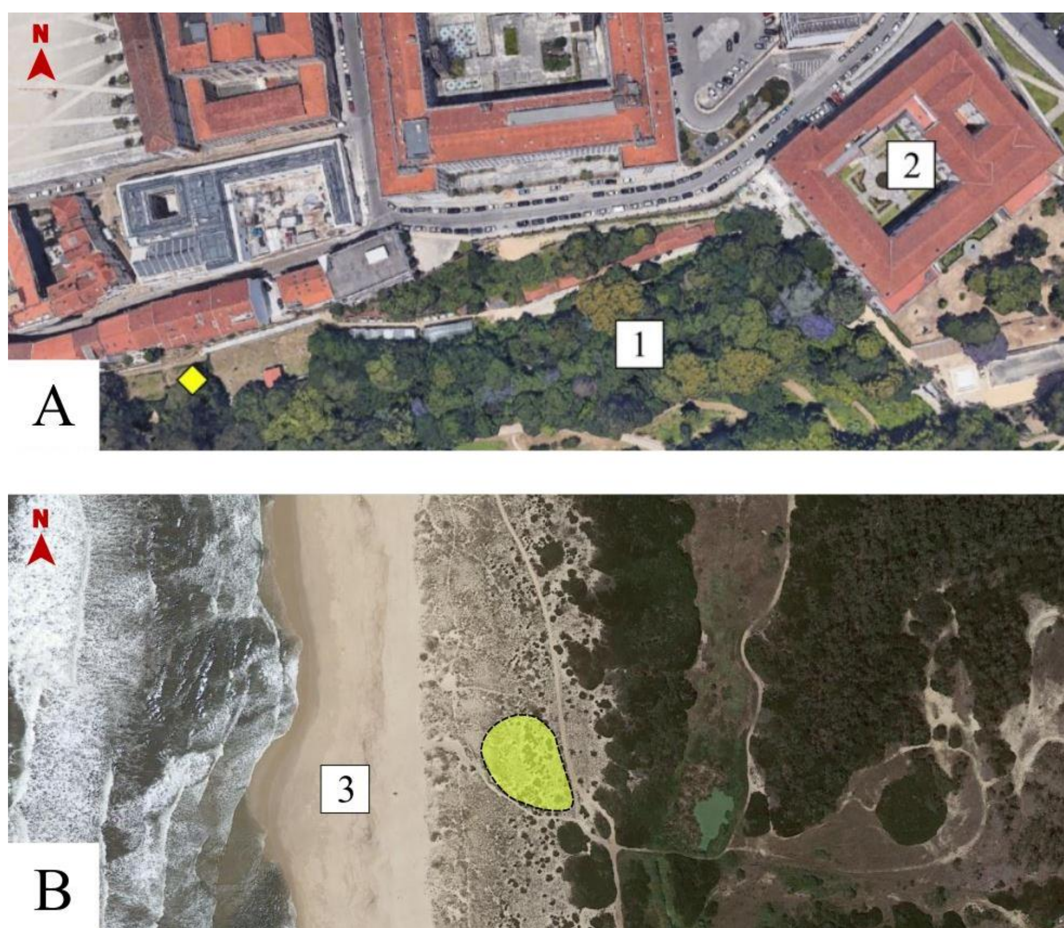

Figure 8. A - Tamarillo trees location, represented by the yellow diamond. **(1)** Botanical Garden of UC; **(2)** Departamento de Ciências da Vida of UC.
B – Portuguese-Crowberry bushes location, represented by the yellow area. **(3)** Quiaios beach. *(source: google.com/earth)*

Material processing

The leaves were freeze-dried and milled into a fine powder (≤ 1 mm granulometry) using a blender and a coffee grinder (Qilive Q.5684 and Qilive 870873, respectively), totaling a weight of 208.88 g of milled tamarillo material and 521.23 g of milled crowberry material, that accounts for 24.4 % and 38.6 % of the original tamarillo and crowberry material fresh weight, respectively. The milled material was stored in plastic bags at room temperature **(Fig. 9)**.

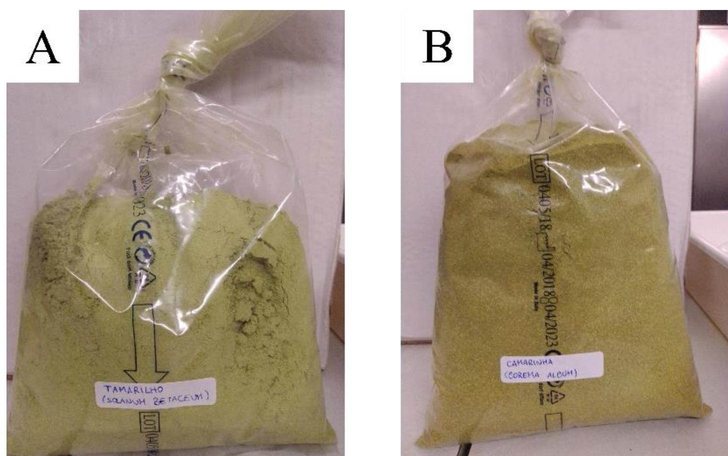

Figure 9. Plant milled material. **(A)** *S. betaceum*; **(B)** *C. album*.

2.2. *P. cinnamomi* cultures and growth medium

P. cinnamomi was freshly propagated from pre-established *in vitro* cultures (courtesy of João Martins) and collected as needed throughout the study. The oomycete was propagated in plastic Petri dishes with Potato Dextrose Agar (PDA) medium (OXOID, LabMal) that consist of a mixture of potato extract, glucose and agar. *P. cinnamomi* was left to grow in the dark at room temperature.

2.3. Plant extract preparation

Compound extraction using ethanol

Both tamarillo material (TAM) and crowberry material (CAM) were weighed in a 2 L erlenmeyer flask and mixed with ethanol 70 %. 76.7 g of milled TAM material and 100.3 g of milled CAM material were mixed with 1L of ethanol **(Fig. 10A)**. The mixtures were then stirred overnight (O/N) on a magnetic stirrer at 110 rpm. On the next day, the mixture supernatants were decanted via vacuum filtration (VWR VP-86, AVANTOR) **(Fig. 10B)**. To maximize the

compound extraction, the three aforementioned steps were repeated two more times, resulting in three total ethanol extractions for each plant material. The ethanol extract was stored in Schott bottles **(Fig. 10C)** and the solid biomass residue was stored at 4 ºC.

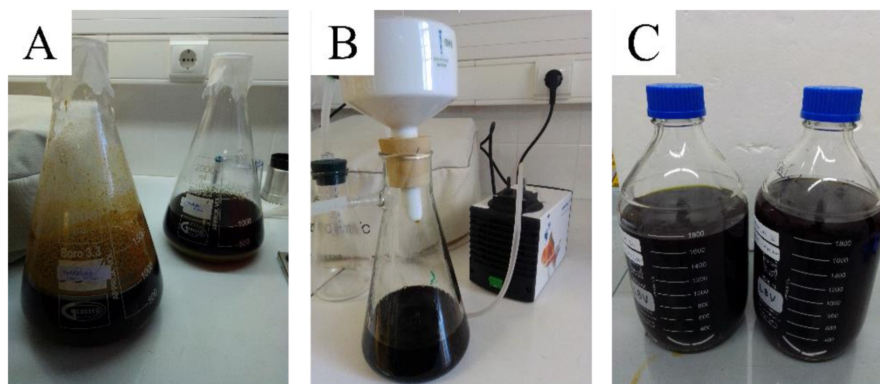

Figure 10. Preparation of the ethanol extracts. **(A)** Mix of milled plant material with ethanol; **(B)** Vacuum filtration; **(C)** The final TAM and CAM ethanol extracts.

Liquid phase and solid biomass - Ethanol and chloroform extract preparation

To evaporate the ethanol from the extract solution, a rotary evaporator (BÜCHI R-200) was used with a 40º C heating bath (BÜCHI B-490) **(Fig. 11A)**. The extract evaporation was done 175 mL at a time in a 500 mL round flask. Due to the substantial volume of ethanol extract obtained from the prior extraction, the ethanol evaporation was proven to be cumbersome and time consuming. Both TAM and CAM ethanol evaporated extracts differ greatly from the ethanol extracts before evaporation, having a cloudy appearance due to the suspension of non-water-soluble compounds **(Fig. 11B)**. After evaporation, the evaporated ethanol extracts were freeze-dried until constant weight and labeled 0-EtOH.

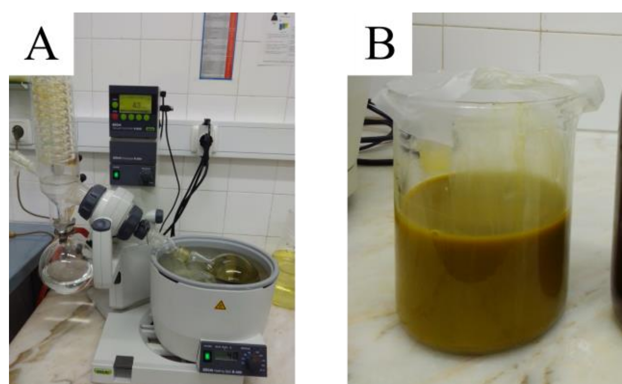

Figure 11. Evaporation of the EtOH extracts. **(A)** Rotary evaporator apparatus; **(B)** CAM ethanol extract appearance after evaporation.

The initial solid biomasses were subject to an extraction using chloroform only. Chloroform was added to the biomass and left O/N at around 180 rpm. On the next day the chloroform was separated via gravity filtration and the solid residue was stored at 4 ºC. Finally, the chloroform extract was freeze-dried until constant weight and labeled CHL-2.

In the end we were left with two distinct fractions of each TAM and CAM extracts, the solid biomass residue chloroform extract (labeled CHL-2) and the solid ethanol extract (labeled 0-EtOH). The freeze-dried TAM and CAM ethanol extracts were stored in falcon tubes and the solid biomass fractions were stored in an airtight bag in the dark at room temperature.

Liquid phase - Fractionation using Liquid Biphasic Systems (LBS)

To prepare the next extracts we resorted to fractionation of the TAM and CAM ethanol extracts using LBS. The solvents used were n-hexane, chloroform, ethyl acetate and n-butanol, in this order. The predicted compounds removed by each solvent are shown in the **Table 10** below, along each solvent density compared to water.

Table 10. Solvents used in LBS and predicted compounds removed in each fraction.

Solvent	Chemical formula	Density (kg/m^3)	Compounds removed
n-Hexane	$CH_3(CH_2)CH_3$	655.0	Non-polar compounds
Chloroform	$CHCl_3$	1476.8	Pigments
Ethyl Acetate	$C_4H_8O_2$	902.0	Biological active compounds
n-Butanol	C_4H_9OH	810.0	Other hydrocarbons
Water	H_2O	997.0	-

8 g (TAM) and 10 g (CAM) of freeze-dried ethanol extract (labeled 0-EtOH) were weighed into 250 mL Schott bottles. 100 mL deionized water was then added to each extract and stirred vigorously to solubilize/suspend the extract as much as possible. (1) 100 mL of n-hexane was then added and the (2) solution was stirred for roughly 30 min at >200 rpm to avoid the formation of layers and ensure an homogenous mixing. (3) The stirred mixtures were then transferred to a separation funnel and left to rest until separate layers were formed. **(Fig. 12)** (4) Both layers were collected into separate bottles, the organic phase (labeled 1-HEX fraction) into a 100 mL amber bottle and the aqueous phase into a Schott bottle. Because water is denser than n-hexane (see **Table 10** above), the bottom layer will be the aqueous phase, and the top layer the organic phase. This will be true for the remaining separations except during the chloroform separation, due to it being denser than water, thus creating an inverted layer order.

To prepare the next fractions, we added each solvent in succession to the previously collected aqueous phase, repeating the separation process through steps 1-4 changing the solvent added in each subsequent separation. All the aforementioned procedures were repeated for both our freeze-dried TAM and CAM ethanol extracts. In the end, we are left with four different fractions (labeled 1-HEX, 2-CHL, 3-ETA and 4-BUT) and a remainder aqueous solution (labeled 5-AQU) of each of our TAM and CAM initial ethanol extracts.

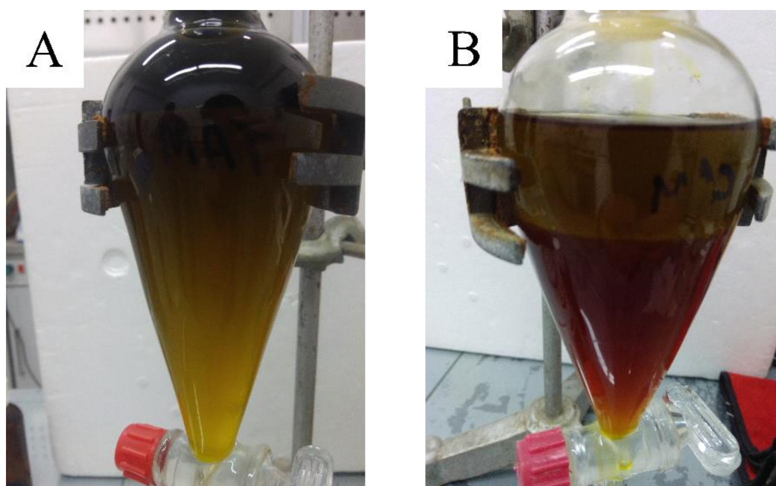

Figure 12. Liquid biphasic system (LBS) fraction separation. **(A)** Beginning of the separation (TAM); **(B)** Fractions fully separated (CAM).

The solvents were then evaporated from the extracts using the rotary evaporator at 40 ºC. Due to the high boiling point of n-butanol, its fraction was mixed evenly with water to produce an azeotropic mixture and lower its boiling point before evaporation. The final evaporated fractions were then reconstituted with acetone and water and left to rest up to a week in the hotte, to evaporate the acetone from solution at room temperature. Finally, all fractions were freeze-dried until constant weight and stored in amber flasks. The lyophilized aqueous solutions (5-AQU) were stored in falcon tubes. The entire extract preparation steps are schematized in the **Fig. 13**.

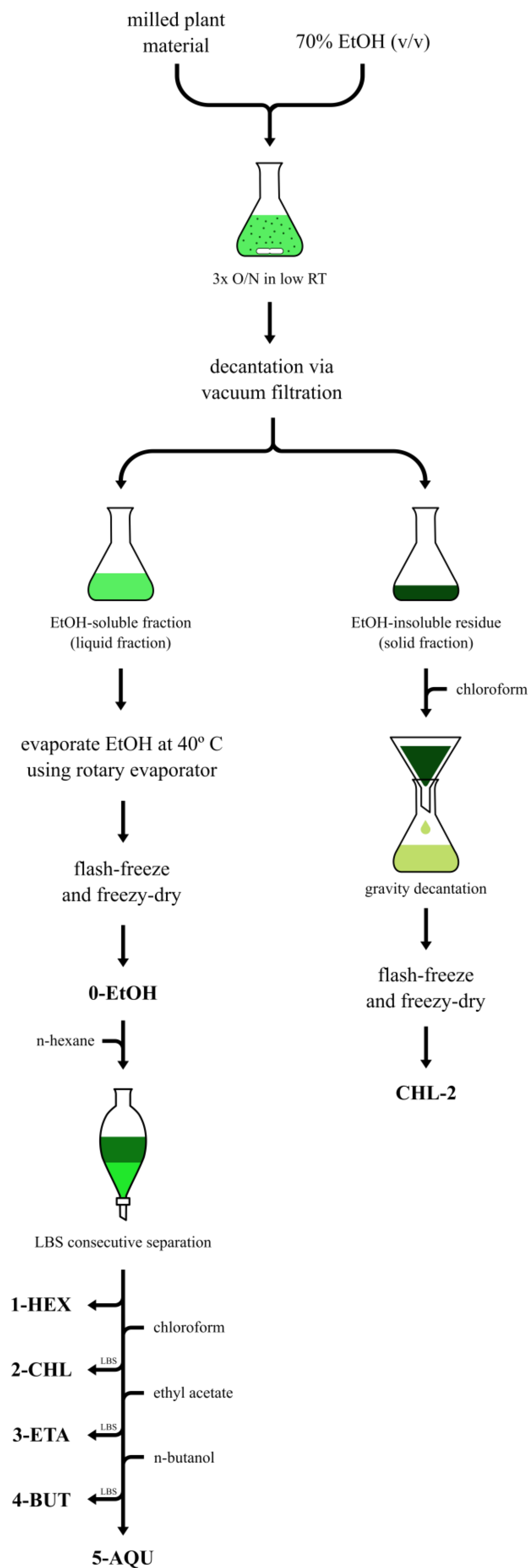

Figure 13. Extract preparation step-by-step scheme.

2.4. FTIR –Fourier-Transform Infrared Spectroscopy

The chemical characterization of all the TAM and CAM freeze-dried fractions was done via Fourier-Transform Infrared Spectroscopy (FTIR), using a FTIR spectrometer (VERTEX 70, BRUKER) in junction with an attenuated total reflectance attachment (ATR) **(Fig. 14)**. FTIR analysis is an effective technique for the chemical analysis of a given sample (Baker et al., 2014), being a fast, inexpensive and non-destructive procedure (Durak & Depciuch, 2020). This technique consists in the collection and reading of spectral data over a wide spectral range to identify the functional chemical groups present in the sampled material (Talari et al., 2017). These functional groups absorb different wavelengths depending on their type and chemical bond, that can be detected via spectroscopy and analyzed in the resulting spectra graph. Although non-compound specific, the identification of the samples molecular functional groups via FTIR is helpful to narrow the identification of the molecules present in the given sample.

Figure 14. FTIR spectrometer with ATR attachment.

FTIR data analysis and tentative attributions

To analyze each fraction, a small portion of dried material was placed above the spectrometer laser and the ATR was closed to tightly compress the material and ensure a reliable reading. The fraction samples were analyzed without further preparation and the spectra data was recorded in the range of 4000 cm^{-1} to 400 cm^{-1}. The absorbance spectra were treated with H_2O compensation and CO_2 compensation (atmospheric compensation) and converted to text files in OPUS (v. 7.5; BRUKER OPTIK), imported into MATLAB (v. R2020b; MATHWORKS, Natick, MA, USA), baseline adjusted using Automatic Weighted Least Squares (algorithm dim: 2 across columns; polynomial order: 2), and then vector normalized (Norm: Returns a vector of unit length; length=1). The absorbance spectra were finally cropped to fingerprint region of 1800 cm^{-1} to 800 cm^{-1}.

2.5. Multiwell Activity Assays

Treatment growth and test model

The effects of the extract fractions on *P. cinnamomi* growth were tested via multiwell activity assays (MAA) using 24-well multiwell plates with 16 mm diameter wells (COSTAR, Corning Inc.), in order to determine each fraction minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC). Many different fraction concentrations were tested in a total of six assays, testing three replicates per concentration. The oomycete growth medium used was PDB, and the fractions were prepared with water. The extract concentrations tested were honed in each subsequent assay, according to the results obtained in the previous ones. The first assay was tentatively conducted at concentrations of 10, 5, 2.5 and 1.25 mg/mL for each fraction but, due to the chemical properties of the different compounds removed in each fraction, not all fractions could successfully solubilize at this concentration, being diluted at higher ratios accordingly. As the extract and PDB medium are mixed 1:1 in the wells, both were priorly prepared at 2x concentration. Each treatment fraction plus PDB solution was mixed outside of the wells and then equally divided between three wells to conduct the three replicates per treatment.

As previously noted, not all of the fractions are equally soluble in water, making it impossible to test some of the extract fractions at high concentrations without the addition of DMSO, such as the n-hexane, chloroform and ethyl acetate fractions, which were tested at much lower concentrations when compared to the maximum tested concentrations of ethanol, n-butanol and aqueous fractions which are highly soluble in water. The solid residue chloroform fraction (2-CHL) was extremely hard to solubilize in water and was deemed not worthy of further testing in the present study. As expected, the addition of DMSO affected the oomycete growth, as this solvent disruptive effect in biological cells is well documented, even at exceedingly low concentrations (Rammler & Zaffaroni, 1967; Jelke & Oertel, 1990; Galvao et al., 2014). Additionally, some other tests were conducted without PDB medium, such as extract only tests and extract plus DMSO.

Control treatments were conducted solely with PDB medium or water. A negative control assay (C-) was prepared using the commercial fungicide *Aliette Flash* (BAYER), where five concentrations were tested: 10, 5, 2.5, 1.25 and 0.75 mg/mL. *Aliette Flash* active compound consists of phosphonic acid or fosetyl-Al (Bayer, 2017), a compound belonging into the phosphite category of compounds known to effectively inhibit *P. cinnamomi* growth. This fungicide was the only substance to be tested in negative control treatments, as no purified

phosphite compounds were available to test. A positive control (C+) was conducted with PDB 1x medium only. The PDB medium was initially sterilized via autoclaving, and all the extracts, water and fungicide were filtered during the assays to prevent contamination. The extracts, water and fungicide were filtered through 0.22 μm polyethersulfone (PES) syringe filters. Finally, the oomycete was left to grow for 7 days in the dark at room temperature. The MAA test model is schematized in the **Fig. 15** below.

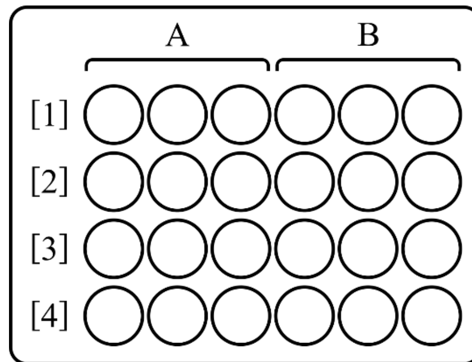

Figure 15. Multiwell activity assays (MAA) test model. **(1-4)** Different tested concentrations (from highest to lowest); (**A, B)** Different extracts.

Post treatment growth (PTG)

After the seven days of MAA treatment, several selected *P. cinnamomi* material (mainly material which failed to grow at a given fraction concentration) was transferred to regular petri dishes with PDA medium to assess the oomycete post treatment growth (PTG test). This additional treatment let us detect if a given concentration corresponds to a lethal concentration or an inhibitory concentration. If *P. cinnamomi* fails to grow at a given MAA tested concentration but subsequentially grow in PTG, the tested concentration is determined to be an inhibitory concentration. If *P. cinnamomi* fails to grow in the MAA and in PTG, the tested concentration is determined to be a lethal concentration. The material transfer was done by suctioning the growth medium mixture out of the selected treatment well, rinsing the leftover oomycete material with distilled water two to three times using a pipette, and transferring the material to a separate petri dish with PDA medium only using a regular tongs. The material was left to grow in the dark at room temperature for a minimum of five days. The PTG test model is schematized in the **Fig. 16**.

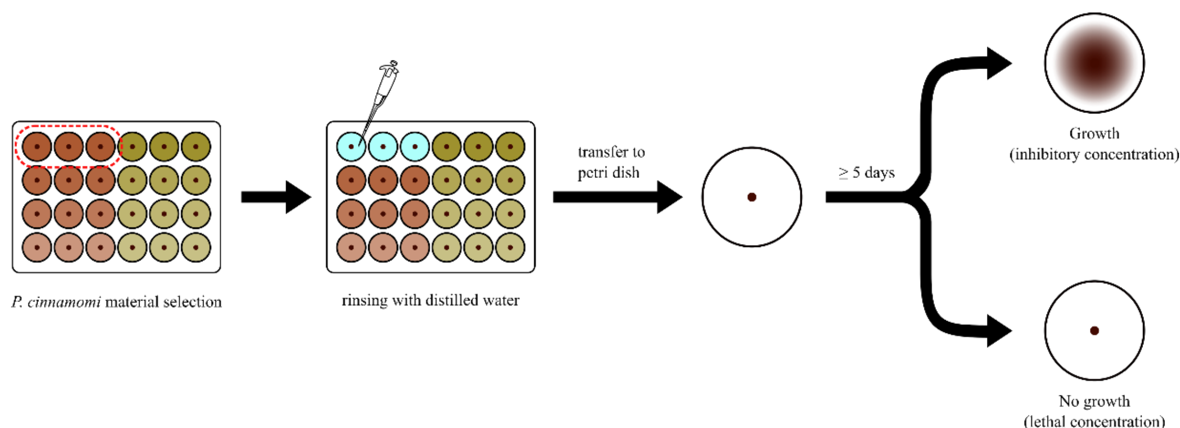

Figure 16. Post treatment growth (PTG) test model.

2.6. Extract Fraction Activity Assays

Assay test model

Only three extract fractions from each plant species leaves remained leftover from the previous multiwell MAA, the ethanol extract, the n-butanol fraction and the aqueous fraction. From these remaining material, additional activity assays were conducted with the n-butanol and aqueous fractions to precisely measure their influence on *P. cinnamomi* hyphal growth area (HGA). The ethanol extract was very difficult to solubilize in water at the desired concentrations, and for consistency's sake it was not tested in this assay. The extract fractions activity assays (EFAA) were performed in plastic petri dishes with 9 cm diameter, with the same PDA medium used in the oomycete propagation. Four concentrations of each species n-butanol and aqueous fractions were tested: 10 mg/mL (1), 5 mg/mL (2), 2.5 mg/mL (3) and 1.25 mg/mL (4). As the fractions are mixed with PDA medium at 1:1 ratio in the petri dishes, the fractions and PDA medium concentration were previously prepared at 2x the desired concentration. Concentrations 2, 3 and 4 were prepared by sequential water dilutions, starting from the original concentration.

15 mL of each fraction were mixed with 15 mL of PDA 2x medium in small Erlenmeyer flasks, distributed evenly in Petri dishes and left to solidify. A total of three replicates per extract concentration were conducted. Each Petri dish totaled roughly 10 mL of fraction plus PDA. Three control (C+) replicates were prepared using only PDA 1x medium. As a matter of interest, three additional C+ replicates were prepared with leftover PDA 2x medium. Finally, small equal circles of *P. cinnamomi* were cut and collected from our previous cultures, placed at the center of the prepared Petri dishes. The water, PDA medium and laboratory material used were initially sterilized via autoclaving to prevent contamination. The EFAA test model is schematized in the **Fig. 17**.

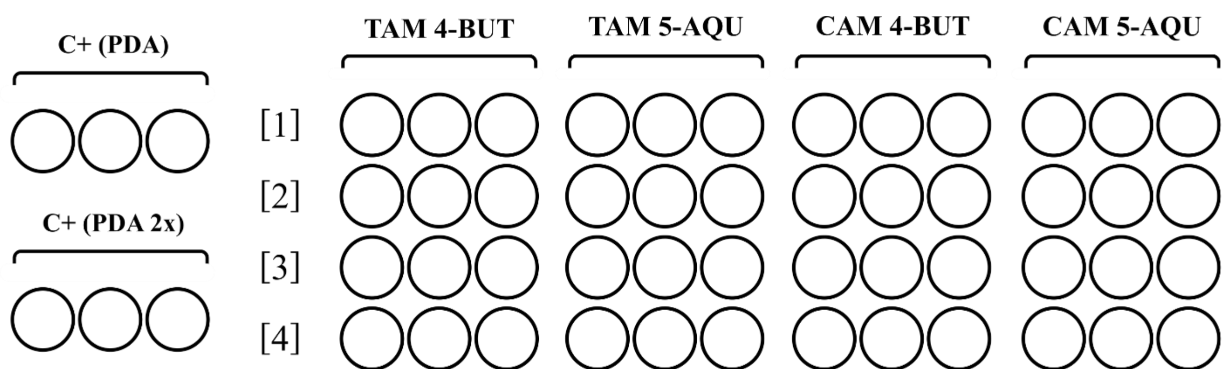

Figure 17. Extract fraction activity assays (EFAA) test model. **(1-4)** Different concentrations.

Growth area and statistical analysis

P. cinnamomi was left to grow for 5 days in the dark at room temperature. After treatment, each individual Petri dish was photographed from top view at a height of 7.5 cm, in the same position and light conditions with the help of a camera stand and a regular smartphone camera **(Fig. 18)**. *P. cinnamomi* mycelium hyphal growth area was measured and analyzed using ImageJ1 software (NIH) (Hartig, 2013) **(Fig. 19)** and the results are expressed in cm^2. Growth reduction was calculated from the three replicates in accordance with Royse & Ries (1978), using the formula: GR = (TG-CG)/CG *100, where GR, TG and CG stand for "growth reduction", "treatment growth" and "control growth", respectively.

The obtained growth area data was subject to one-way analysis of variance (ANOVA) and *post hoc* analysis using Tuckey test, both with p = 0.05. Tuckey test p-values were corrected according to Bonferroni (1936) using the formula α = P/N, where P and N note the "p significance" and "number of comparisons" between groups, respectively. The statistical analysis was conducted using the Data Analysis ToolPak add-on of Microsoft Excel in accordance with Salkind (2015).

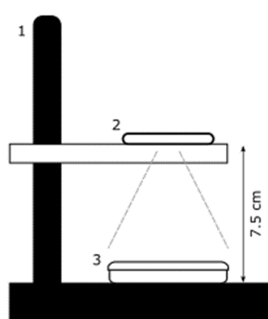

Figure 18. Photo capture model. **(1)** Camera stand; **(2)** Camera; **(3)** Petri dish.

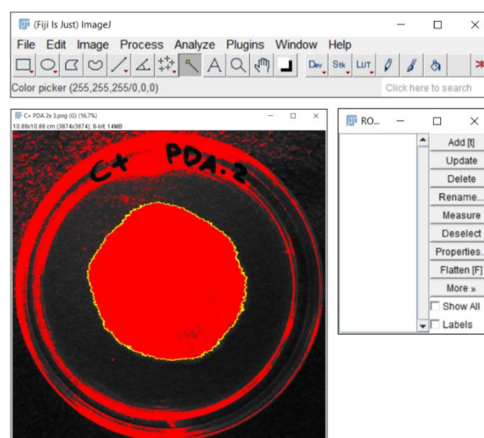

Figure 19. ImageJ1 software interface with a loaded and processed image.

2.7. Trichome observation and scanning electron microscopy

Sampled material

A total of four *S. betaceum* varieties leaf material were sampled for SEM observation: Two varieties based on the fruit color (red-type and orange-type), and two *in vitro* varieties (C9 and C12) (courtesy of Dr. Sandra Correia). Red-type and orange-type varieties were sampled from leaves of the same tamarillo trees from which we previously collected leaves for extract preparation planted at the Botanical Garden of UC. The two *in vitro* genotypes were pre-established seedlings grown in test tubes of unknown age **(Fig. 20)**. A single variety of *C. album* leaf material was observed, sampled from plant material collected at Praia de Quiaios.

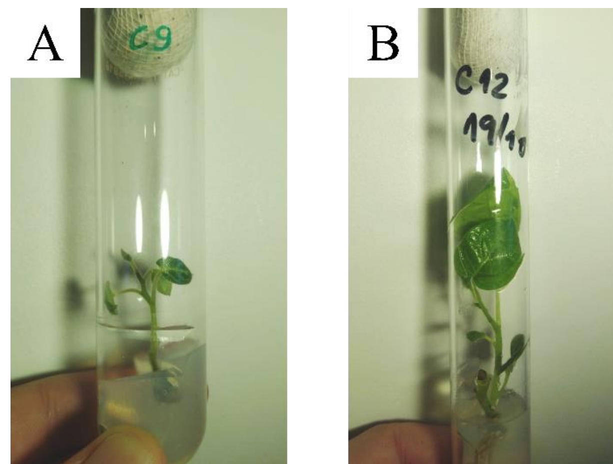

Figure 20. *In vitro* tamarillo varieties observed in SEM. **(A)** C9 variety; **(B)** C12 variety.

Scanning electron microscopy

Scanning electron microscopy took place at *Instituto Biomédico de Investigação, Luz e Imagem* (IBILI) using a variable pressure scanning electron microscope (Flex SEM 1000, HITACHI). Samples were prepared by cutting small square segments of leaf blade and several cross-sections along the midrib, placed on carbon stickers above metallic stubs and observed without further preparation. The observations were conducted at 10.0 kV in freeze conditions (-20 °C).

3. RESULTS

3.1. Multiwell Activity Assays

The treatments tested in the multiwell activity assays (MAA) can be divided into three major categories, named here as "standard" treatments (extract fraction plus PDB medium only), "conditioned" treatments (any treatment conducted using the extract fraction with/without DMSO and/or PDB medium) and control treatments. It is important to give this distinction between "standard" and "conditioned" treatments, as in both species the oomycete growth is significantly influenced by whether or not DMSO and PDB medium are present in the treatment. All individual MAA results and test models can be found in **Appendix 1-6**.

3.1.1. *Solanum betaceum* MAA

Standard treatments

In the standard treatments with *S. betaceum* fractions, the n-butanol fraction inhibited *P. cinnamomi* growth, at concentrations of 18 mg/mL. The oomycete also failed to grow in a concentration of 3.33 mg/mL with the ethyl acetate fraction in two of the three total replicates conducted. *P. cinnamomi* material treated with the ethyl acetate fraction at 3.33 mg/mL and with the n-butanol fraction at 18 mg/mL were subjected to post-treatment growth tests (PTG). *P. cinnamomi* tested with the ethyl acetate fraction at 3.33 mg/mL and the n-butanol fraction at 18 mg/mL successfully grew in PTG tests. All the remaining treatments conducted with *S. betaceum* fractions failed to inhibit *P. cinnamomi* growth at tested concentrations. All results from the standard treatments with *S. betaceum* fractions are shown in **Table 11** and the compiled tamarillo fractions MIC/MLC is found in the **Table 12**.

Table 11. MAA and PTG results in standard treatments with *S. betaceum* fractions.

Fraction	C (mg/mL)	Growth	PTG
1-HEX (n-Hexane)	1.43	+	
	0.715	+	
	0.358	+	
	0.1788	+	
2-CHL (Chloroform)	1	+	
	0.5	+	
	0.25	+	
	0.125	+	
3-ETA (Ethyl acetate)	3.33	+	+
	1.665	+	
	0.833	+	
	0.416	+	
4-BUT (n-Butanol)	18	-	+
	10.95	+	
	10.90	+	
	10.85	+	
	10.80	+	
	10.75	+	
	10.50	+	
	10.25	+	
	10	+	
	9	+	
	5	+	
	4.5	+	
	2.5	+	
	2.25	+	
	1.25	+	
5-AQU (Aqueous)	18	+	
	16	+	
	14	+	
	12	+	
	10	+	
	5	+	
	2.5	+	
	1.25	+	
0-EtOH (Ethanol)	18	+	
	16	+	
	14	+	
	12	+	
	10	+	
	5	+	
	2.5	+	
	1.25	+	

\+ Growth
- No growth

Table 12. *S. betaceum* fractions MIC and MLC.

Leaf material	Fraction	MIC (mg/mL)	MLC (mg/mL)
Tamarillo (*S. betaceum*)	Ethanol	> 18	-
	n-Hexane	> 1.43	-
	Chloroform	> 1	-
	Ethyl Acetate	> 3.33	-
	n-Butanol	10.95 - 18	> 18
	Aqueous	> 18	-

Conditioned treatments

In the conditioned tests with *S. betaceum* extracts, two main types of treatments were tested, the combination of DMSO plus fraction with PDB medium and DMSO plus fraction without PDB medium. A third type of treatment was conducted with n-butanol fraction exclusively between 11-14 mg/mL, without DMSO and PDB. Three of the conducted treatments successfully inhibited *P. cinnamomi* growth, DMSO 0.25 % plus n-hexane 1.5 mg/mL without PDB, DMSO 5 % plus ethyl acetate 6 mg/mL with PDB and DMSO 0.5 % plus ethyl acetate 6 mg/mL without PDB. All the treatments with the standalone n-butanol fraction inhibited *P. cinnamomi* growth. Further PTG tests were conducted in oomycete material tested with n-butanol at 11 and 12 mg/mL, in which the material successfully grew. The results from the conditioned treatments conducted with *S. betaceum* fractions are shown in **Table 13**.

Table 13. MAA results in conditioned treatments with *S. betaceum* fractions.

Fraction	C (mg/mL)	Observations	Growth	PTG
1-HEX (n-Hexane)	3	DMSO 5 %	+	
	1.5	DMSO 5 %	+	
	1.5	DMSO 0.25 % (without PDB)	-	
	0.75	DMSO 5 %	+	
	0.75	DMSO 0.25 % (without PDB)	+	
	0.375	DMSO 5 %	+	
	0.375	DMSO 0.25 % (without PDB)	+	
	0.1875	DMSO 0.25 % (without PDB)	+	
2-CHL (Chloroform)	2	DMSO 5 %	+	
	1	DMSO 5 %	+	
	1	DMSO 0.25 % (without PDB)	+	
	0.5	DMSO 5 %	+	
	0.5	DMSO 0.25 % (without PDB)	+	
	0.25	DMSO 5 %	+	
	0.25	DMSO 0.25 % (without PDB)	+	
	0.125	DMSO 0.25 % (without PDB)	+	
3-ETA (Ethyl acetate)	6	DMSO 5 %	-	
	6	DMSO 0.5 % (without PDB)	-	
	3	DMSO 5 %	+	
	1.5	DMSO 5 %	+	
	0.75	DMSO 5 %	+	
4-BUT (n-Butanol)	14	without PDB	-	
	13	without PDB	-	
	12	without PDB	-	+
	11	without PDB	-	+

+ Growth
- No growth

3.1.2. *Corema album* MAA

Standard treatments

The ethanol, n-hexane, chloroform and ethyl acetate fractions successfully inhibited *P. cinnamomi* growth at some given concentration. Both the n-hexane and the chloroform fractions inhibited *P. cinnamomi* growth at a concentration of 1.75 mg/mL. The ethanol and ethyl acetate fractions inhibited the oomycete growth at 1.25 mg/mL and 0.7 mg/mL, respectively. Oomycete material treated with the chloroform fraction at 1.25 mg/mL and above, with the ethyl acetate fraction at 1.25 mg/mL and above (except the 1.5 mg/mL treatment), with the n-butanol fraction between 10-10.75 mg/mL and with the ethanol extract at 0.7 mg/mL and above, were all subject to additional PTG tests. Regarding the PTG of material treated with the chloroform fraction, only the oomycete material priorly treated at 1.25 mg/mL grew. No *P. cinnamomi* material treated with the ethyl acetate fraction grew during PTG tests. During PTG tests in material treated with the n-butanol fraction, only the oomycete material treated at 1.5 mg/mL failed to grow. In PTG tests conducted with *P. cinnamomi* material previously treated with the ethanol extract, material treated between 0.7-1.1 mg/mL failed to grow and only the material treated at 1.25 and 2.5 mg/mL grew. All results from the standard treatments with *C. album* fractions are shown in **Table 14** and the compiled tamarillo fractions MIC/MLC is found in the **Table 15**.

Table 14. MAA and PTG results in standard treatments with *C. album* fractions. Conflicting results between MAA and PTG tests are presented in red.

Fraction	C (mg/mL)	Growth	PTG
1-HEX (n-Hexane)	4	-	
	2	-	
	1.75	-	
	1.50	+	
	1.25	+	
	1	+	
	0.5	+	
2-CHL (Chloroform)	2.5	-	-
	2	-	-
	1.75	-	-
	1.50	+	-
	1.25	+	+
	0.625	+	
	0.313	+	
3-ETA (Ethyl acetate)	2	-	-
	1.75	-	-
	1.50	-	
	1.25	-	-
	1	+	
	0.5	+	
	0.25	+	
4-BUT (n-Butanol)	18	a	
	16	a	
	14	a	
	12	a	
	10.75	a	+
	10.50	a	-
	10.25	a	+
	10	+	+
	5	+	
	2.5	+	
	1.25	+	
5-AQU (Aqueous)	18	+	
	16	+	
	14	+	
	12	+	
	10	+	
	5	+	
	2.5	+	
	1.25	+	
0-EtOH (Ethanol)	2.5	-	+
	1.25	-	+
	1.1	-	-
	1	-	-
	0.9	-	-
	0.8	-	-
	0.7	-	-
	0.625	+	
	0.3125	+	

+ Growth
- No growth
a Unknown, indiscernible

Table 15. *C. album* fractions MIC and MLC.

Leaf material	Fraction	MIC (mg/mL)	MLC (mg/mL)
Crowberry (*C. album*)	Ethanol	0.625 - 0.7	0.7
	n-Hexane	1.75	-
	Chloroform	1.5 - 1.75	1.75
	Ethyl Acetate	1.00 - 1.25	1.25
	n-Butanol	> 10	> 10.75
	Aqueous	> 18	-

Conditioned treatments

A single conditioned test was conducted using the n-hexane fraction, at a concentration of 0.25 mg/mL without PDB, in which *P. cinnamomi* successfully grew. Several treatments without PDB were conducted with the n-butanol fraction starting at a concentration of 11 mg/mL. No oomycete growth was observed in any of these conditioned treatments using the n-butanol fraction. The n-butanol fraction treatments conducted at 11 and 12 mg/mL were subject to further PTG tests, in which they failed to grow. The results from the conditioned treatments conducted with *C. album* fractions are shown in **Table 16**.

Table 16. MAA and PTG results in conditioned tests with *C. album* fractions.

Fraction	C (mg/mL)	Observations	Growth	PTG
1-HEX	0.25	without PDB	+	
4-BUT	18	without PDB	-	
	16	without PDB	-	
	14	without PDB	-	
	13	without PDB	-	
	12	without PDB	-	-
	11	without PDB	-	-

\+ Growth
- No growth

3.1.3. *Aliette Flash* and DMSO MAA

The negative control treatments conducted using *Aliette Flash* successfully inhibited *P. cinnamomi* growth, even at the lowest concentration tested of 0.75 mg/mL. No further PTG tests were conducted in the material tested with *Aliette Flash*. Additional control tests were conducted using DMSO only to assess the compound standalone effects on *P. cinnamomi* growth. The oomycete is able to grow in DMSO concentrations as high as 2.5 % and fails to grow at 5 % DMSO concentration, with similar results observed in the PTG tests conducted in this material. Both treatments results are found in the **Table 17**.

Table 17. MAA and PTG results in *Aliette Flash* and DMSO treatments. *Aliette Flash* and DMSO concentrations are noted in mg/mL and in percentage (%), respectively.

Fraction	C	Growth	PTG
Aliette Flash	10	-	
	5	-	
	2.5	-	
	1.25	-	
	0.75	-	
DMSO	5	-	-
	2.5	+	+
	2.0	+	+
	1.5	+	+
	0.5	+	+

+ Growth
- No growth

3.2. Extract Fraction Activity Assays

The final determined treatment growth (TG) is presented alongside each treatment growth reduction (GR) when compared to control growth (CG). TG is noted in cm^2, and GR is noted both in cm^2 and percentage (%). GR_{mean} and its associated standard deviation (StdDev) are obtained from each treatment three replicates GR in percentage (%). Statistically significant differences between treatments are noted as different letters in the result graphs. The complete HFA growth area results can be found in **Appendix 7**. The full ANOVA and Tuckey test data from the EFAA can be found in **Appendix 8-19**.

3.2.1. Control EFAA

P. cinnamomi grew on average 22.8 cm^2 in the control treatment with PDA, reaching a maximum hyphal growth area (HGA) of 23.1 cm^2 and a minimum HGA of 22.6 cm^2. *P. cinnamomi* grew to an average of 16.2 cm^2 in PDA 2x control treatments, with a maximum HGA of 18.8 cm^2 and a minimum HGA of 13.8 cm^2 **(Table 18 and Fig. 21)**.

Table 18. EFAA control treatments hyphal growth area results.

Control	CG (cm^2)	CG_{mean} (cm^2)	StdDev
PDA	22.679	22.813	0.218
	22.639		
	23.121		
PDA 2x	16.054	16.198	2.060
	18.789		
	13.750		

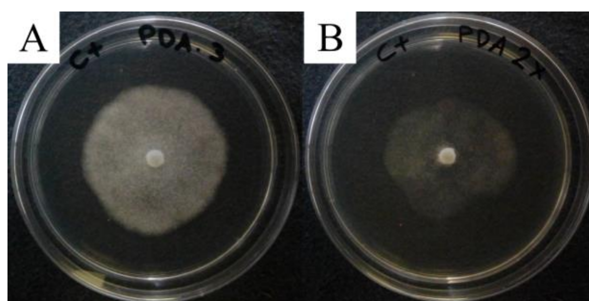

Figure 21. Control treatments growth after 5 days. **(A)** PDA; **(B)** PDA 2x.

3.2.2. *Solanum betaceum* EFAA

S. betaceum n-butanol fraction decreases *P. cinnamomi* HGA by 7.67 ± 3.39 % in the 1.25 mg/mL treatment, 41.57 ± 8.12 % in the 2.5 mg/mL treatment, 64.35 ± 4.06 % in the 5 mg/mL treatment and 75.90 ± 1.18 % in the 10 mg/mL treatment. Statistical analysis shows that the oomycete HGA is significantly different between 1.25, 2.5 and 10 mg/mL treatments, and not significantly different between 2.5-5 mg/mL and 5-10 mg/mL **(Fig. 22 and 23)**.

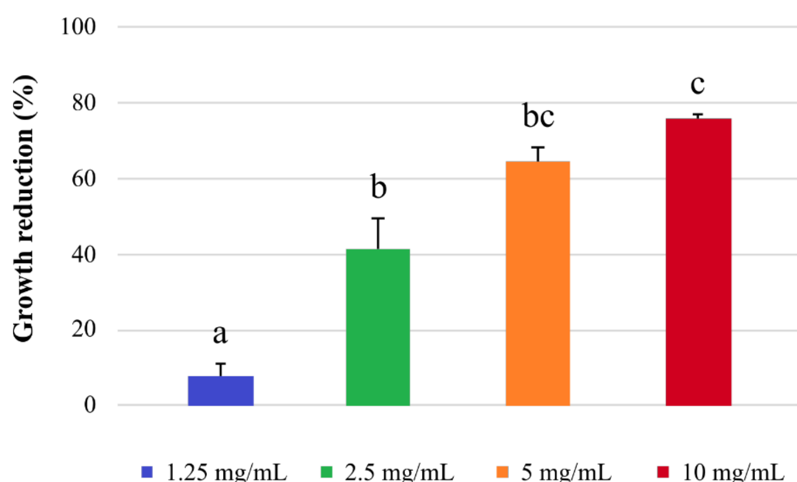

Figure 22. *S. betaceum* n-butanol fraction GR in percentage.

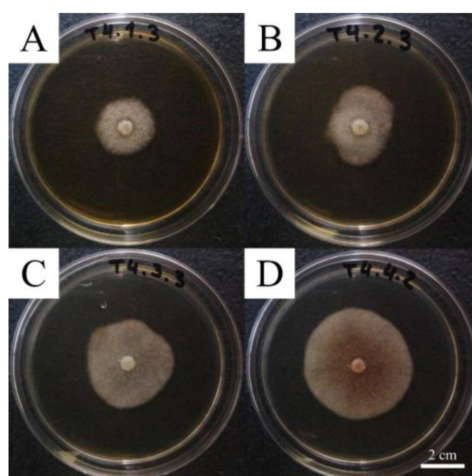

Figure 23. *S. betaceum* n-butanol fraction treatments. A single replicate from the total three is shown. **(A)** 10 mg/mL; **(B)** 5 mg/mL; **(C)** 2.5 mg/mL; **(D)** 1.25 mg/mL.

S. betaceum aqueous fraction reduces *P. cinnamomi* HGA by 8.60 ± 10.35 % in the 1.25 mg/mL treatment, 22.36 ± 2.58 % in the 2.5 mg/mL treatment, 36.53 ± 7.07 % in the 5 mg/mL treatment and 53.00 ± 3.41 % in the 10 mg/mL treatment. One of the 1.25 mg/mL treatment replicates shows a HGA increase of 6.60 % was detected when compared to the control. The statistical analysis shows that there were no significant differences between the HGA of 1.25, 2.5 and 5 mg/mL treatments, as well as between 5-10 mg/mL, but the HGA of the 1.25 and 2.5 mg/mL treatments are significantly different from 10 mg/mL **(Fig. 24 and 25)**.

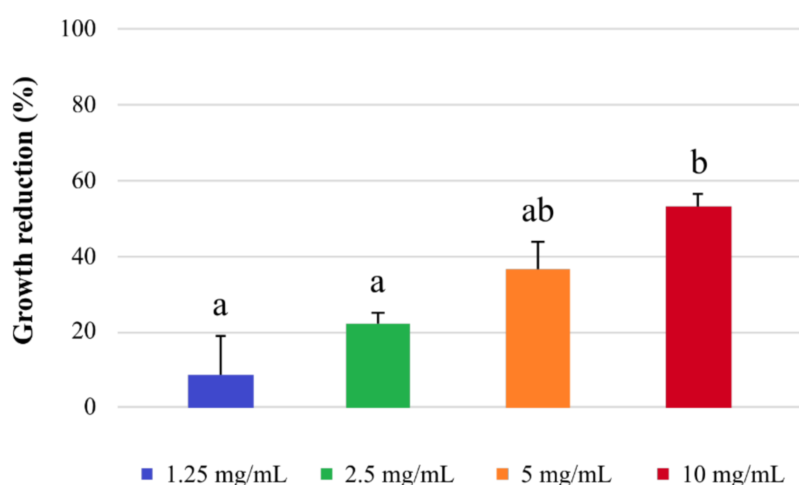

Figure 24. *S. betaceum* aqueous fraction GR in percentage.

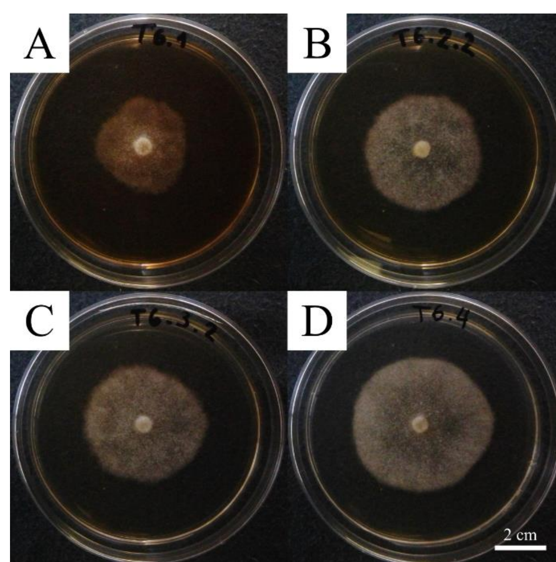

Figure 25. *S. betaceum* aqueous fraction treatments. A single replicate from the total three is shown. **(A)** 10 mg/mL; **(B)** 5 mg/mL; **(C)** 2.5 mg/mL; **(D)** 1.25 mg/mL.

3.2.3. *Corema album* EFAA

C. album n-butanol fraction decreases *P. cinnamomi* HGA by 65.81 ± 4.41 % in the 1.25 mg/mL treatment, 81.24 ± 2.16 % in the 2.5 mg/mL treatment, 89.37 ± 1.14 % in the 5 mg/mL treatment and 100 % in the 10 mg/mL treatment, where it completely inhibits oomycete growth. There were no statistically significant differences between 1.25-2.5 mg/mL treatments HGA, but all the remaining treatments are significantly different amongst themselves **(Fig. 26 and 27)**.

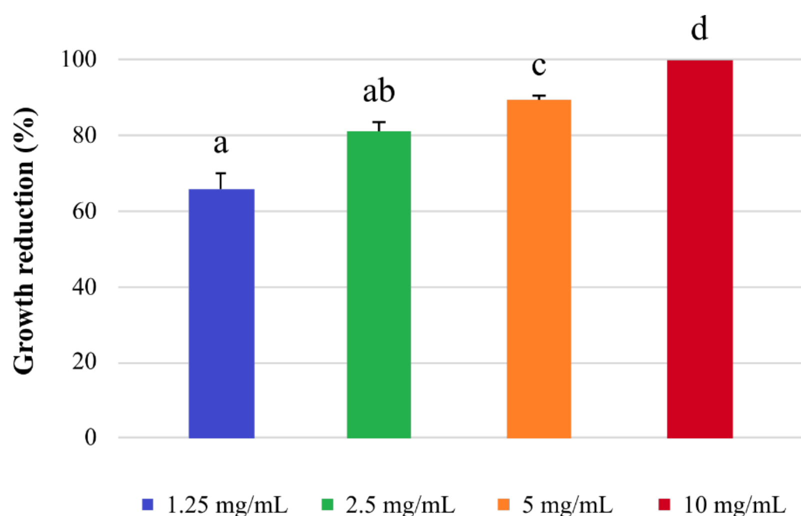

Figure 26. *C. album* n-butanol fraction GR in percentage.

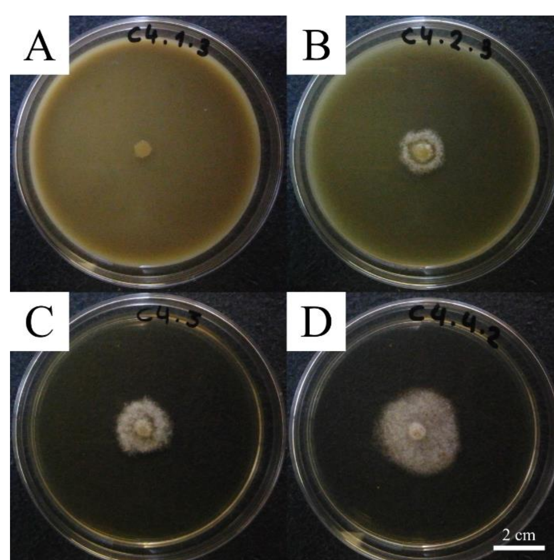

Figure 27. *C. album* n-butanol fraction treatments. A single replicate from the total three is shown. **(A)** 10 mg/mL; **(B)** 5 mg/mL; **(C)** 2.5 mg/mL; **(D)** 1.25 mg/mL.

C. album aqueous fraction increases or diminishes *P. cinnamomi* HGA depending on the treatment concentration. When compared to the control, the treatment at 1.25 mg/mL increases HGA by 9.02 ± 2.37 %. *P. cinnamomi* HGA decreases by 1.49 ± 14.60 % in the 2.5 mg/mL treatment, 32.94 ± 2.76 % in the 5 mg/mL treatment and 57.89 ± 2.58 % in the 10 mg/mL treatment. There were no statistically significant differences between 1.25-2.5 mg/mL and 2.5-5 mg/mL treatments, being the remaining treatments significantly different amongst each other **(Fig. 28 and 29)**.

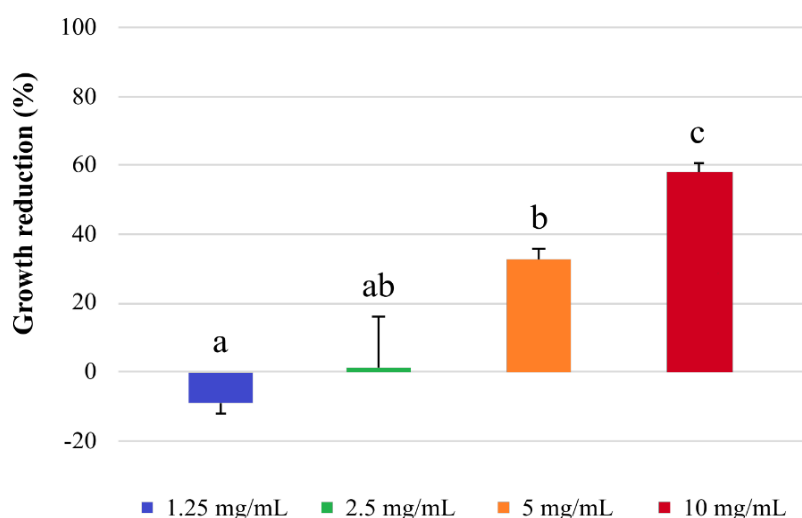

Figure 28. *C. album* aqueous fraction GR in percentage.

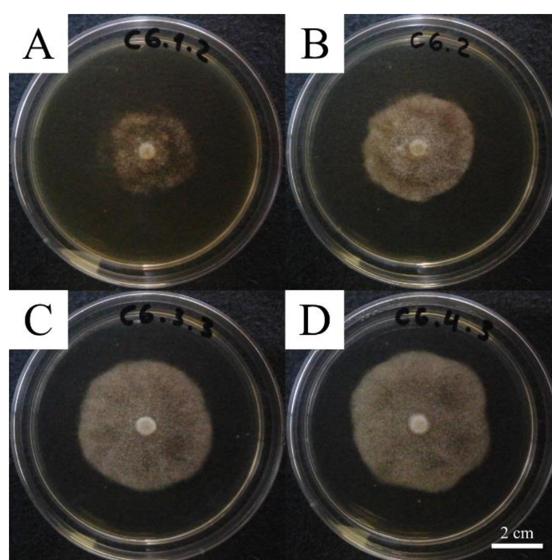

Figure 29. *C. album* aqueous fraction treatments. A single replicate from the total three is shown. **(A)** 10 mg/mL; **(B)** 5 mg/mL; **(C)** 2.5 mg/mL; **(D)** 1.25 mg/mL.

3.3. FTIR analysis

Only the FTIR spectra obtained from fractions which were able to influence *P. cinnamomi* growth during the MAA and the fractions used in the EFAA will be thoroughly analyzed. The attributions go in accordance with Martin et al. (2019) and Martin et al. (2021). The FTIR spectra of all the remaining fractions that are not presented can be found in **Appendix 20-25**.

3.3.1. *Solanum betaceum* FTIR analysis

n-Butanol fraction spectrum

The n-butanol fraction spectrum presents many narrow and pronounced peaks throughout the entire fingerprint region. The most prominent peak occurs at 1596 cm^{-1} (*a*) which suggests the presence of polyphenols in the fraction. Next, the peak at 1260 cm^{-1} (*c*) could be found with a shoulder protruding at 1282 cm^{-1} (*b*), both corresponding to the presence of bending hydroxyl (OH) groups, suggesting the presence of polysaccharides such as fructose or the presence of cutin. Right beside this peak, two more paired strong and narrow peaks could be found at 1178 cm^{-1} (*d*) and 1160 cm^{-1} (*e*), both associated with the presence of antisymmetric stretching in C-O-C groups corresponding to ester compounds. Finally, one of the more prominent peaks was detected at the start threshold of the fingerprint region, at 809 cm^{-1} (*j*). This final peak may indicate the presence of triterpenoid compounds. The peaks at 1115 (*f*) cm^{-1}, 1067 cm^{-1} (*g*) and 1040 cm^{-1} (*h*) are also worth noting. These peaks suggest the presence of stretching symmetric C-O-C, stretching C-O and C-C groups, each corresponding to ester and glycosidic bonds, likely linked to polysaccharides compounds, respectively **(Fig. 30)**.

Aqueous fraction spectrum

The aqueous fraction FTIR spectrum presents significantly less peaks in comparison with the previous n-butanol fraction. Nonetheless, the same strong peak appears in the aqueous fraction, at 1596 cm^{-1} (*a*) which notes the presence of polyphenols. The peak at 1040 cm^{-1} (*h*) is very pronounced and corresponds to C-C groups associated with the existence of polysaccharides in the aqueous fraction. This peak also has two prominent shoulders, one at 1067 cm^{-1} (*g*) and one at 989 cm-1 (*i*), associated with the presence of esters and sucrose, respectively. Two smaller peaks should also be noted: the first at 1260 cm^{-1} (*c*) with a shoulder at 1282 cm^{-1} (*b*) associated with the presence of polysaccharides, which also appeared much more pronounced in the n-butanol fraction, and the second peak at 809 cm^{-1} (*j*) pointing to the presence of triterpenoids **(Fig. 31)**.

Table 19. FTIR analysis and peak values of different functional groups obtained from the n-butanol and aqueous leaf fractions of *S. betaceum*. Assignments based on Martin et al. (2019) and Martin et al. (2021).

	Wavenumber (cm^{-1})	Reference	Biomass	Feature	Tentative Assignment
a	1596	1609 (Martin et al., 2019)	Crowberry fruit (outer skin)	ν(C═C)ring	Polyphenols
b	1282	1277 (Martin et al., 2019)	Crowberry fruit (outer skin)	δ(OH)	Fructose/ Polysaccharides; cutin
c	1260	1262 (Martin et al., 2021)	Tamarillo fruit (peel)	δ(O-H)	-
d	1178	1175 (Martin et al., 2021)	Tamarillo fruit (outer skin)	ν asym(C-O-C)ester	-
e	1160	1160 (Martin et al., 2021)	Tamarillo fruit (inner skin)	ν asym(C-O-C)ester	-
f	1115	1111 (Martin et al., 2021)	Tamarillo fruit (inner skin)	ν sym(C-O-C)ester	-
g	1067	1068 (Martin et al., 2021)	Tamarillo fruit (peel)	ν(C-O-C)glycosidic	-
h	1040	1038 (Martin et al., 2021)	Tamarillo fruit (seed and inner skin)	ν(C-O) /ν(C-C)	Polysaccharides, pectins
i	989	995 (Martin et al., 2021)	Tamarillo fruit (peel)	ν(CO) / ν(CC)ring	Sucrose
j	809	808 (Martin et al., 2019)	Crowberry fruit (outer skin)	-	Triterpenoids

Abbreviations: **sym** symmetric; **asym** antisymmetric; **ν** stretching; **δ** bending; **ρ** plane; **oρ** out-of-plane.

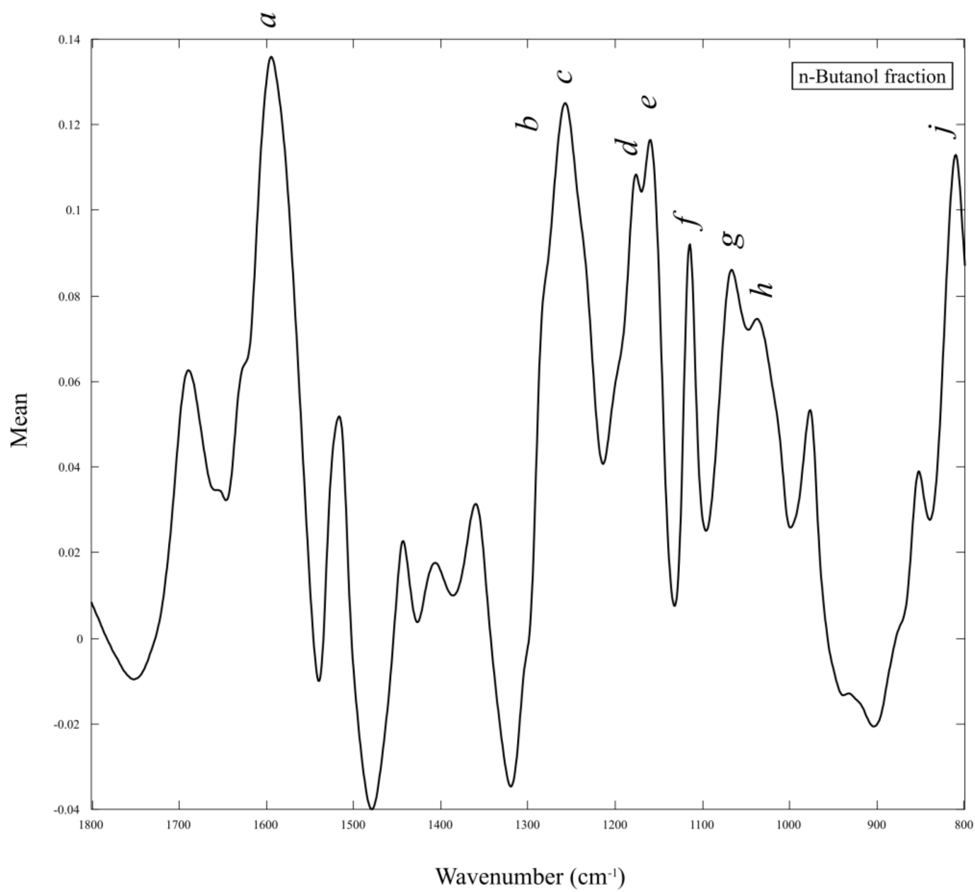

Figure 30. FTIR absorbance spectrum of *S. betaceum* n-butanol fraction.

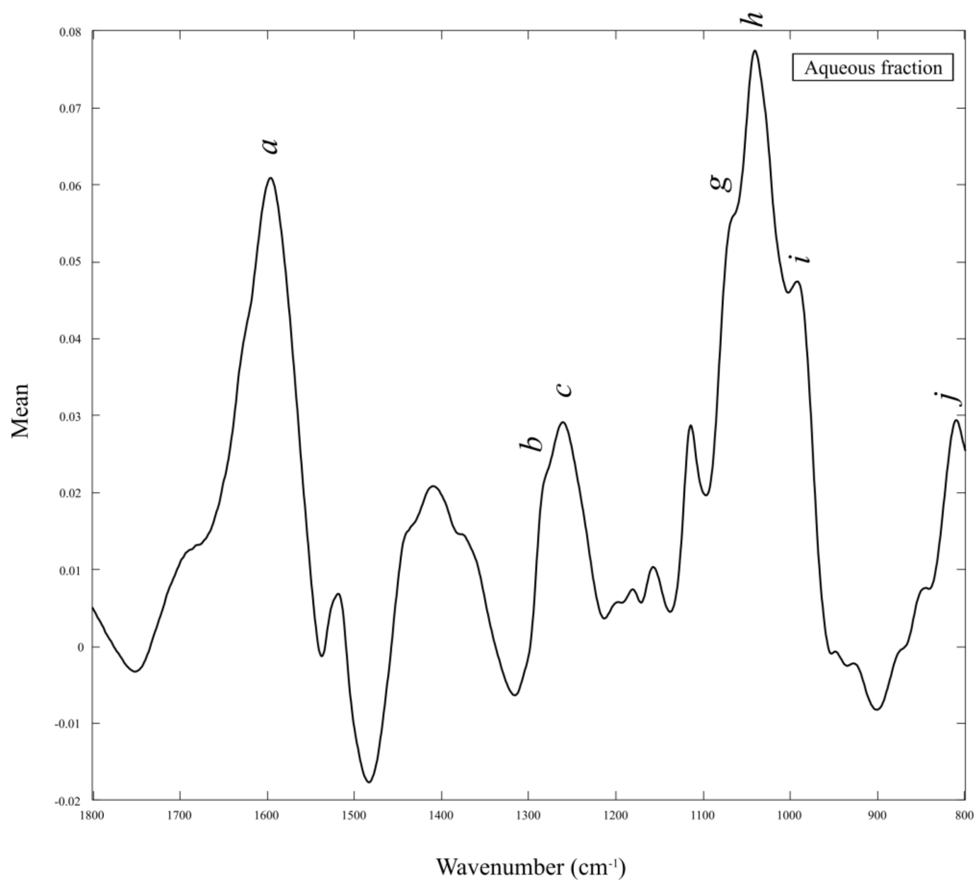

Figure 31. FTIR absorbance spectrum of *S. betaceum* aqueous fraction.

3.3.2. *Corema album* FTIR analysis

Ethanol extract spectrum

The ethanol extract spectrum presents two notable peak clusters around the 1600 cm^{-1} and 1000 cm^{-1} regions. In the first region there are two peaks at 1632 cm^{-1} (*a*) and 1603 cm^{-1} (*b*). The peak at 1632 cm^{-1} corresponds to antisymmetric stretching bonds in COO^- groups, suggesting the presence of acids in the fraction. It may also correspond to the presence of water molecules. The 1603 cm^{-1} region does not have any assignments in Martin et al. (2019) and Martin et al. (2021). Nonetheless, previous works by Ahmad et al. (2016) in *H. bacciferum* flowers assign the peak region to stretches in C-C ring bonds, suggesting the presence of aromatic compounds. In the second peak cluster of the ethanol extract spectrum there are four main peaks: three stronger peaks at 1065 cm^{-1} (*h*), 1041 cm^{-1} (*i*) and 1030 cm^{-1} (*j*) associated with stretching glycosidic bonds in ester groups (C-O-C), and a weaker peak at 986 cm^{-1} (*k*) that points the existence of stretching linear chains of carbons (C-C) that may denote the presence of polysaccharides **(Fig. 32)**.

n-Hexane fraction spectrum

The n-hexane fraction spectrum has many narrow peaks with accentuated dips in between them. Two noteworthy peaks can be observed at 1632 cm^{-1} (*a*) and 1159 cm^{-1} (*e*). The first peak can also be found in the ethanol extract spectrum and corresponds to antisymmetric stretching bonds in COO^- groups, and the second peak is associated with stretching CO and CH bonds, noting the presence of phenolic compounds and polysaccharides such as pectins **(Fig. 33)**.

Chloroform fraction spectrum

The chloroform fraction spectrum depicts two strong peak clusters and many narrow and prominent peaks. The first cluster hold two peaks at 1632 cm^{-1} (*a*) and 1603 cm^{-1} (*b*) akin to the ethanol extract spectrum. The peak at 1632 cm^{-1} corresponds to COO^- groups and the peak at 1603 cm^{-1} corresponds to the presence of aromatic compounds. The second peak cluster has three peaks: 1208 cm^{-1} (*d*) which suggests the existence of bending CCH bonds associated with lipid compounds, 1159 cm^{-1} (*h*) representing the presence of stretching bonds in CO and CH groups which suggest the presence of pectins and phenolic compounds, and 1140 cm^{-1} (*f*) associated with stretching glycosidic bonds in COC groups. Two narrow peaks should be pointed out, one at 1448 cm-1 (*c*) that may be associated with the presence of lipids and one at 818 cm-1 (*l*) noting the presence of triterpenoids **(Fig. 34)**.

Ethyl acetate fraction spectrum

Three very strong peaks could be seen in the ethyl acetate fraction spectrum. As in aforementioned fractions spectra, two of the major ethyl acetate fraction peaks are found at 1632 cm^{-1} (*a*) and 1603 cm^{-1} (*b*), which assignments were already extensively mentioned in the previous fractions, and the third major peak is found at 1140 cm^{-1} (*f*) that is related to the presence of phenolic compounds and polysaccharides like pectins. Several relatively strong peak clusters can also be observed around peaks at 1208 cm-1 (*d*) and 1065 cm-1 (*h*), which suggest the presence of lipids and glycosidic bonds in ester groups (COC). A fourth major peak is found at 818 cm^{-1} (*l*) pointing to the existence triterpenoid compounds **(Fig. 35)**.

n-Butanol fraction spectrum

The n-butanol fraction spectrum showed a narrow lone peak at 1603 cm^{-1} (*b*) correspondent to the presence of C-C ring related to aromatic compounds. An even stronger three peak cluster can be observed in the lower fingerprint threshold, consisting of the peaks at 1041 cm^{-1} (*i*), 1030 cm^{-1} (*j*) and 986 cm^{-1} (*k*), all associated with the presence of polysaccharides. A protruding shoulder at 1116 cm^{-1} (*g*) also suggests the presence of polysaccharides. The peak a 818 cm^{-1} (*l*) noting the existence of triterpenoids is also somewhat strong, but smaller than in previous fractions **(Fig. 36)**.

Aqueous fraction spectrum

The aqueous fraction spectrum greatly differs from all the other fraction spectra. The peaks are wide and smooth, with two noteworthy peaks at 1603 cm^{-1} (*b*) and 1030 cm^{-1} (*j*), which point the presence of aromatic compounds and the presence of stretching bonds in linear chains of carbons (C-C), respectively. One strong shoulder can be observed adjacent to the later peak at 986 cm^{-1} (*k*) which corresponds to stretching CO bonds depicting to the presence of polysaccharides **(Fig. 37)**.

Table 20. FTIR analysis and peak values of different functional groups obtained from ethanol, n-hexane, chloroform, ethyl acetate , n-butanol and aqueous leaf fractions of *C. album*.

Wavenumber (cm^{-1})		Reference	Biomass	Feature	Tentative assignment	
a	1632	1632 (Martin et al., 2021)	Tamarillo fruit (peel)	ν asym (COO^-), H_2O	- ; water	
b	1603	1600 (Ahmad et al., 2016)	*Heliotropium bacciferum* flower	C-C stretch in ring	Aromatic compounds	*
c	1448	1444 (Martin et al., 2019)	Crowberry fruit (inner skin)	δ(CH_2) / δ(CH_3)glucosydic	Lipids	
d	1208	1214 (Martin et al., 2019)	Crowberry fruit (outer skin)	δ(CCH)	Lipids	
e	1159	1153 (Martin et al., 2021)	Tamarillo fruit (skin)	ν(CO) / ν(CH)	Pectins Phenolic compounds	
f	1140	1145 (Leite et al., 2018)	*Physalis angulata* stem	-	Pectins	*
g	1116	1111 (Martin et al., 2019)	Crowberry fruit (inner skin)	ν(COC)glycosidic	-	
h	1065	1065 (Martin et al., 2021)	Tamarillo fruit (skin)	ν(C-O-C)glycosidic	-	
i	1041	1047 (Martin et al., 2019)	Crowberry fruit (outer skin)	ν(COC)glycosidic	-	
j	1030	1032 (Martin et al., 2019)	Crowberry fruit (inner skin)	ν(C─C)linear chains	Polysaccharides	
k	986	976 (Martin et al., 2019)	Crowberry fruit (outer skin)	ν(CO)	Polysaccharides	
l	818	808 (Martin et al., 2019)	Crowberry fruit (outer skin)	-	Triterpenoids	

Abbreviations: **sym** symmetric; **asym** antisymmetric; **ν** stretching; **δ** bending; **ρ** plane; **τ** twisting; **ω** wagging.

* Due to the wavenumbers relevance in the fractions spectra, and the assignments not being found in the previous studies of Martin et al. (2019) and Martin et al. (2021), from where the present study tentative assignments are drawn from, this exceptional attributions were taken in accordance with previous studies in *H. bacciferum* flowers by Ahmad et al. (2016) and *P. angulata* stems by Leite et al. (2018).

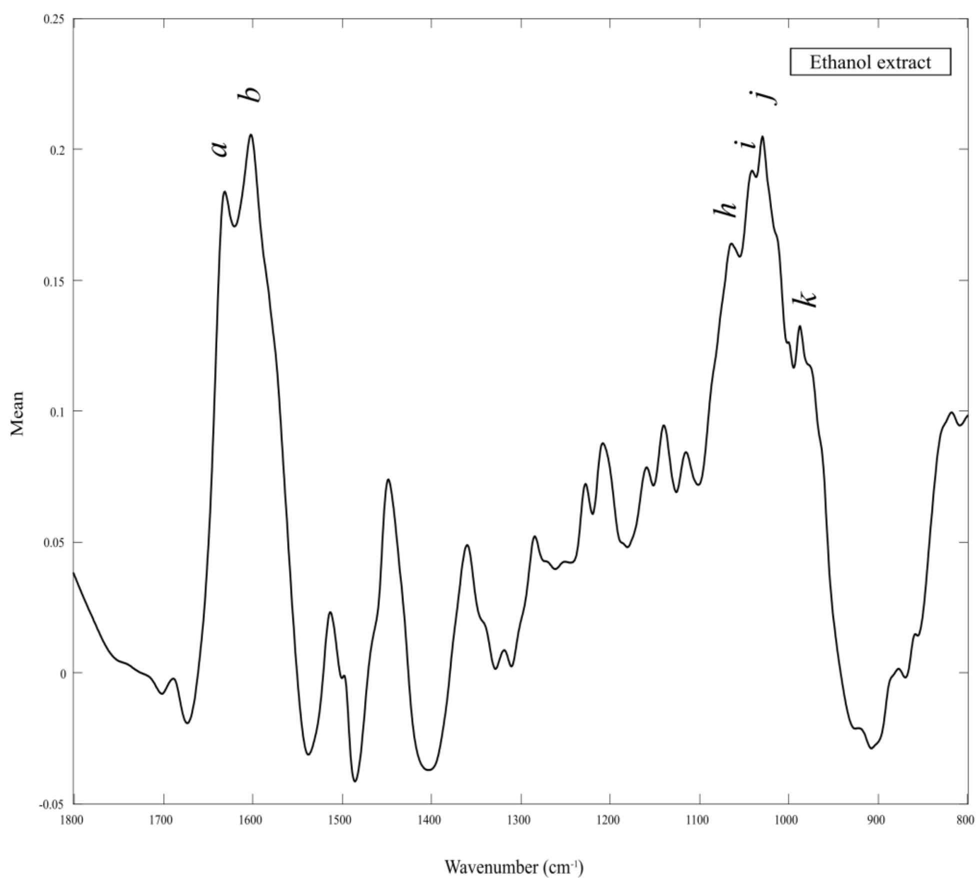

Figure 32. FTIR absorbance spectrum of *C. album* ethanol extract.

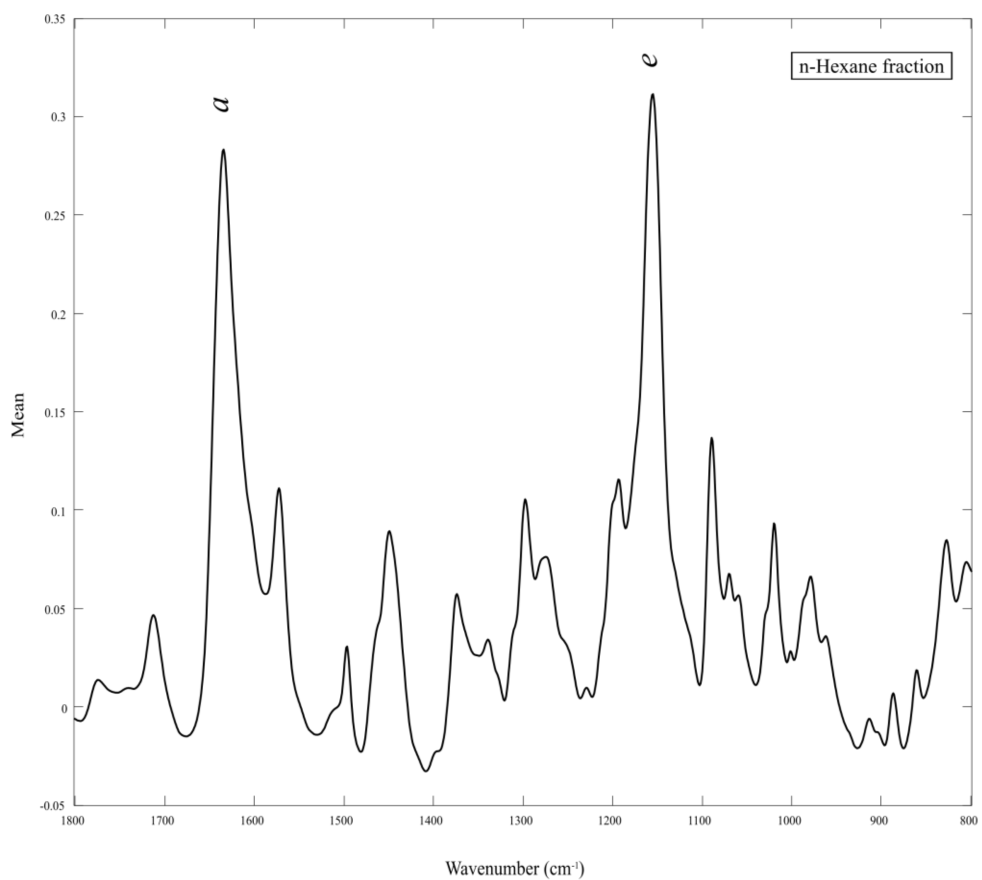

Figure 33. FTIR absorbance spectrum of *C. album* n-hexane fraction.

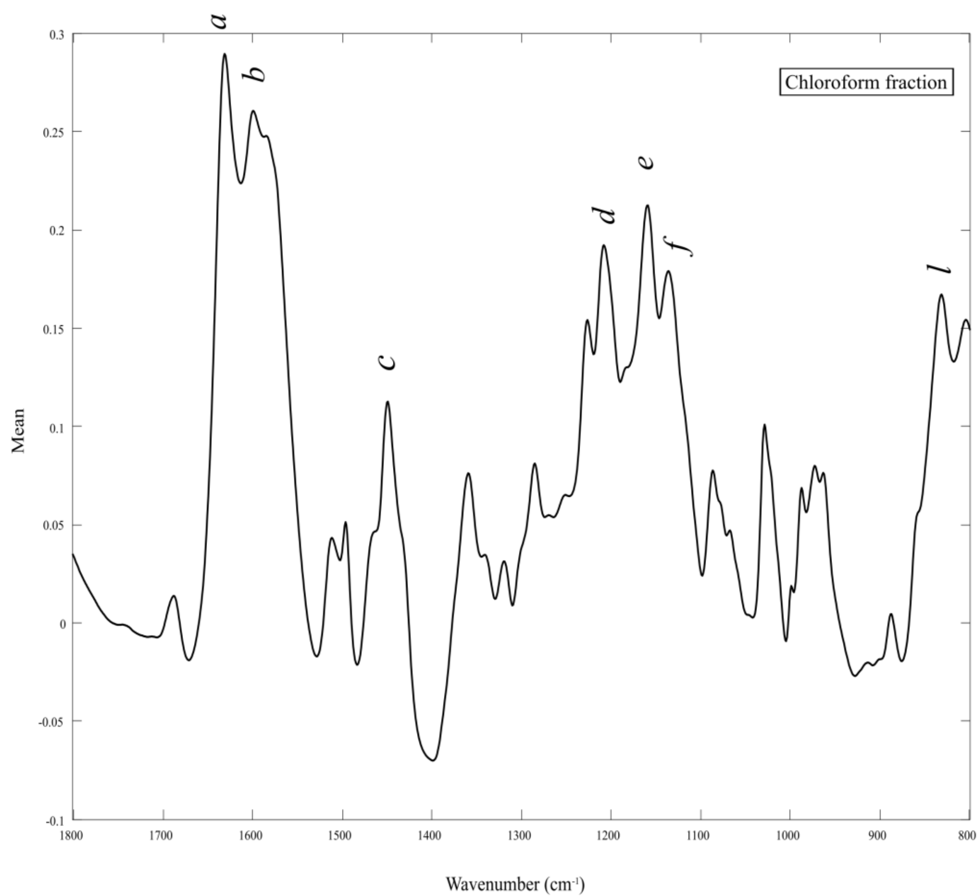

Figure 34. FTIR absorbance spectrum of *C. album* chloroform fraction.

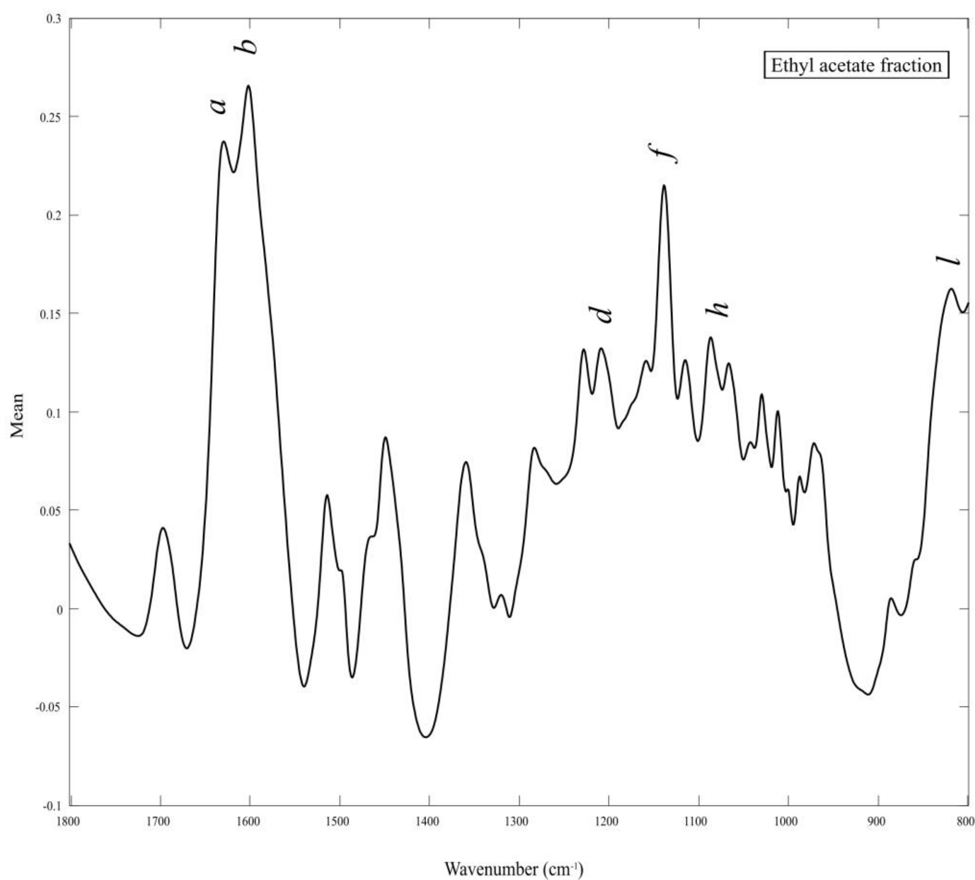

Figure 35. FTIR absorbance spectrum of *C. album* ethyl acetate fraction.

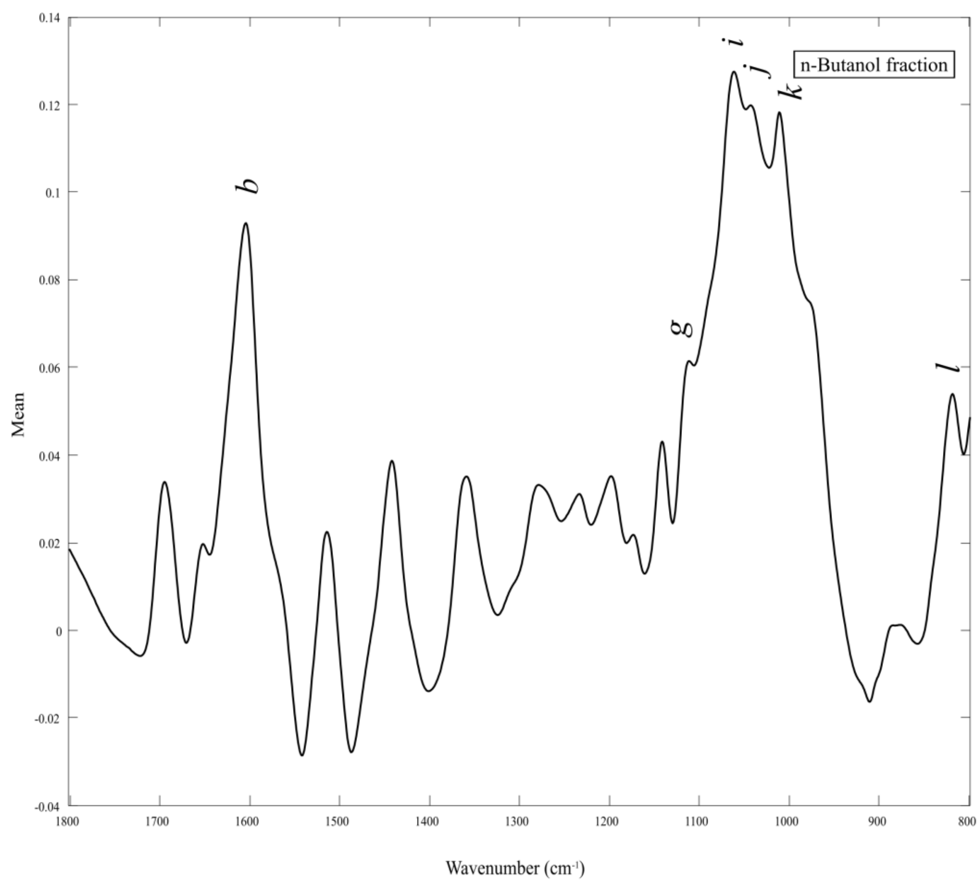

Figure 36. FTIR absorbance spectrum of *S. C. album* n-butanol fraction.

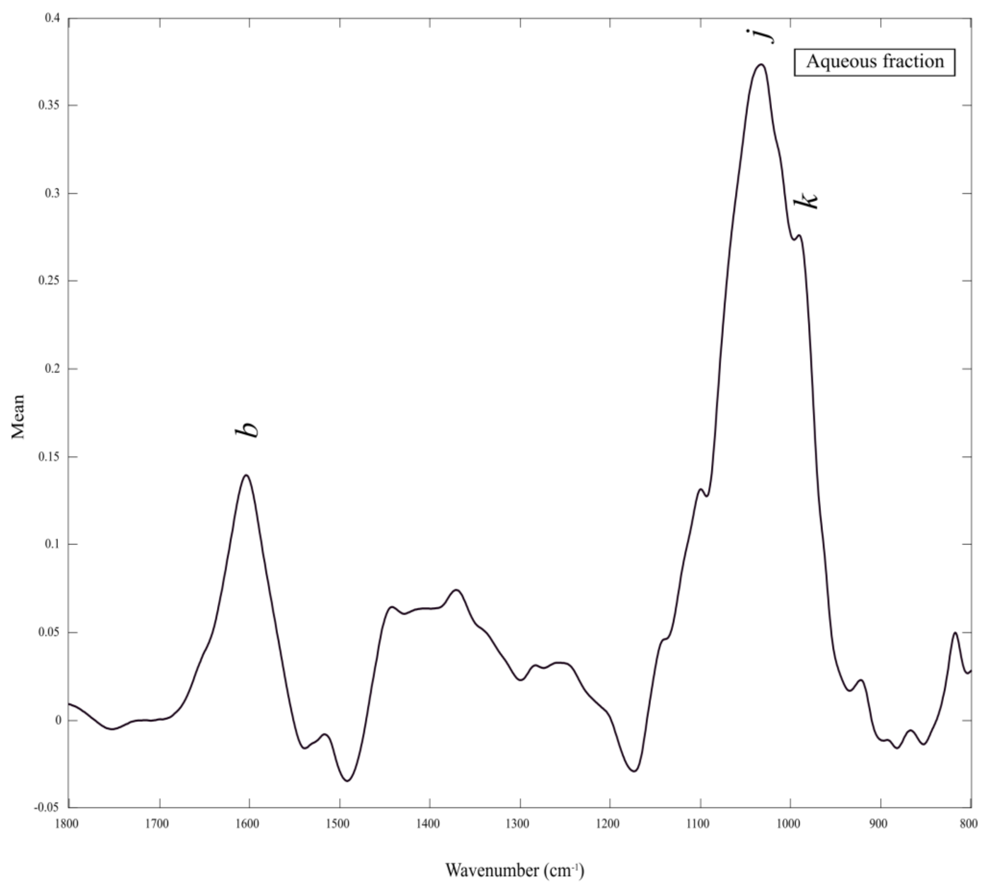

Figure 37. FTIR absorbance spectrum of *C. album* aqueous fraction.

3.4. Trichome identification

3.4.1. *Solanum betaceum* trichomes

Three types of trichomes can be observed in red-type and orange-type of tamarillo leaves, identified in this study as type A1, A2 and B: Type A1 are uniseriate capitate long glandular trichomes with four coplanar secretory cells at their tip (***e.g.:*** **Fig. 39B**), type A2 are short capitate glandular trichomes, also with four coplanar secretory cells (***e.g.:*** **Fig. 38A**) and type B are very long uniseriate apiculate non-glandular trichomes (***e.g.:*** **Fig. 38C**). A third type of glandular trichome was observed in the C9 and C12 *in vitro S. betaceum* varieties, identified here as type A3 This type is a very long uniseriate capitate glandular trichome, possibly with a single secretory cell at its tip (***e.g.:*** **Fig. 41A**).. In all the tamarillo varieties observed, the most numerous trichome is the non-glandular type B, and the indumentum is significantly denser in the abaxial leaf surface and along the leaves midrib and veins. C9 and C12 varieties have much less trichomes than the red and orange-type varieties.

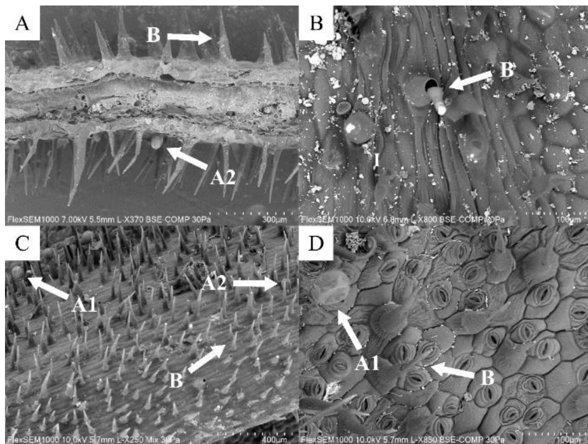

Figure 38. Red-type *S. betaceum* leaf trichomes observation in SEM. **(A)** Transversal cut; **(B)** Adaxial leaf surface; **(C, D)** Abaxial leaf surface.

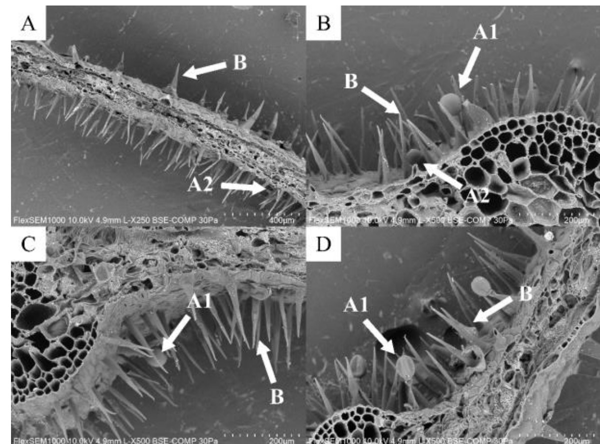

Figure 39. Orange-type *S. betaceum* leaf trichomes observation in SEM. **(A-D)** Transversal cut.

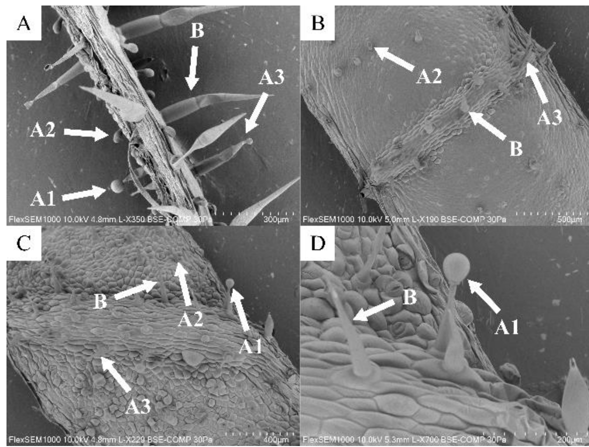

Figure 40. C9 *S. betaceum in vitro* variety leaf trichomes observation in SEM. **(A)** Transversal cut; **(B)** Adaxial leaf surface; **(C, D)** Abaxial leaf surface.

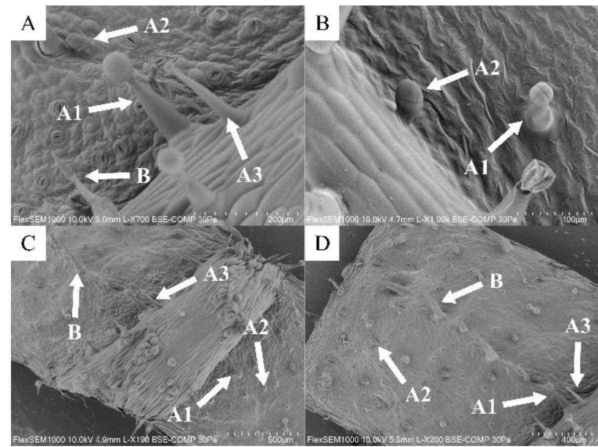

Figure 41. C12 *S. betaceum in vitro* variety leaf trichomes observation in SEM. **(A, B)** Transversal cut; **(C, D)** Adaxial leaf surface.

3.4.2. *Corema album* trichomes

Three type of trichome can be observed in *C. album*, identified in present study as type C1, C2 and D: Type C1 are long uniseriate capitate glandular trichomes **(*e.g.:* Fig. 42F)**, type C2 are short capitate glandular trichomes **(Fig. 42G)**, and type D are long uniseriate non-glandular trichome which covers the vast majority of the abaxial leaf surface **(*e.g.:* Fig 42E)**. Type C1 and C2 are very sparce, especially type C2, which can only be observed once in all SEM captured observations. *C. album* adaxial leaf surface is completely glabrous **(Fig. 42D)**.

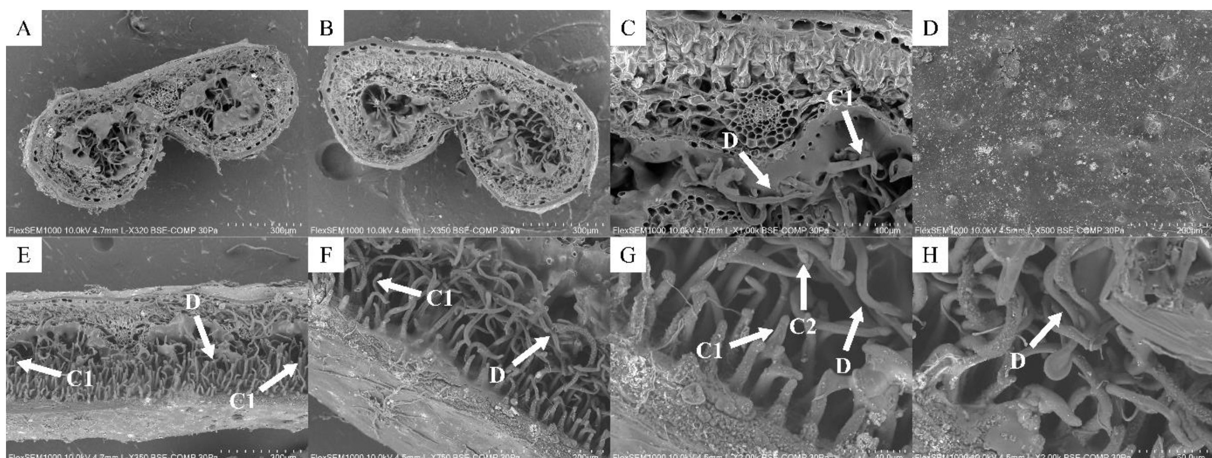

Figure 42. *C. album* leaf trichomes observation in SEM. **(A-C)** Transversal cut; **(D)** Adaxial leaf surface; **(E-H)** Abaxial leaf surface.

4. DISCUSSION

P. cinnamomi is a hazardous phytopathogen responsible for several of plant diseases across the globe and have a broad affinity to many plants hosts. The consequences onset by these diseases can be severely detrimental to the habitats were the pathogen hosts inhabit, as plants hold a fundamental role at the bottom of food chains (Power, 1992) and can provide shelter to many other coexistent species (Bossenbroek et al., 1977; Fukui, 2001). The oomycete can also infect plenty of plant crops and garden species, which have an important economic value. Given the lack of favorable and effective measures to control the propagation of *P. cinnamomi*, the search and study of definitive and environmentally friendly solutions is of utmost importance. A possible solution to this matter is the use of plant extracts as a cheap and low residual alternative to the use of potentially harmful pesticides (Hossain et al., 2017; Laxmishree & Nandita, 2017). In the present study, *S. betaceum* and *C. album* fractioned leaf extracts were assessed *in vitro* conditions to test anti-oomycete activity in *P. cinnamomi* growth. To the best of my knowledge, this is the first report in the anti-oomycete activity of *S. betaceum* and *C. album* leaf extracts against *P. cinnamomi*.

Results analysis - S. betaceum fractions

The n-butanol fraction was the only *S. betaceum* fraction which showed some kind of significant inhibitory activity against *P. cinnamomi* during the MAA. The n-butanol fraction inhibited *P. cinnamomi* growth at 18 mg/mL in standard treatments and at 11 mg/mL and above without PDB in conditioned treatments. Despite these results, no clear conclusions can be drawn regarding the MIC/MLC of the n-butanol fraction other than it is between 10.95-18 mg/mL, as the gap between this tested concentrations is very high, and the absence of growth medium highly compromises the oomycete growth. Nonetheless, the n-butanol fraction held promising results during EFAA growth reduction tests, causing a reduction of 41.57 ± 8.12 % in *P. cinnamomi* hyphal growth area (HGA) at concentrations as low as 2.5 mg/mL, and significantly reduces *P. cinnamomi* HGA by 75.90 ± 1.18 % at 10 mg/mL. The polyphenols and terpenoid compounds present in the n-butanol fraction are likely involved in the anti-oomycete activity observed, as the anti-oomycete activity of both compounds classes are well documented (*e.g.:* Shim et al., 2009; Damian Badillo et al., 2010; Madrid et al., 2015; Montenegro et al., 2019). The presence of polysaccharides may also play a role in the bioactivity of the n-butanol fraction. Although few, some studies have already been published

which report the direct antimicrobial activity of polysaccharides (Paris et al., 2019). Like in the MAA, the n-butanol fraction and PDA mixture also formed a precipitate during the EFAA, but in this treatments the precipitate starts forming at a concentration of 5 mg/mL, approximately at half the concentration at which the precipitate formed during MAA. This is likely due to the medium used being PDA and not PDB. The *S. betaceum* ethyl acetate fraction inhibited *P. cinnamomi* growth in two of the three replicates conducted at 3.33 mg/mL during the MAA standard treatments. Despite the inhibition observed in two of the replicates at this concentration, we could not consider the 3.33 mg/mL as the MIC of the ethyl acetate fraction, as one of the replicates did grow. The fraction plus PDB medium solution used in the treatments was primarily mixed outside of the wells and then equally divided between the multiwells, which means that there were no possible treatment differences between each replicate and being plausible that the remaining two replicates failed to grow as a result of other factors such as material handling carelessness which might have compromised *P. cinnamomi* cultures. Curiously, *P. cinnamomi* is able to grow at 3 mg/mL plus DMSO 5 % during the ethyl acetate fraction conditioned treatments. The fact that the oomycete is able to grow at this comparatively similar concentration with the presence of DMSO seems to support the aforementioned statement. Previous studies conducted with various solanaceous plants report similar results to the ones observed in *S. betaceum* fractions during the present study. Muto et al. (2006) reports that nightshade (*Solanum nigrum*) roots n-butanol extract is effective at inhibiting conidial germination in the fungal pathogen *Alternaria brassicicola*, and studies carried out by Khan et al. (2011) conducted with night-blooming jasmine (*Cestrum nocturnum*) shows that the whole plant n-butanol extract have an high antimicrobial activity against several fungal and bacterial pathogenic strains. No *P. cinnamomi* growth inhibition was noted during the remaining standard treatments conducted with the *S. betaceum* ethanol, n-hexane, chloroform and aqueous fractions, so no further conclusions can be made about the mentioned fractions MIC/MLC. The lack of activity of the ethanol extract may be explained by the concentration of the extract compounds, which were too diluted in the ethanol extract and became more concentrated in the n-butanol fraction. Additionally, the aqueous fraction EFAA results reveals that the fraction is not very effective at reducing *P. cinnamomi* HGA, especially when compared to the other *S. betaceum* fraction of n-butanol tested in EFAA. The aqueous fraction is approximately half as effective as the n-butanol fraction regarding *P. cinnamomi* growth reduction (GR), with a minimum reduction of 8.60 ± 10.35 % in the 1.25 mg/mL treatment and a maximum reduction of 53.00 ± 3.41 % in the 10 mg/mL treatment. It is important to highlight that the aqueous fraction concentrations tested during EFAA present little statistically significant differences

amongst themselves and the 1.25 and 5 mg/mL treatments shows highly variable results, so much so that one of the 1.25 mg/mL treatment replicates actually increases *P. cinnamomi* HGA by 6.60 % when compared to the control HGA mean. This outlier result may be due to various reasons, but the difference in fraction plus growth medium mixture can be excluded, as the treatment growth solutions were prepared prior to their addition and distribution to the Petri dishes. A possible explanation may be the hormesis effect. Hormesis is highly specific between the active agent and the exposed species (Calabrese et al., 2019) and its effects are apparent and widely reported in oomycetes and fungi alike. For instance, studies by Kato et al. (1990) and Zhang et al. (1997) report that the common fungicide hymexazol stimulates growth at low concentrations in *Phytophthora* spp.. Likewise, studies by Fenn & Coffey (1984) conducted with phosphorus acid show that the compound boosts the growth of *Pythium ultimum* and *Pythium myriotylum* at low doses. In present study, the exposure to low concentrations of the supposedly inhibitory aqueous fraction may trigger a compensatory stress response in *P. cinnamomi* and promote its growth, with further testing being needed. In contrast with the previously assessed n-butanol fraction, a higher concentration of polysaccharides than polyphenols can be observed in the aqueous fraction, with a seemingly similar concentration of terpenoid compounds. A substantial decrease in ester compounds is also observed, which suggests a reduction in the fraction lipid content. Both chemical composition changes are to be expected, given that polysaccharides and terpenoids are relatively soluble in water (due to hydrogen bonding), while polyphenols are only sparingly soluble and lipids are hydrophobic, both more soluble in non-polar solvents such as n-butanol. This reduction in polyphenols may be influencing the inhibitory activity of the aqueous fraction in relation with the n-butanol fraction, again suggesting that *P. cinnamomi* is likely showing a higher sensitivity towards the polyphenol content rather than the polysaccharide compounds.

Results analysis - C. album fractions

The standard treatments conducted using *C. album* extract fractions held much more interesting results. We were able to assess most of the tested fractions MIC/MLC and draw clearer conclusions regarding the fractions inhibitory capacity. Unfortunately, several conflicting results arose from PTG tests when compared to the corresponding MAA treatments results, which will be discussed throughout. The n-hexane fraction inhibits *P. cinnamomi* growth starting at 1.75 mg/mL, indicating that the fraction MIC may possibly be 1.75 mg/mL. However, this conclusion cannot be fully supported, as the lethality of this concentration was not assessed with additional PTG tests. The treatments conducted with the chloroform fraction

reveal that 1.75 mg/mL is lethal to *P. cinnamomi* and likely is the chloroform fraction MLC, considering that the material treated at 1.75 mg/mL and above failed to grow during PTG tests. This indicates that the chloroform MIC has to be between 1.50-1.75 mg/mL. Curiously, the oomycete material treated at 1.50 mg/mL, which did grow during the MAA treatments, failed to grow in the succeeding PTG tests. This might be a result of carelessness in material handling or rinsing during the oomycete transfer (*e.g.:* tongs being too hot; ineffective fraction rinsing) from the multiwell to the Petri dish in which PTG were conducted. The ethyl acetate fraction inhibits *P. cinnamomi* growth starting at 1.25 mg/mL. This inhibited oomycete growth (except the 1.50 mg/mL treated material) was subsequently subject to PTG tests in which it also failed to grow, concluding that 1.25 mg/mL is the ethyl acetate fraction MLC and that the fraction MIC is within 1.00-1.25 mg/mL. Not many conclusions can be drawn from the *C. album* n-butanol fraction treatments. Despite its high solubility in water, which allows the testing of relatively high concentrations, the mixture of fraction plus PDA medium form a dense precipitate in the solution over time starting at 10.25 mg/mL, making it impossible to observe *P. cinnamomi* hyphae growth and draw results from these treatments. Nonetheless, further PTG tests were conducted with oomycete material treated with the n-butanol fraction between 10-10.75 mg/mL. This testing brought contradicting results, with oomycete material treated with 1.75 mg/mL successfully growing, but material treated with 1.5 mg/mL failing to do so. Yet again, this irregular result is likely to be a result of *P. cinnamomi* material handling sloppiness. The only conclusion that can be drawn from the n-butanol MAA treatments is that, despite the lack of observable results, 10.75 mg/mL cannot be the fraction MLC, as *P. cinnamomi* material treated at this concentration successfully grew in PTG tests. The n-butanol fraction was also used in EFAA treatments, where we obtained interesting results. From all the EFAA fractions tested, it was the most effective fraction at reducing *P. cinnamomi* HGA, with an impressive reduction of 65.81 ± 4.41 % in the lowest concentration tested of 1.25 mg/mL treatment and completely inhibiting *P. cinnamomi* growth in the 10 mg/mL treatment. These are interesting results, as the concentration of 10 mg/mL failed to inhibit the pathogen growth during the MAA treatments but successfully inhibited it during the EFAA treatments. Only the growth medium used differ in the different assays, with PDB being used in MAA and PDA being used in EFAA. Being the only difference amongst treatments, the growth medium seems to influence *P. cinnamomi* ability to grow and may have synergistic properties with the extract, being further research needed to confirm this assessment. Additionally, the *C. album* n-butanol fraction was tested in conditioned treatments between 11-18 mg/mL without PDB, successfully inhibiting oomycete growth in all treatments. Further PTG tests conducted with *P. cinnamomi* material

treated at 11 and 12 mg/mL without PDB reveal that these concentrations are lethal to the oomycete. On a side note, no precipitate is formed during the *C. album* conditioned n-butanol fraction treatments without PDB, akin to what occurred in the standard treatments at the same tested concentrations. This is likely due to the absence of PDB medium, which forms the precipitate by reacting with the n-butanol fraction. The aqueous fraction did not show any relevant results, failing to inhibit *P. cinnamomi* growth even at concentrations as high as 18 mg/mL. This result suggest that the aqueous fraction MIC is far from being reached. The fraction was also used during EFAA tests, where it showed modest results at 5 and 10 mg/mL, with an oomycete HGA reduction of 32.94 ± 12.76 % and 57.89 ± 2.58 %, respectively. Surprisingly, the aqueous fraction boosts *P. cinnamomi* HGA in all the 1.25 mg/mL replicates by 9.02 ± 2.37 % increase, and in one 2.5 mg/mL replicate, which increase HGA by 13.00 %. The possible explanation to this growth stimulation may be the hormesis effect, which was previously explained in similar results observed in the *S. betaceum* aqueous fraction (see *Results analysis - S. betaceum fractions*). Inhibiting *P. cinnamomi* growth starting at 0.7 mg/mL, the ethanol extract has the lowest inhibitory concentration of all tested fractions in the entire present study, showing impressive results. The oomycete material which failed to grow was subject to PTG tests, in which it also failed to grow, suggesting that 0.7 mg/mL is the MLC of the ethanol extract and that its MIC must be between 0.625-0.7 mg/mL. Oomycete material treated at 1.25 and 2.5 mg/mL in the MAA did grow during PTG tests. These two outliers are particularly strange, as the usual outlier results occur when oomycete material fails to grow in between successful results and can be reasonably justified with material handling carelessness. Most of the *C. album* fractions possess acids (presence of negatively charged carboxyl groups), aromatic compounds and ester compounds in their composition, which are likely the compounds responsible for the inhibition of *P. cinnamomi* growth. The anti-oomycete activity of various acid compounds is well documented against *Phytophthora* spp. (*e.g.:* Lee et al., 2004; Son et al., 2008), as well as in aromatic compounds (*e.g.:* Montenegro & Madrid, 2019; McKee et al., 2020). Due to the non-specific chemical characterization conducted in this study, no further assessments can be made of which compounds class are present in the *C. album* fractions. The detected acid may be related to a plethora of different compounds which possess COO^- groups, and the aromatic compounds can be related to phenolic compounds or alkaloids (aromatic ring), which are highly different in terms of their bioactivity, with further research being needed to identify the specific compounds present. The detection of triterpenoids in several of the *C. album* fractions also corroborates with the detection of aromatic compounds.

The *C. album* n-butanol and aqueous fractions showed a relatively small anti-oomycete activity during the MAA treatments, when compared to the remaining tested fractions. Nonetheless, the n-butanol fraction inhibition shown during the EFAA treatments is astonishing, effectively halting *P. cinnamomi* growth at 10 mg/mL. The fraction possesses a high concentration of aromatic compounds, polysaccharides and triterpenoids, akin to most of the remaining *C. album* fractions. Further tests should had been conducted using the other fractions to fully assess the compounds anti-oomycete activity, but the lack of crude extract and the fractionation yield conditioned the number of possible tests and fraction material we could use during the present study.

DMSO and growth medium influence on P. cinnamomi

The conditioned treatments conducted with both species fractions seem to indicate that the PDB has substantially more influence in oomycete growth than the DMSO, as the PDB presence allow *P. cinnamomi* to grow in treatments with up to a 10x greater DMSO percentage. This is to be expected as the PDB medium supply nutrients which sustain the oomycete growth. *P. cinnamomi* is capable of growing in several of the conditioned treatments conducted with DMSO 5 %, but the control treatments show that DMSO 5 % by itself is lethal to *P. cinnamomi*. This observation suggests that DMSO and our extract fractions may have synergistic properties amongst themselves, but this assessment cannot be fully supported by the present study results alone, being further research needed.

Taken together, these results show that there are substantial differences between the two species fractions regarding the inhibition of *P. cinnamomi*. *C. album* fractions are significantly more effective in the control of the pathogen growth, especially the ethanolic initial extract, which is seemingly as effective at low concentrations as the fungicide *Aliette Flash*. The *C. album* ethanol extract successfully halts *P. cinnamomi* growth at 0.7 mg/mL, similarly to the lowest tested concentration of *Aliette Flash* of 0.75 mg/mL. It is relevant to point out that the determined MIC/MLC of our extract fraction may not be the exact concentrations, as they may be found between the known tested concentrations in which *P. cinnamomi* grew and failed to do so, such as the *C. album* n-hexane fraction MIC, that may actually be found within 1.50-1.75 mg/mL. To precisely find the MIC/MLC of a given fraction, exhaustive concentration honing tests must be conducted. Furthermore, additional precise treatments can always be made in accordance with preceding results, resulting in a seemingly endless number of possible tests

in search of the exact MIC/MLC concentrations. The limited fraction material available to test in the present study strictly conditions the number of testing that can be handled, so concentration honing tests had to be reasonably conducted. The Portuguese-crowberry belongs to the Ericaceae family, a particularly susceptible family to *P. cinnamomi* which causes a vast array of root diseases in the family species (Moreira & Martins, 2005; Robin et al., 2012; Newhook, 2020), thus the increased extract effectiveness against *P. cinnamomi* is an interesting and unexpected result. Recent studies conducted with *Arbutus unedo*, another Ericaceae, by Martins et al., (2021) report similar results using the species leaf extracts, proving its effectiveness in reducing *P. cinnamomi* growth.

Trichomes

The identification of trichomes in tamarillo was strongly based on trichome morphological characteristics described by Luckwill (1943), which thoroughly described and identified the trichome morphology of *Lycopersicon*. Luckwill (1943) classifies *Lycopersicon* trichomes as type I-VII, being types I, IV, VI and VII glandular trichomes and types II, III, V non-glandular trichomes. The trichome types observed in this study share many morphological similarities and can be compared with the trichomes types described by Luckwill (1943). Type A1, A2 and A3 glandular trichomes are similar to the type VI, VII and IV respectively, and the type B non-glandular trichomes resemble type III trichomes. *S. betaceum* presents much more non-glandular trichomes than glandular trichomes. The glandular to non-glandular trichomes ratio seems to be largely dependent of abiotic and biotic environment factors such as water availability (Lauter & Munns, 1986). Plants need to spend water in the production and maintenance of glandular trichomes and their exudates, which may be disadvantageous in hotter environments (Lauter & Munns, 1986; Van Dam et al., 1999), and favor the production of non-glandular trichomes. The production of non-glandular trichomes can also be related to herbivorous insects population density (Gibson, 1979). In all the tamarillo genotypes observed, the indumentum is significantly denser in the abaxial leaf page and along the leaves midrib and veins. This density difference between the adaxial and abaxial leaf pages may be due to the trichomes role in stomata protection against damaging UV radiation (Grammatikopoulos et al., 1994) or transpiration control. Brewer et al. (1991) and Fernández et al. (2014b) report that higher trichome density in the abaxial leaf surface result in a lower surface water retention, which decreases stomatal occlusion and promotes CO_2 leaf exchanges in *Glycine max* and *Quercus ilex* leaves, respectively. Further studies should be conducted to access the role of *S. betaceum* trichomes in plant defense and water retention. C9 and C12 varieties have much less

trichomes than the red and orange-type varieties. This observation was expected, as the leaves from the *in vitro* genotypes are much younger than the leaves observed from the red and orange-type, and the *in vitro* plantlets are not as exposed to biotic and abiotic factors, which stimulate trichome production. The A3 type found in the *in vitro* genotypes may be a result of mutations that condition trichome development or deviations in the formation of type A1 trichomes. We cannot assume with certainty that this type of trichomes have a single secretory cell, as a bundle of cells may be encapsuled in a membrane, visually appearing as a single cell. In all four *S. betaceum* variants trichomes were significantly denser in the abaxial leaf surface and along the midrib and nervures. Three types of trichomes were observed in all tamarillo varieties, one type of needle-like (or apiculate) multicellular non-glandular trichomes, and two types of multicellular glandular trichomes, one being long and the other being short, both with four coplanar conspicuous secretory cells. These types of trichomes can be observed in different stages of maturation, leading to morphological differences such as size and number of cells. These observations go in accordance with observations made by Mahlberg (1985).

C. album presents a large number of long non-glandular trichomes and only a small number of glandular trichomes are observed. The presence of a thick mucilage at the abaxial surface suggests that a substantial amount of glandular trichomes should be present, from which the mucilage would secrete from, but only a very small number of glandular trichomes were observed, possibly due to the thick mucilage covering them. The adaxial surface is completely glabrous, where no trichomes are present. From a personal observation, the C1 type trichomes are morphologically similar to the D type trichomes. The D type trichomes are long non-glandular trichomes, seemingly unicellular. The C1 type are long glandular multicellular trichomes and the C2 type are short glandular multicellular trichomes. These observations go in accordance with observations made in Antunes et al. (2018). The compounds tested in the present study may be present either in the leaf cells themselves or in the thick mucilage present, with further studies being needed to fully assess the compounds origin.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The study of the activity of plant secondary metabolites such as plant stress tolerance give way to the discovery of potential new applications to these compounds and generate an increasing interest in these compounds research and production. The promising results obtained provide a first approach and contribute to future research concerning the identification and purification of the tested fractions compounds which can be further applied in selective breeding programs to develop more resistant cultivars.. The present study shows that tamarillo and crowberry leaves may be useful materials to produce natural plant products to control *P. cinnamomi* infections and its associated diseases, and possibly contribute to the discovery of an environmentally-friendly alternative to phosphites and their derivatives pesticides, which currently are the best (and worst) treatment solution to *P. cinnamomi* control.

Given the present study results obtained with the *C. album* crude ethanol extract and the n-butanol fraction, it would be of interest to further research these specific fractions composition and assess *P. cinnamomi* sensitivity towards these compounds. Moreover, the studied fractions effectiveness should also be subsequentially tested *in vivo*, which also let us evaluate if the fractions are toxic to the plant organisms subjected to the treatment and if external factors not present in *in vitro* testing influence the fractions effectiveness.

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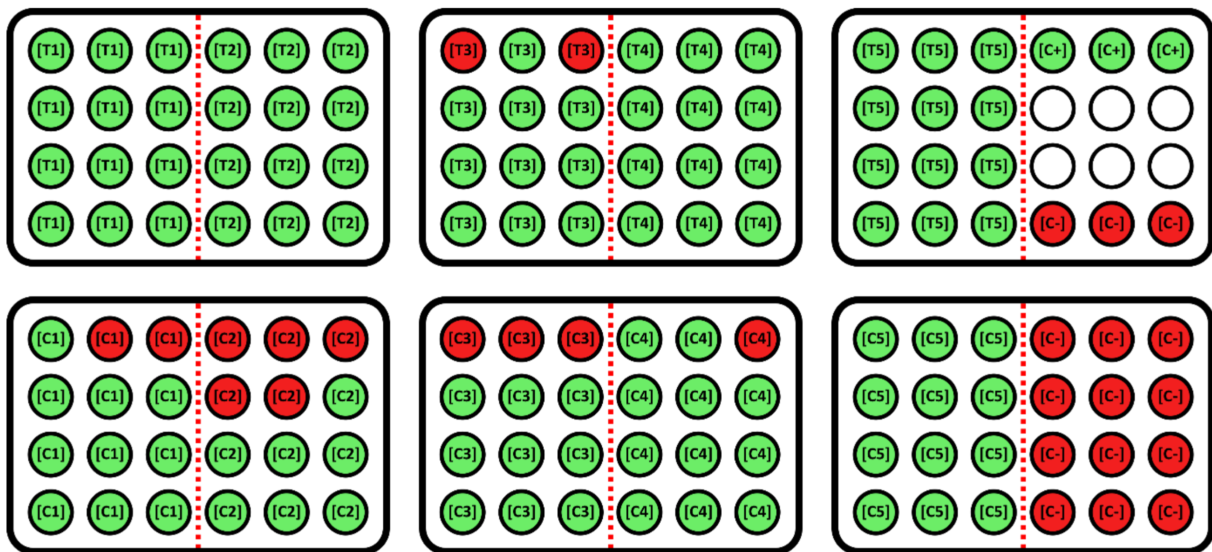
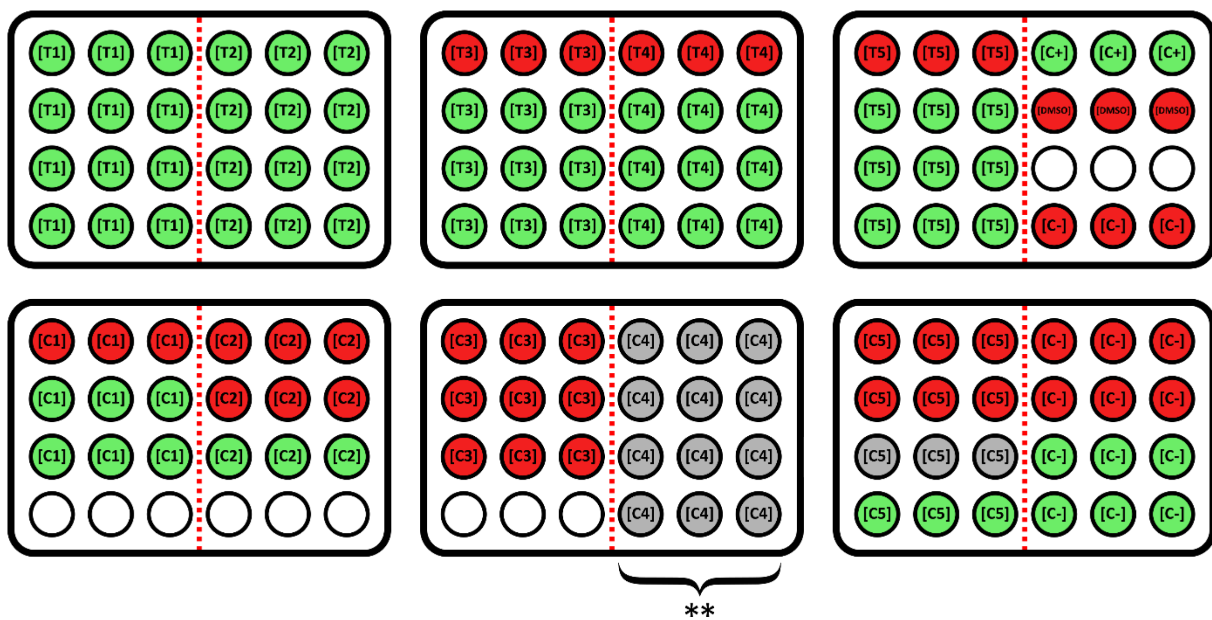
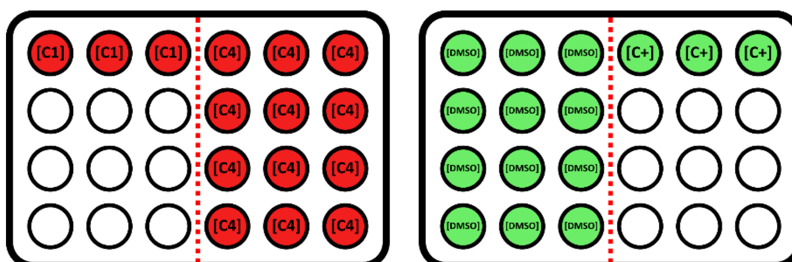
APPENDIXES



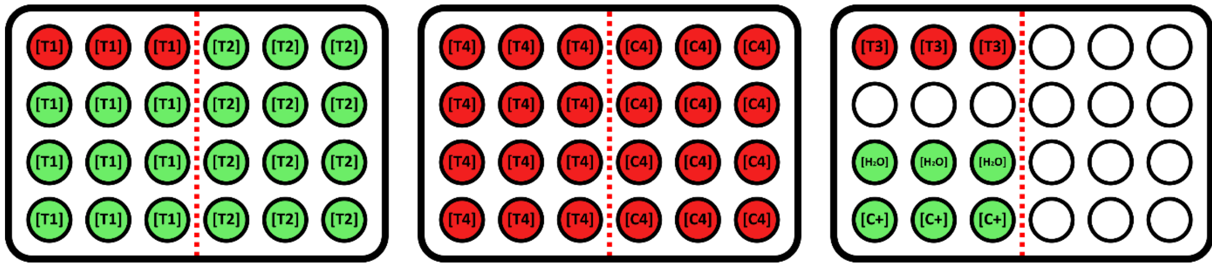
Appendix 1. Multiwell activity assay #1 growth results. **Green** – Growth; **Red** – No growth.



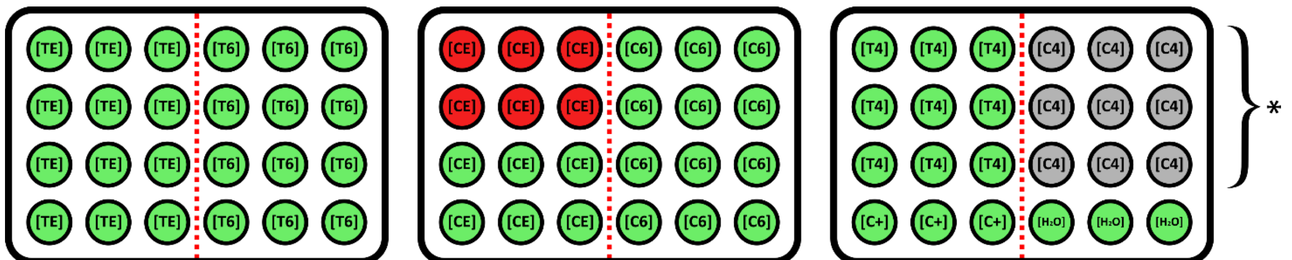
Appendix 2. Multiwell activity assay #2 growth results. ** A dense precipitate was found and hyphal growth could not be observed. **Green** – Growth; **Red** – No growth.



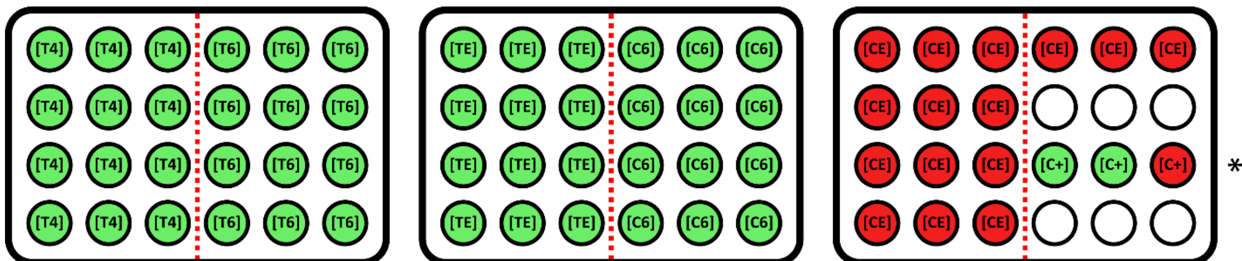
Appendix 3. Multiwell activity assay #3 growth results. **Green** – Growth; **Red** – No growth.



Appendix 4. Multiwell activity assay #4 growth results. **Green** – Growth; **Red** – No growth.



Appendix 5. Multiwell activity assay #5 growth results. * A dense precipitate was found and hyphal growth could not be observed. **Green** – Growth; **Red** – No growth.



Appendix 6. Multiwell activity assay #6 growth results. * Control replicate did not grow.
Green – Growth; **Red** – No growth.

Appendix 7. Extract fraction activity assay (EFAA) growth area results.

Treatment	C (mg/mL)	Area (cm^2)
Tamarillo n-Butanol fraction (4-BUT)	10	5,381
		5,499
		6,003
	5	6,582
		8,133
		8,792
	2.5	10,389
		14,851
		13,329
	1.25	21,063
		21,336
		19,574
Tamarillo Aqueous fraction (5-AQU)	10	11,113
		9,304
		10,723
	5	14,479
		17,561
		13,876
	2.5	18,897
		17,713
		17,595
	1.25	24,318
		20,852
		18,576
Crowberry n-Butanol fraction (4-BUT)	10	0
		0
		0
	5	2,424
		2,000
		2,624
	2.5	3,998
		5,157
		4,279
	1.25	6,888
		9,331
		7,830
Crowberry Aqueous fraction (5-AQU)	10	9,608
		9,913
		8,539
	5	15,327
		13,978
		15,299
	2.5	25,777
		17,663
		22,474
	1.25	25,971
		24,871
		24,786
PDA	-	22,679
		22,639
		23,121
PDA 2x	-	16,054
		18,789

13,750

Appendix 8. *S. betaceum* 4-BUT EFAA treatment growth (TG) and growth reduction (GR).

C (mg/mL)	TG (cm^2)	GR (cm^2)	GR (%)	GR_{mean} (%)	StdDev
1.25	21.063 21.336 19.574	1.750 1.477 3.239	7.671 6.474 14.198	7.671	3.39
2.5	10.389 14.851 13.329	12.424 7.962 9.484	54.460 34.901 41.573	41.573	8.12
5	6.582 8.133 8.792	16.231 14.680 14.021	71.148 64.349 61.461	64.349	4.06
10	5.381 5.499 6.003	17.432 17.314 16.810	76.413 75.895 73.686	75.895	1.18

Appendix 9. *S. betaceum* 4-BUT EFAA (one-way ANOVA; p=0.05).

	SS	df	MS	F	P-value	F_{crit}
Between groups	7688.3297	3	2562.7766	71.6997	**0.00000401**	4.0662
Within groups	285.9454	8	35.7432	-	-	-
Total	7974.2751	11	-	-	-	-

Appendix 10. *S. betaceum* 4-BUT EFAA *post hoc* Tuckey test (p=0.05). Significative or non-significative differences between groups are marked as green and red, respectively.

Groups	1.25 vs 2.5		1.25 vs 5		1.25 vs 10		2.5 vs 5		2.5 vs 10		5 vs 10	
	1.25	**2.5**	**1.25**	**5**	**1.25**	**10**	**2.5**	**5**	**2.5**	**10**	**5**	**10**
Average	9.4478	43.6447	9.4478	65.6526	9.4478	75.3313	43.6447	65.6526	43.6447	75.3313	65.6526	75.3313
Variance	17.2814	98.8585	17.2814	24.7358	17.2814	2.0970	98.8585	24.7358	98.8585	2.0970	24.7358	2.0970
N	3	3	3	3	3	3	3	3	3	3	3	3
P. variance	58.0700	-	21.0086	-	9.6892	-	61.7971	-	50.4778	-	13.4164	-
H. mean	0	-	0	-	0	-	0	-	0	-	0	-
df	4	-	4	-	4	-	4	-	4	-	4	-
t Stat	-5.4961	-	-15.0183	-	-25.9225	-	-3.4288	-	-5.4622	-	-3.2363	-
P one-tail	0.0027	-	0.0001	-	0.0000	-	0.0133	-	0.0027	-	0.0159	-
t_{crit} one-tail	2.1318	-	2.1318	-	2.1318	-	2.1318	-	2.1318	-	2.1318	-
P two-tail	0.0053	-	0.0001	-	0.0000	-	0.0266	-	0.0055	-	0.0318	-
t_{crit} two-tail	2.7764	-	2.7764	-	2.7764	-	2.7764	-	2.7764	-	2.7764	-

Bonferroni correction: α = p significance/nº comparisons = 0.05/6 = 0.00833

Appendix 11. *S. betaceum* 5-AQU EFAA treatment growth (TG) and growth reduction (GR).

C (mg/mL)	TG (cm²)	GR (cm²)	GR (%)	GR_{mean} (%)	StdDev
1.25	24.318 20.852 18.576	-1.505 1.961 4.237	-6.597 8.596 18.573	8.596	10.35
2.5	18.897 17.713 17.595	3.916 5.100 5.218	17.166 22.356 22.873	22.356	2.58
5	14.479 17.561 13.876	8.334 5.252 8.937	36.532 23.022 39.175	36.532	7.07
10	11.113 9.304 10.723	11.700 13.509 12.090	51.287 59.216 52.996	52.996	3.41

Appendix 12. *S. betaceum* 5-AQU EFAA (one-way ANOVA; p=0.05).

	SS	df	MS	F	P-value	F_{crit}
Between groups	3668.6164	3	1222.8721	18.59197	**0.00057789**	4.0662
Within groups	526.1938	8	65.7742	-	-	-
Total	4194.8102	11	-	-	-	-

Appendix 13. *S. betaceum* 5-AQU EFAA *post hoc* Tuckey test (p=0.05). Significative or non-significative differences between groups are marked as green and red, respectively.

Groups	1.25 vs 2.5		1.25 vs 5		1.25 vs 10		2.5 vs 5		2.5 vs 10		5 vs 10	
	1.25	2.5	1.25	5	1.25	10	2.5	5	2.5	10	5	10
Average	6.8572	20.7981	6.8572	32.9096	6.8572	54.4996	20.7981	32.9096	20.7981	54.4996	32.9096	54.4996
Variance	160.6480	9.9628	160.6480	75.0707	160.6480	17.4154	9.9628	75.0707	9.9628	17.4154	75.0707	17.4154
N	3	3	3	3	3	3	3	3	3	3	3	3
P. variance	85.3054	-	117.8593	-	89.0317	-	42.5167	-	13.6891	-	46.2431	-
H. mean	0	-	0	-	0	-	0	-	0	-	0	-
df	4	-	4	-	4	-	4	-	4	-	4	-
t Stat	-1.8486	-	-2.9391	-	-6.1840	-	-2.2749	-	-11.1560	-	-3.8884	-
P one-tail	0.0691	-	0.0212	-	0.0017	-	0.0426	-	0.0002	-	0.0089	-
t_{crit} one-tail	2.1318	-	2.1318	-	2.1318	-	2.1318	-	2.1318	-	2.1318	-
P two-tail	0.1382	-	0.0424	-	0.0035	-	0.0853	-	0.0004	-	0.0177	-
t_{crit} two-tail	2.7764	-	2.7764	-	2.7764	-	2.7764	-	2.7764	-	2.7764	-

Bonferroni correction: α = p significance/nº comparisons = 0.05/6 = 0.00833

Appendix 14. *C. album* 4-BUT EFAA treatment growth (TG) and growth reduction (GR).

C (mg/mL)	TG (cm^2)	GR (cm^2)	GR (%)	GR_{mean} (%)	StdDev
1.25	6.888	15.925	69.807	65.677	4.41
	9.331	13.482	59.098		
	7.83	14.983	65.677		
2.5	3.998	18.815	82.475	81.243	2.16
	5.157	17.656	77.394		
	4.279	18.534	81.243		
5	2.424	20.389	89.374	89.374	1.14
	2	20.813	91.233		
	2.624	20.189	88.498		
10	0	22.813	100	100	0
	0	22.813	100		
	0	22.813	100		

Appendix 15. *C. album* 4-BUT EFAA (one-way ANOVA; p=0.05).

	SS	df	MS	F	P-value	F_{crit}
Between groups	2003.1307	3	667.7102	70.01962	**0.00000439**	4.0662
Within groups	76.2884	8	9.5360	-	-	-
Total	2079.4190	11	-	-	-	-

Appendix 16. *C. album* 4-BUT EFAA *post hoc* Tuckey test (p=0.05). Significative or non-significative differences between groups are marked as green and red, respectively.

Groups	1.25 vs 2.5		1.25 vs 5		1.25 vs 10		2.5 vs 5		2.5 vs 10		5 vs 10	
	1.25	**2.5**	**1.25**	**5**	**1.25**	**10**	**2.5**	**5**	**2.5**	**10**	**5**	**10**
Average	64.8607	80.3708	64.8607	89.7018	64.8607	100	80.3708	89.7018	80.3708	100	89.7018	100
Variance	29.1700	7.0234	29.1700	1.9508	29.1700	0	7.0234	1.9508	7.0234	0	1.9508	0
N	3	3	3	3	3	3	3	3	3	3	3	3
P. variance	18.0967	-	15.5604	-	14.5850	-	4.4871	-	3.5117	-	0.9754	-
H. mean	0	-	0	-	0	-	0	-	0	-	0	-
df	4	-	4	-	4	-	4	-	4	-	4	-
t Stat	-4.4654	-	-7.7127	-	-11.2690	-	-5.3950	-	-12.8289	-	-12.7708	-
P one-tail	0.0056	-	0.0008	-	0.0002	-	0.0029	-	0.0001	-	0.0001	-
t_{crit} one-tail	2.1318	-	2.1318	-	2.1318	-	2.1318	-	2.1318	-	2.1318	-
P two-tail	0.0111	-	0.0015	-	0.0004	-	0.0057	-	0.0002	-	0.0002	-
t_{crit} two-tail	2.7764	-	2.7764	-	2.7764	-	2.7764	-	2.7764	-	2.7764	-

Bonferroni correction: α = p significance/nº comparisons = 0.05/6 = 0.00833

Appendix 17. *C. album* 5-AQU EFAA treatment growth (TG) and growth reduction (GR).

C (mg/mL)	TG (cm^2)	GR (cm^2)	GR (%)	GR_{mean} (%)	StdDev
1.25	25.971 24.871 24.786	-3.158 -2.058 -1.973	-13.843 -9.021 -8.649	-9.021	2.37
2.5	25.777 17.663 22.474	-2.964 5.150 0.339	-12.993 22.575 1.486	1.486	14.60
5	15.327 13.978 15.299	7.486 8.835 7.514	32.815 38.728 32.937	32.937	2.76
10	9.608 9.913 8.539	13.205 12.900 14.274	57.884 56.547 62.570	57.884	2.58

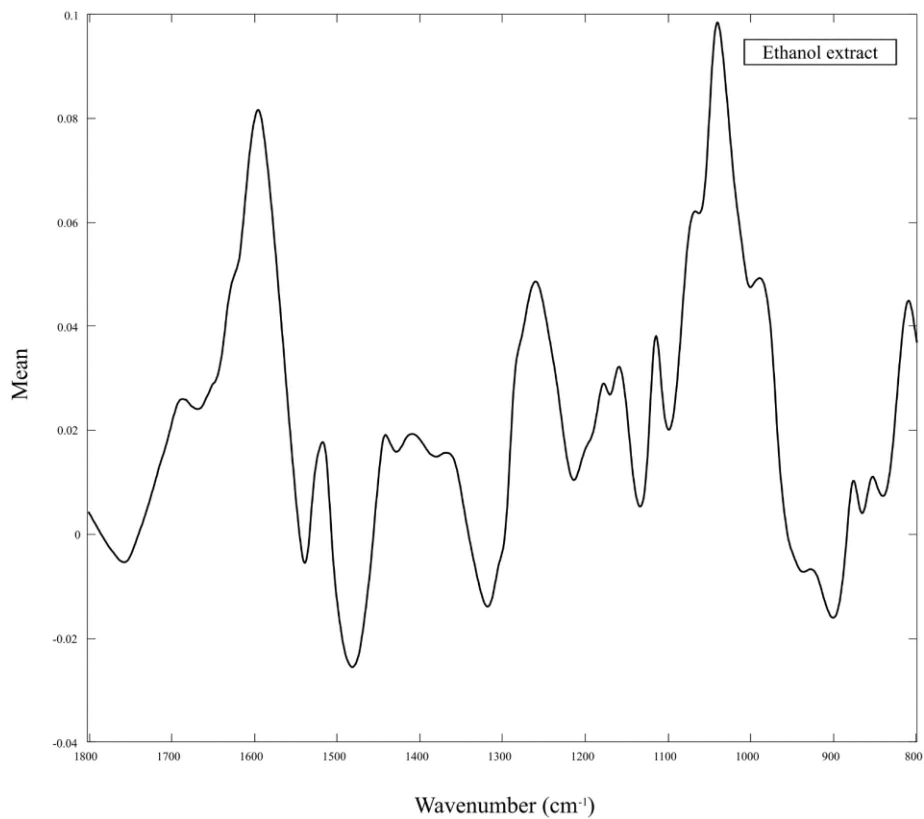
Appendix 18. *C. album* 5-AQU EFAA (one-way ANOVA; p=0.05).

	SS	df	MS	F	P-value	F_{crit}
Between groups	8775.2421	3	2925.0807	33.45633	**0.00007089**	4.0662
Within groups	699.4385	8	87.4298	-	-	-
Total	9474.6806	11	-	-	-	-

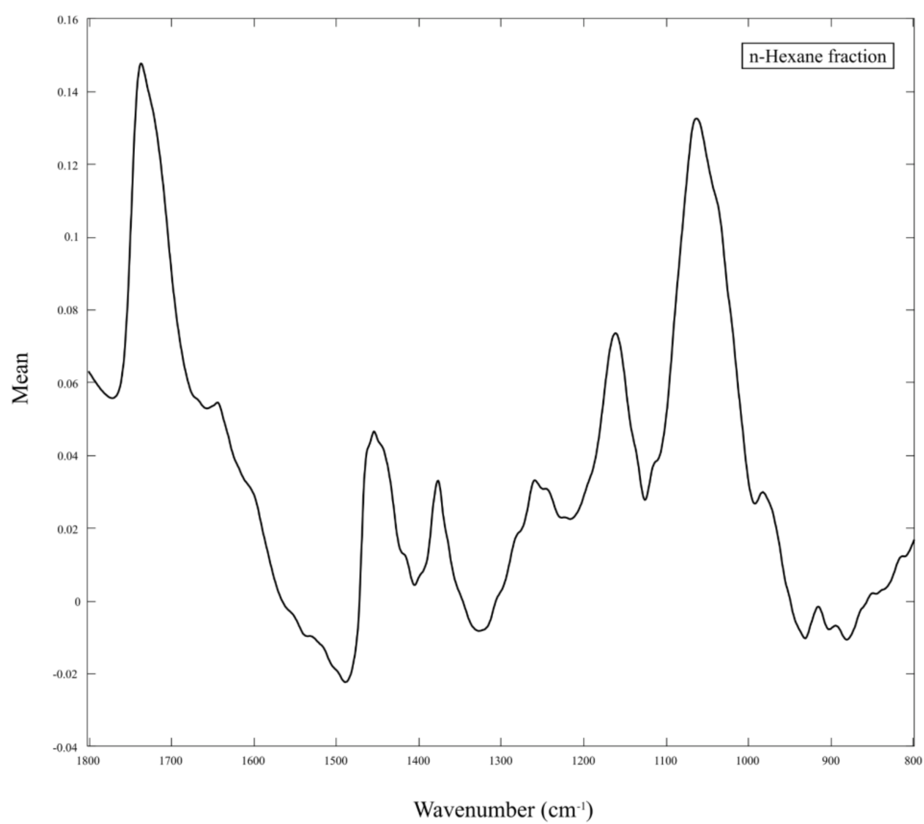
Appendix 19. *C. album* 5-AQU EFAA *post hoc* Tuckey test (p=0.05). Significative or non-significative differences between groups are marked as green and red, respectively.

Groups	1.25 vs 2.5		1.25 vs 5		1.25 vs 10		2.5 vs 5		2.5 vs 10		5 vs 10	
	1.25	2.5	1.25	5	1.25	10	2.5	5	2.5	10	5	10
Average	-10.5042	3.6894	-10.5042	34.8266	-10.5042	59.0000	3.6894	34.8266	3.6894	59.0000	34.8266	59.0000
Variance	8.3951	319.9020	8.3951	11.4188	8.3951	10.0034	319.9020	11.4188	319.9020	10.0034	11.4188	10.0034
N	3	3	3	3	3	3	3	3	3	3	3	3
P. variance	164.1485	-	9.9069	-	9.1993	-	165.6604	-	164.9527	-	10.7111	-
H. mean	0	-	0	-	0	-	0	-	0	-	0	-
df	4	-	4	-	4	-	4	-	4	-	4	-
t Stat	-1.3568	-	-17.6388	-	-28.0660	-	-2.9629	-	-5.2744	-	-9.0462	-
P one-tail	0.1232	-	0.0000	-	0.0000	-	0.0207	-	0.0031	-	0.0004	-
t_{crit} one-tail	2.1318	-	2.1318	-	2.1318	-	2.1318	-	2.1318	-	2.1318	-
P two-tail	0.2464	-	0.0001	-	0.0000	-	0.0414	-	0.0062	-	0.0008	-
t_{crit} two-tail	2.7764	-	2.7764	-	2.7764	-	2.7764	-	2.7764	-	2.7764	-

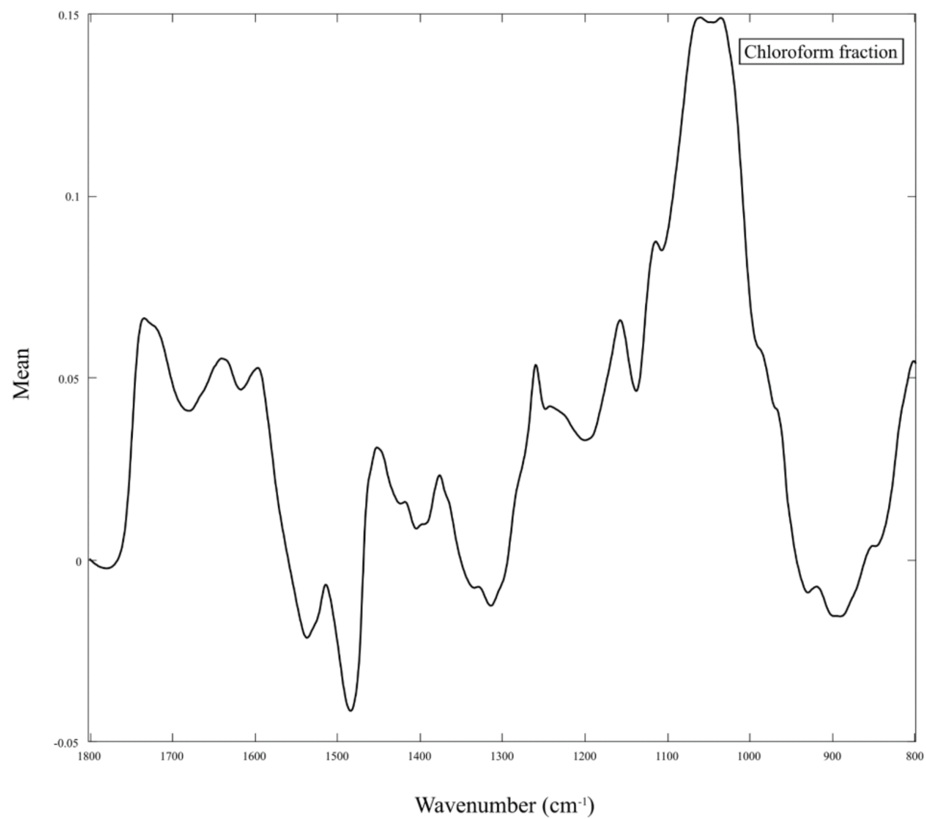
Bonferroni correction: α = p significance/nº comparisons = 0.05/6 = 0.00833



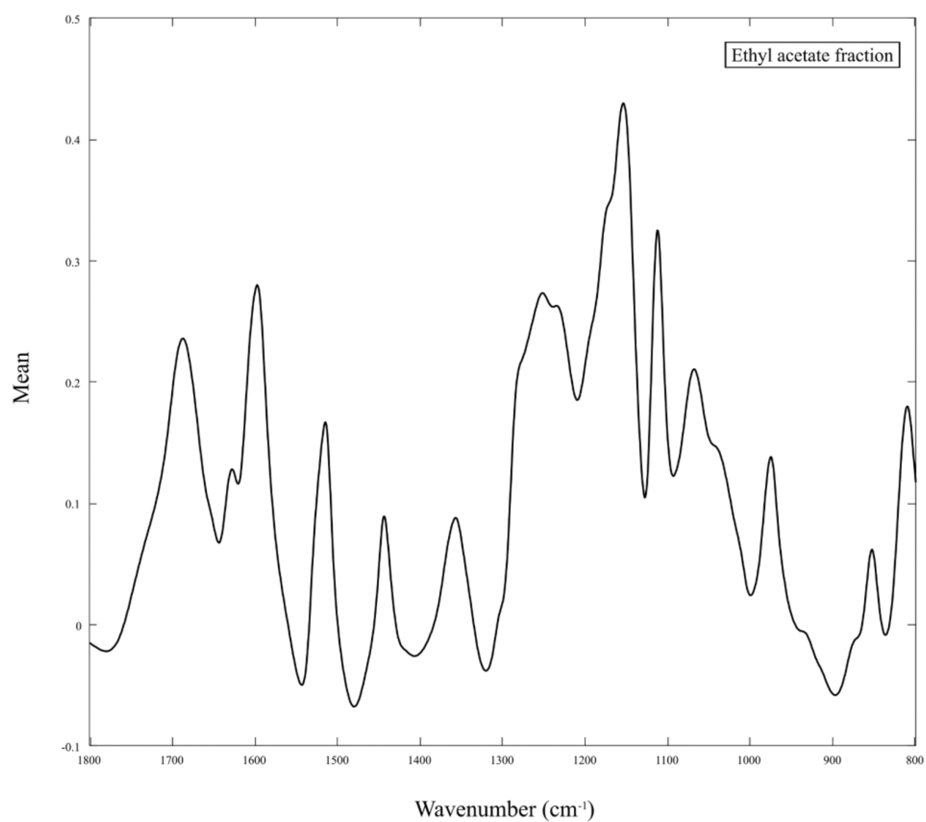
Appendix 20. FTIR absorbance spectrum of *S. betaceum* ethanol extract.



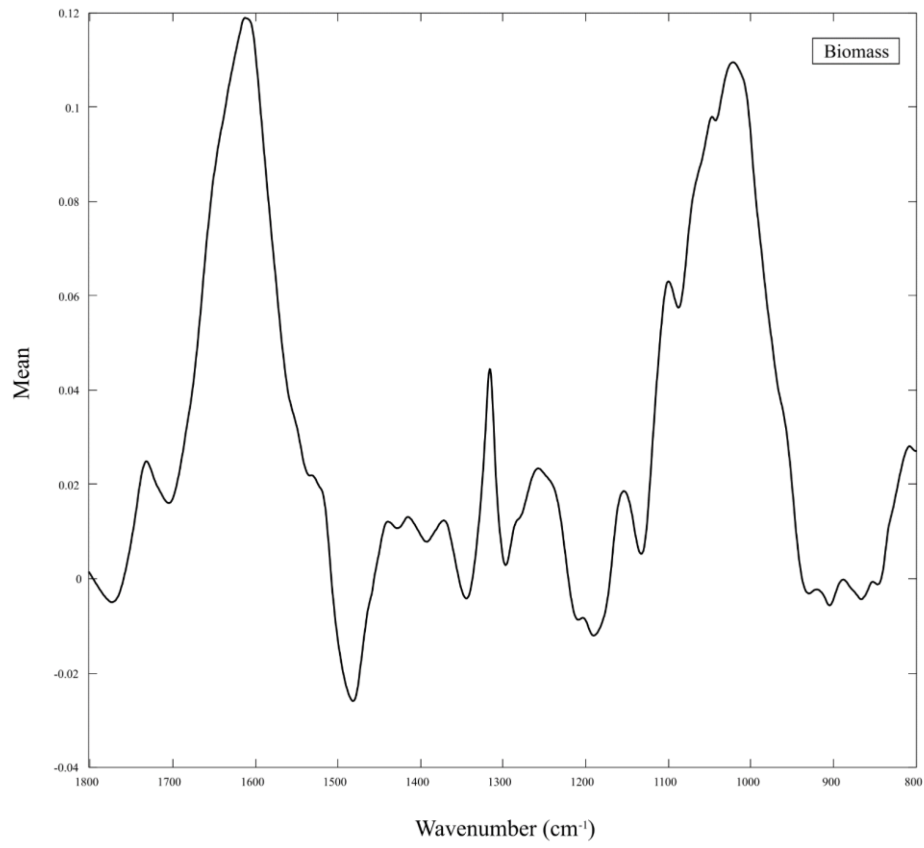
Appendix 21. FTIR absorbance spectrum of *S. betaceum* n-hexane fraction.



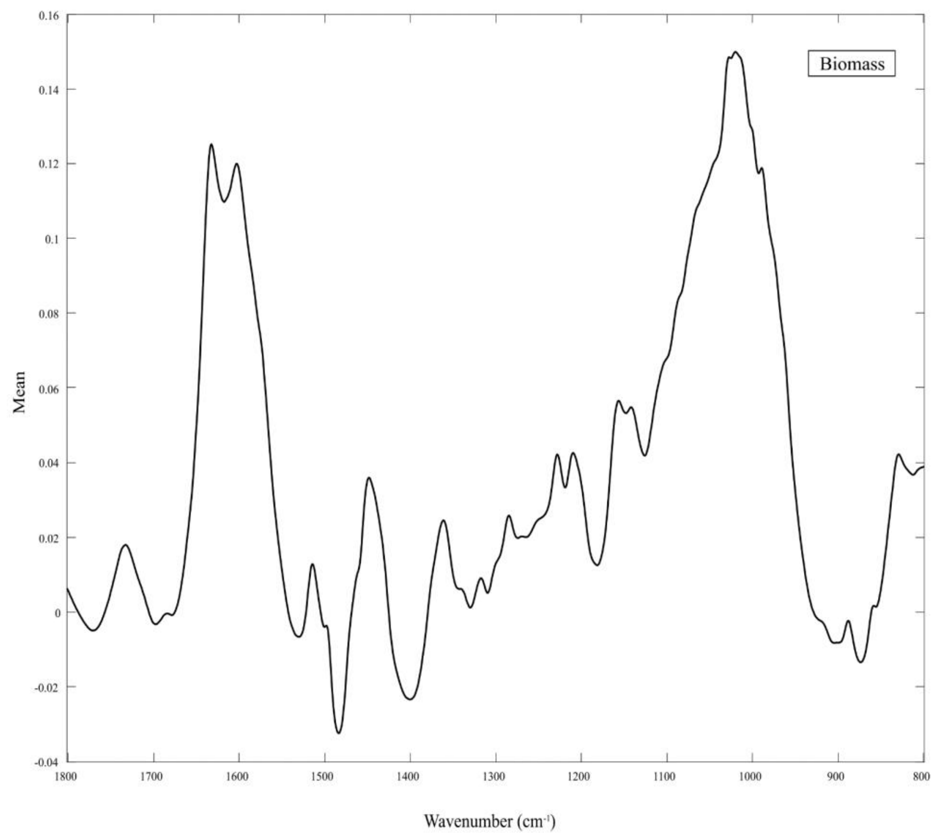
Appendix 22. FTIR absorbance spectrum of *S. betaceum* chloroform fraction.



Appendix 23. FTIR absorbance spectrum of *S. betaceum* ethyl acetate fraction.



Appendix 24. FTIR absorbance spectrum of *S. betaceum* biomass.



Appendix 25. FTIR absorbance spectrum of *C. album* biomass.