Inhibitory activity of propolis and essential oils against oomycete pathogens important in aquaculture

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Doctoral thesis / Disertacija

2022

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Food Technology and Biotechnology / Sveučilište u Zagrebu, Prehrambeno-biotehnološki fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:159:175344

University of Zagreb

Faculty of Food Technology and Biotechnology

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INHIBITORY ACTIVITY OF PROPOLIS AND ESSENTIAL OILS AGAINST OOMYCETE PATHOGENS IMPORTANT IN AQUACULTURE

DOCTORAL DISSERTATION

Supervisor: PhD, Ana Bielen, Associate Professor
PhD, Maja Dent, Assistant Professor

Zagreb, 2022

Sveučilište u Zagrebu

Prehrambeno-biotehnološki fakultet

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INHIBICIJSKO DJELOVANJE PROPOLISA I ETERIČNIH ULJA NA OOMICETNE PATOGENE ZNAČAJNE ZA SLATKOVODNU AKVAKULTURU

DOKTORSKI RAD

Mentori: izv. prof. dr. sc. Ana Bielen
doc. dr. sc. Maja Dent

Zagreb, 2022.

This dissertation was prepared in the Laboratory for Biology and Microbial Genetics and the Laboratory for Analytical Chemistry at the Faculty of Food Technology and Biotechnology, University of Zagreb, as part of the Croatian Science Foundation project "Interactions of freshwater pathogenic oomycetes and the environment – InteractOomyc" (UIP-2017-05-6267, PI: Assoc. Prof. Ana Bielen, PhD), under the supervision of Assoc. Prof. Ana Bielen, PhD and Assist. Prof. Maja Dent, PhD. Chemical composition of essential oils and fluid propolis formulations was determined in the Laboratory for Drying Technologies and Monitoring of Biologically Active Compounds (Faculty of Food Technology and Biotechnology, University of Zagreb) and in the Department of Chemistry (Faculty of Chemical Technology, University of Split). Molecular docking analyses were performed at the Directorate of Cold Water Fisheries Research, Indian Council of Agricultural Research, Uarakhand, India, in collaboration with Ritesh Shantilal Tandel, PhD and Raja Aadil Hussain Bhat, PhD.

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CURICULUM VITAE

Assoc. Prof. Ana Bielen, PhD, has been working in the Laboratory for Biology and Microbial Genetics at the Faculty of Food Technology and Biotechnology, University of Zagreb, since 2003. She received her PhD in molecular microbiology from the Faculty of Science, University of Zagreb in 2011.

Her scientific interest is in microbial ecology of pathogenic freshwater oomycetes with focus on development of molecular methods for their detection and monitoring as well as environmentally friendly methods for their control in aquaculture.

To date, she has led three national and one international scientific project and collaborated on ten national and three international projects. Currently, she is the PI of the founding research project of the Croatian Science Foundation "Interactions of Freshwater Pathogenic Oomycetes and the Environment" (InteractOomyc). She has co-authored 31 scientific articles in internationally recognised journals in the field of environmental science and molecular microbiology, most of them in the first and second quartile. She presented the results of her research at a number of scientific conferences. She has conducted parts of her research at prestigious foreign institutions (Graz University of Technology, Austria; University of Pau, France; Helmholtz Centre Munich, Germany) and participated in numerous national and international courses.

She has supervised 3 diploma and 8 final theses, as well as 3 student research projects awarded with the Rector's Award at the University of Zagreb. She is co-author of two university textbooks "Methods in Molecular Biology" and "Handbook of Biology 1". She is a member of several professional societies and is particularly active in the Croatian Microbiological Society, where she held the position of Secretary from 2018 to 2019. She has participated in the organisation of several international scientific conferences and was the editor of two international books of abstracts. She received the Award of the Society of University Teachers and Other Scientists in Zagreb for Young Scientists and Artists in 2009, the first prize in the competition "ddPCR challenge" in 2019 and the Award of the Faculty of Food Technology and Biotechnology for an outstanding contribution to research in 2020. She is continuosly involved in science communication and popularisation.

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CURRICULUM VITAE

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The dissertation topic was accepted at the 10th regular session of the Faculty Council of the Faculty of Food Technology and Biotechnology, University of Zagreb in the academic year 2020/2021 held on 16th July, 2021.

BASIC DOCUMENTATION CARD

University of Zagreb **PhD thesis**
Faculty of Food Technology and Biotechnology
Postgraduate study in Biotechnology and Bioprocess Engineering, Food Technology and Nutrition
UDK: 582.244.1:638.135:664.1.038.8:665.525-032.25(043.3)
Scientific Area: Biotechnical Sciences
Scientific Field: Biotechnology

INHIBITORY ACTIVITY OF PROPOLIS AND ESSENTIAL OILS AGAINST OOMYCETE PATHOGENS IMPORTANT IN AQUACULTURE

Anđela Miljanović, mag. ing. techn. aliment.

Thesis performed at the Laboratory for Biology and Microbial Genetics and the Laboratory for Analytical Chemistry at the Faculty of Food Technology and Biotechnology

Supervisors: PhD Ana Bielen, Associate Professor and PhD Maja Dent, Assistant Professor

Short abstract
Saprolegnia parasitica and *Aphanomyces astaci* are oomycete pathogens that cause significant economic losses in salmonid and crayfish aquaculture. In this dissertation, inhibition of their growth and reproduction by sage, rosemary and bay laurel essential oils and fluid propolis formulations was investigated, as environmentally friendly alternatives to the toxic chemicals currently used. Considering the high cost of essential oil production, the isolation of essential oil from bay laurel, sage and rosemary leaves was optimised to increase the yield. Ultrasonic and reflux extraction hydrodistillation pretreatments increased the essential oil yield by up to 60%, while the overall quality of the oil remained largely unchanged. In contrast, the use of cell wal degrading enzymes as hydrodistillation pretreatments did not affect the yield. Next, it was shown that the tested essential oils and propolis formulations strongly inhibited the motility and germination of zoospores and the growth of the mycelium of both pathogens, albeit with differences in efficacy depending on the substance, oomycete species and life cycle stage. The fluid propolis formulations most potently inhibited mycelial growth of *A. astaci*, while the sage essential oil most strongly inhibited zoospore germination and mycelium growth of *S. parasitica* and zoospore germination of *A. astaci*. Although the results point to several compounds as the basis of the inhibitory effect, such as camphor in sage and chrysin in propolis, the observed anti-oomycetic activity is probably due to the synergistic effect of many different bioactive components present at low concentrations. Overall, the results of this doctoral thesis provide a starting point for further *in vivo* testing of the use of essential oils and propolis formulations in freshwater aquaculture as an environmentally acceptable control of diseases caused by oomycetes.

Number of pages: 118
Number of figures:3
Number of tables: 1
Number of references: 178
Number of attachments: 6
Original in: English

Key words: *Aphanomyces astaci*, *Saprolegnia parasitica*, bay laurel, rosemary, sage, fluid propolis formulations
Date of the thesis defense: 14th April, 2022.
Reviewers:
1. PhD Verica Dragović-Uzelac, Full Professor
2. PhD Lidija Šver, Associate Professor
3. PhD Marin Ježić, Assistant Professor
4. PhD Tomislav Vladušić, Assistant Professor

Thesis deposited in: Library of Faculty of Food Technology and Biotechnology, Kačićeva 23, Zagreb, National and University Library, Hrvatske bratske zajednice bb, Zagreb, and University of Zagreb, Trg Republike Hrvatske 14, Zagreb.

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu **Doktorski rad**
Prehrambeno-biotehnološki fakultet
Sveučilišni poslijediplomski studij Biotehnologija i bioprocesno inženjerstvo, prehrambena tehnologija i nutricionizam
UDK: 582.244.1:638.135:664.1.038.8:665.525-032.25(043.3)
Znanstveno područje: Biotehničke znanosti
Znanstveno polje: Biotehnologija

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Anđela Miljanović, mag. ing. techn. aliment.

Rad je izrađen u Laboratoriju za biologiju i genetiku mikroorganizama i Laboratoriju za analitičku kemiju Prehrambeno-biotehnološkog fakulteta Sveučilišta u Zagrebu

Mentori: izv. prof. dr. sc. Ana Bielen i doc. dr. sc. Maja Dent

Kratki sažetak disertacije:
Saprolegnia parasitica i *Aphanomyces astaci* su patogeni iz skupine vodenih plijesni (Oomycota) koji uzrokuju značajne ekonomske štete u akvakulturi salmonida i rakova. U ovoj disertaciji istražena je njihova inhibicija eteričnim uljima kadulje, ružmarina i lovora te tekućim pripravcima propolisa, kao ekološki prihvatljivim alternativama toksičnim kemikalijama koje se trenutno koriste. Obzirom na visoku cijenu proizvodnje eteričnih ulja, s ciljem povećanja prinosa optimizirani su uvjeti izolacije eteričnog ulja iz listova kadulje, lovora i ružmarina. Predtretmani ultrazvukom i klasičnom ekstrakcijom uz refluks povećali su prinos eteričnog ulja do 60 %, pri čemu je kvaliteta eteričnog ulja ostala uglavnom nepromijenjena. Nasuprot tomu, primjena enzima koji razgrađuju staničnu stijenku kao predtretman hidrodestilaciji nije povećala prinos ulja. Nadalje, pokazano je da su testirani uzorci eteričnih ulja i propolisa snažno inhibirali pokretljivost i klijavost zoospora i rast micelija oba patogena, ali s razlikama u djelotvornosti ovisno o uzorku, vrsti patogena te fazi životnog ciklusa. Tekuće formulacije propolisa najsnažnije su inhibirale rast micelija vrste *A. astaci*, dok je eterično ulje kadulje najjače inhibiralo klijavost zoospora i rast micelija vrste *S. parasitica* te zoospore vrste *A. astaci*. Iako rezultati ukazuju na nekoliko komponenti kao osnovu inhibicijskog učinka, primjerice kamfor u kadulji i krizin u propolisu, demonstrirano inhibicijsko djelovanje je vjerojatno posljedica sinergističkog učinka većeg broja različitih bioaktivnih komponenti prisutnih u niskim koncentracijama. Zaključno, rezultati ovog doktorskog rada predstavljaju polazište za daljnja *in vivo* istraživanja primjene eteričnih ulja i propolisa u slatkovodnoj akvakulturi u svrhu ekološki prihvatljive kontrole bolesti uzrokovanih vodenim plijesnima.

Broj stranica: 118
Broj slika: 3
Broj tablica: 1
Broj literaturnih navoda: 178
Broj priloga: 6
Jezik izvornika: engleski

Ključne riječi: *Aphanomyces astaci*, *Saprolegnia parasitica*, lovor, ružmarin, kadulja, tekući pripravci propolisa
Datum obrane: 14. travnja 2022.
Stručno povjerenstvo za obranu:
1. prof. dr. sc. Verica Dragović-Uzelac
2. izv. prof. dr. sc. Lidija Šver
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Rad je pohranjen u knjižnici Prehrambeno-biotehnološkog fakulteta u Zagrebu, Kačićeva 23, Zagreb, Nacionalnoj i sveučilišnoj knjižnici, Hrvatske bratske zajednice bb, Zagreb i na Sveučilištu u Zagrebu, Trg Republike Hrvatske 14, Zagreb.

EXTENDED SUMMARY

Saprolegnia parasitica, the causative agent of saprolegniosis in salmonids, and *Aphanomyces astaci*, the causative agent of crayfish plague, are oomycete pathogens that cause significant economic losses in aquaculture. Since chemicals harmful to humans and the environment are used for their prevention and control in aquaculture, new environmentally friendly methods need to be developed. In this context, the essential oils of Mediterranean wild plants sage, bay laurel and rosemary, as well as propolis formulations, are rich in bioactive components and have a number of beneficial properties. Although their inhibitory activity against a wide range of microbes has already been demonstrated, their inhibitory potential against oomycetes has been much less studied.

The objectives of this dissertation were: (i) to determine the optimal conditions for the isolation of the essential oils of Mediterranean wild plants sage, bay laurel and rosemary, in order to increase the yield, (ii) to determine the chemical composition of the essential oils and fluid propolis formulations, and (iii) to determine the inhibitory potential of the essential oils and propolis formulations against the mycelial growth and zoospores of the pathogenic oomycetes *S. parasitica* and *A. astaci*.

Since one of the main obstacles to the mass application of essential oils in aquaculture is the high production cost, the application of different hydrodistillation pretreatments was tested to increase the essential oil yield. Ultrasonic pretreatment and pretreatment by classical reflux extraction, as environmentally friendly and economically justifiable approaches, resulted in a significant increase in essential oil yield by 40 – 64%, while the chemical composition remained largely unchanged, as confirmed by statistical analysis of gas chromatography-mass spectrometry (GC-MS) results. In contrast, pretreatment by classical reflux extraction with the addition of cell wall-degrading enzymes did not significantly increase the yield of essential oils.

Next, GC-MS and ultra-performance liquid chromatography combined with tandem mass spectrometry (UPLC/MS-MS) showed that the essential oils and fluid propolis preparations were rich in bioactive components. Their inhibitory potential against the life stages of model pathogenic oomycetes was analysed *in vitro*, and EC_{50} values (concentrations of tested samples leading to 50% inhibition) of mycelial growth and germination of zoospores, as well as MIC values (minimum inhibitory concentrations) of zoospore motility were determined. The binding of selected components of propolis, i.e., chrysin, pinocembrin, cinnamic acid and

apigenin, to oomycete proteins that play a role in pathogenesis was simulated using the molecular docking method. All tested samples had strong inhibitory activity, but it depended on the type of tested sample, oomycete species and life cycle stage (mycelium or zoospores). Propolis samples showed the strongest inhibitory effect on the growth of the mycelium of *A. astaci*, while sage essential oil most strongly inhibited the zoospores and mycelium of *S. parasitica* and the zoospores of *A. astaci*. Despite the fact that the results point to some molecules as the basis of the inhibitory effect, such as camphor in sage and chrysin in propolis, the demonstrated inhibitory effect is most likely due to the synergy of a number of bioactive components present at low concentrations.

Based on all the above, the results of this dissertation represent the first step in the development of the use of essential oils and propolis to control oomycete diseases in aquaculture.

PROŠIRENI SAŽETAK

Saprolegnia parasitica, uzročnik saprolegnioze kod salmonidnih riba, i *Aphanomyces astaci,* uzročnik račje kuge, oomicetni su patogeni koji uzrokuju značajne ekonomske gubitke u slatkovodnoj akvakulturi. Budući da se za njihovu prevenciju i suzbijanje koriste kemikalije štetne za ljude i okoliš, potrebno je razviti nove, ekološki prihvatljve metode kontrole patogenih oomiceta u akvakulturi. U tom kontekstu, eterična ulja samoniklog mediteranskog bilja, kadulje, lovora i ružmarina, kao i pripravci propolisa, bogati su bioaktivnim komponentama s brojnim pozitivnim svojstvima. Iako je utvrđeno da djeluju inhibicijski prema mnogim mikrobima, njihova inhibicijska aktivnost prema oomicetima je slabo istražena.

Ciljevi ovoga doktorskog rada bili su: (i) odrediti optimalne uvjete izolacije eteričnih ulja samoniklog mediteranskog bilja, kadulje, lovora i ružmarina, s ciljem povećanja prinosa, (ii) odrediti kemijski sastav eteričnih ulja i tekućih pripravaka propolisa, i (iii) odrediti inhibicijski potencijal eteričnih ulja i pripravaka propolisa na rast micelija te pokretljivost i klijavost zoospora patogenih oomiceta *S. parasitica* i *A. astaci*.

S obzirom da je jedna od glavnih prepreka masovnog korištenja eteričnih ulja u akvakulturi visoka cijena njihove proizvodnje, u ovom je radu testirana primjena različitih predtretmana hidrodestilaciji s ciljem povećanja prinosa ulja. Predtretman ultrazvučnom sondom i predtretman klasičnom ekstrakcijom uz refluks, kao okolišno i ekonomski prihvatljivi pristupi, uzrokovali su značajno povećanje prinosa eteričnog ulja od 40 – 64 %, pri čemu je kemijski sastav bio većinom nepromijenjen, što je potvrđeno statističkim analizama rezultata plinske kromatografije s masenom spektrometrijom (engl. *gas chromatography – mass spectrometry,* GC-MS). Nasuprot tome, predtretman klasičnom ekstrakcijom uz refluks uz dodatak enzima koji razgrađuju staničnu stijenku nije doveo do značajnog povećanja prinosa eteričnih ulja.

Nadalje, primjenom GC-MS i tekućinske kromatografije ultra visoke djelotvornosti spregnute s tandemskom masenom spektrometrijom (engl. *ultraperformance liquid chromatography-tandem mass spectrometry,* UPLC/MS-MS) pokazano je da su korištena eterična ulja i pripravci propolisa bogati bioaktivnim komponentama. *In vitro* testovima analiziran je njihov inhibicijski potencijal prema različitim životnim stadijima patogenih modelnih oomiceta te su određene EC_{50} vrijednosti (koncentracije testiranih uzoraka koje dovode do 50 %-tne inhibicije) rasta micelija i klijavosti zoospora, kao i MIC-vrijednosti (engl. *minimum inhibitory concentration*) pokretljivosti zoospora. Metodom ″molecular docking″ simulirano je vezanje odabranih komponenti propolisa, krizina, pinocembrina, cimetne kiseline i apigenina, na

proteine oomiceta koji imaju ulogu u patogenezi. Svi testirani uzorci imali su snažno inhibicijsko djelovanje, međutim ono je bilo ovisno o tipu uzorka, vrsti patogena i stadiju životnog ciklusa (micelij ili zoospore). Uzorci propolisa pokazali su najsnažnije inhibicijsko djelovanje prema rastu micelija patogena *A. astaci*, dok je eterično ulje kadulje najsnažnije inhibiralo klijavost zoospora i rast micelija vrste *S. parasitica* te klijavost zoospora vrste *A. astaci*. Unatoč tome što su rezultati ukazali na neke molekule kao temelj inhibicijskog djelovanja, poput kamfora iz kadulje i krizina iz propolisa, demonstrirani inhibicijski učinak je najvjerojatnije posljedica sinergije većeg broja bioaktivnih komponenti prisutnih u malim koncentracijama.

Temeljem svega navedenog, rezultati ove disertacije predstavljaju prvi korak u razvoju primjene eteričnih ulja i propolisa za kontrolu oomicetnih bolesti u akvakulturi.

ZAHVALA

Na prvom mjestu zahvaljujem se svojoj mentorici, izv. prof. dr. sc. Ani Bielen, koja mi je pružila priliku da se bavim znanstveno-istraživačkim radom u njezinom timu. Zahvaljujem joj na ukazanom povjerenju, podršci i prijateljstvu te nesebičnom i strpljivom prenošenju znanja tijekom ovih godina. Njen entuzijazam, predanost radu, korektnost i znanstvena znatiželja su mi svakodnevna inspiracija.

Također, veliko hvala mojoj mentorici doc. dr. sc. Maji Dent za sve dobronamjerene savjete i za vođenje kroz kemijski dio eksperimenata te na jako lijepom i ugodnom društvu tijekom boravka s njom u uredu.

Hvala mojim divnim kolegicama i prijateljicama Dori i Dorotei na podršci, pomoći, smijehu, ugodnoj radnoj atmosferi te svim veselim trenutcima, nadam se da će ih u budućnosti biti još puno. S vama je svaka neuspjela sporulacija bila lakša!

Hvala svim kolegama iz Laboratorija za biologiju i genetiku mikoorganizama te Laboratorija za analitičku kemiju za ugodnu suradnju i susretljivost te kolegama iz drugih laboratorija koji su na bilo koji način pomogli pri provođenju eksperimenata.

Od srca hvala članovima Povjerenstva na detaljnom čitanju disertacije te kritičkim komentarima koji su doprinijeli kvaliteti disertacije.

Hvala mojim prijateljima i obitelji, a najviše roditeljima, sestrama i bratu na pomoći i podršci kroz sve životne situacije, pa tako i ovu. Veliko hvala teti Kati što je bezuvjetno bila tu kad god smo ju zatrebali.

Na kraju najveća hvala mojim najmilijima - mome Vlahu i mojoj najdivnijoj djeci, Maris i Anti, koji su mi uljepšali i najnapornije dane te su moja najveća podrška, motivacija i inspiracija. Ovaj rad posvećujem njima...

1. TABLE OF CONTENTS

1. INTRODUCTION

1.1. Oomycetes as pathogens of freshwater animals

Oomycetes (phylum Oomycota, kingdom Chromista), also known as water moulds, are a group of filamentous, heterotrophic, fungal-like microorganisms that include many pathogens of plants and animals (Beakes et al., 2012; Diéguez-Uribeondo et al., 2009). The number of disease outbreaks caused by oomycetes in plant and animal populations is increasing, threatening food security as well as biodiversity of wild species (Fisher et al., 2012; Phillips et al., 2008). Agriculturally important plant pathogenic oomycete species have traditionally received much attention as they pose a global, persistent threat to food security (Abdulkhair, 2021; Fisher et al., 2012; Kamoun et al., 2015). Among them, members of the genus *Phytophthora* have a particularly negative impact. *Phytophthora infestans*, the causal agent of potato late blight, for example, is responsible for the annual loss of more than USD 6 billion worldwide (Haverkort et al., 2008; Fisher et al., 2012). In comparison, animal pathogens are understudied, although several species cause devastating diseases in freshwater ecosystems and the methods to control their spread are extremely limited (Becking et al., 2021; Derevnina et al., 2016; Iberahim et al., 2018; Phillips et al., 2008; van West, 2006). The best-studied representatives of the animal pathogenic freshwater oomycetes are *Aphanomyces astaci* and *Saprolegnia parasitica*, both from the order Saprolegniales. *Aphanomyces astaci* is the causative agent of crayfish plague, a disease that mainly affects native European crayfish species (Jussila et al., 2021; Oidtmann et al., 2002). *Saprolegnia parasitica* causes saprolegniosis in numerous freshwater fish species, including salmonids, important for aquaculture (Gozlan et al., 2014). The number of disease outbreaks caused by oomycetes in aquaculture is increasing due to massive animal production, which is stressful for the animals and thus favours disease development (van den Berg et al., 2013).

The complete life cycle of oomycetes consists of asexual reproduction, which involves the formation of zoosporangia and motile zoospores, and sexual reproduction, which involves the formation of resistant oospores that are morphologically distinct from zoospores (Figure 1; Phillips et al., 2008; van West, 2006). Oospores enhance survival of the pathogen under adverse environmental conditions and increase genetic variability (Beakes and Bartnicki-Garcia, 1989; Diéguez-Uribeondo et al., 2009), although in some species, including *A. astaci*, the sexual stage has not been documented (Bruno and Wood, 1999; Diéguez-Uribeondo et al., 2009; Phillips et al., 2008; Söderhäll and Cerenius, 1992). Zoospores represent the most important infectious phase of the life cycle and are crucial for the spread of the pathogen since they can travel over

long distances (Diéguez-Uribeondo et al., 2009; Lone and Manohar, 2018; Oidtmann et al., 2002; Phillips et al., 2008; Strand, 2013). They are released from the hyphal tips into the surrounding water as zoosporangia grow out of the diseased host tissue (Diéguez-Uribeondo et al., 1994a; 2009; Oidtmann et al., 2002). Infection occurs when the zoospores find the appropriate host through chemotaxis (Cerenius and Söderhäll, 1984; Unestam, 1969), encyst on the surface of the host, e.g. the crayfish exoskeleton or the fish skin, and the cysts germinate into hyphae that invade internal tissues and organs (Bruno and Wood, 1999; Cerenius et al., 1988; Hatai and Hoshia, 1993; Söderhäll and Cerenius, 1998; Willoughby and Roberts, 1994). In some oomycete species, including *A. astaci* and *S. parasitica*, zoospores encyst when they fail to find a suitable host and then release a new zoospore generation. This phenomenon is called repeated zoospore emergence or polyplanetism and increases the probability of finding a suitable host (Cerenius and Söderhäll, 1984; Diéguez-Uribeondo et al., 1994a). The formation of zoosporangia and the release of zoospores can be triggered under laboratory conditions by a lack of nutrients or a sharp drop in temperature (Lawrence et al., 2017). Furthermore, our recent study has shown that parameters related to organic matter in water, especially its aromatic part, can positively influence the sporulation intensity of *S. parasitica* (Pavić et al., 2022).

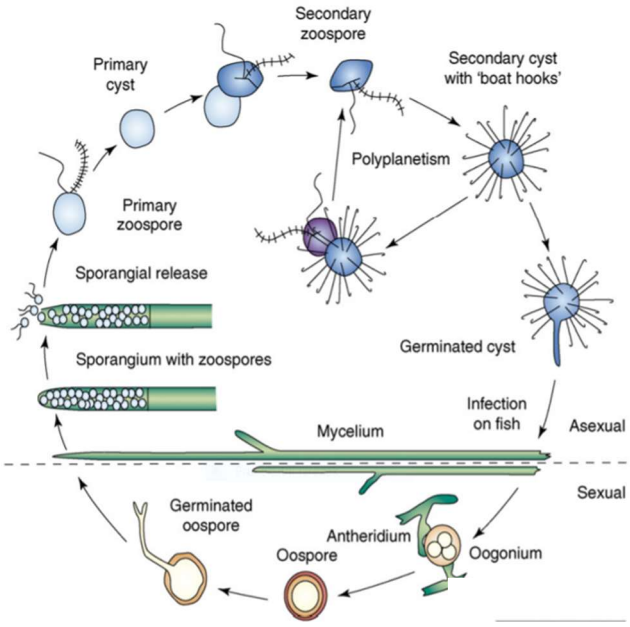

Figure 1. Life cycle of the pathogen *Saprolegnia parasitica* (from Phillips et al., 2008).

1.1.1. *Saprolegnia parasitica*

Species of the genus *Saprolegnia*, including *S. parasitica*, *Saprolegnia australis* and *Saprolegnia diclina*, are usually considered opportunistic secondary pathogens that infect the host under stress conditions (Gozlan et al., 2014; van den Berg et al., 2013). In our recent study, we used droplet digital PCR (ddPCR) to detect *S. parasitica* in environmental DNA (eDNA) samples isolated from water and confirmed the ubiquity of this pathogen in freshwater environments throughout Croatia. Furthermore, skin swabs collected from the surface of injured trout had significantly higher *S. parasitica* loads than healthy fish, which is consistent with its opportunistic lifestyle (Pavić et al., unpublished results).

Saprolegnia spp. cause the disease saprolegniosis in numerous freshwater fish species, affecting eggs, juveniles and adults (Sarowar et al., 2019; Stueland et al., 2005; Thoen et al., 2011). The main symptom of saprolegniosis is the development of a cotton-like tufts of mycelium on the skin of the animal or the surface of the eggs (Figure 2). As the infection progresses, the fish become lethargic and lose their balance, while the embryos in the infected eggs usually die as a result of hyphal rupture of the chorionic membrane (Bruno et al., 2011; Liu et al., 2014). The host range of the pathogen is very broad and includes many fish species such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), Persian sturgeon (*Acipencer persicus*), silver perch (*Bidyanus bidyanus*), channel catfish (*Ictalurus punctatus*), Pacific salmon (*Oncorhynchus nerka*) and Japanese salmon (*Oncorhynchus masu*) (Gozlan et al., 2014). It can also infect crayfish species, especially if the animals are injured (Diéguez-Uribeondo et al., 1994b).

Figure 2. Rainbow trout (*Oncorhynchus mykiss*) (A) and rainbow trout eggs (B) infected with *S. parasitica* (own photos). Oomycete mycelium is visible is on the fish tail (encircled).

Saprolegniosis is a serious problem in salmon and trout farms and hatcheries. Massive infections of eggs are common and entire batches can be lost (Cao et al., 2012; Meyer, 1991; Pavić et al., 2021; Rach et al., 2005; Thoen et al., 2011; van den Berg et al., 2013). This is a significant problem worldwide, typically causing annual economic losses of more than 10%

and occasionally up to 50% (van den Berg et al., 2013; Diéguez-Uribeondo et al., 2007; Rezinciuc et al., 2014).

1.1.2. *Aphanomyces astaci*

Aphanomyces astaci, the causative agent of crayfish plague, was introduced to Europe in the 19th century with North American invasive crayfish species, like signal crayfish (*Pacifstacus leniusculus*), spinycheek crayfish (*Faxonius limosus*) and red swamp crayfish (*Procambarus clarkii*), that are mostly resistant to the disease and therefore act as carriers of the pathogen, spreading it through European waterways (Holdich et al., 2009; James et al., 2017). In contrast, crayfish plague is usually fatal to native European freshwater crayfish, such as aquaculturally important noble crayfish (*Astacus astacus*). However, susceptibility to the disease is also related to host fitness, and even North American crayfish species can succumb to the disease if they live in stressful conditions, such as a high-density farming environment (Aydin et al., 2014; Cerenius et al., 2003; Edsman et al., 2015; Sandström et al., 2014; Thomas et al., 2020). Therefore, all freshwater crayfish are considered susceptible to *A. astaci*, albeit with varying sensitivity depending on species and individual immunity, and crayfish plague is listed as a notifiable animal disease by the World Organisation for Animal Health (OIE, 2019).

During the infection process, the hyphae penetrate the exoskeleton of crayfish and spread throughout the tissues, leading to the development of the fatal disease (Edsman et al., 2015; Kokko et al., 2012). Melanisation is often the first symptom and results from the localised crayfish innate immune response to the hyphal penetration (OIE, 2019) (Figure 3). As the disease progresses, other more serious signs appear, such as daytime activity, loss of limbs or abdominal paralysis (Alderman et al., 1987).

Although the negative effects of *A. astaci* are studied in much more detail in natural ecosystems, the damage caused by this pathogen in astaciculture should be of concern, especially in the production of native European crayfish species, which are highly susceptible to infection by *A. astac*i (Becking et al., 2015; Benavent-Celma et al., 2021; Harlioğlu, 2008; Holdich, 1993; Souty-Grosset and Reynolds, 2009).

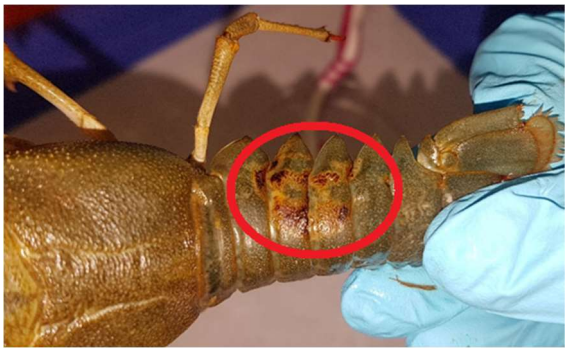

Figure 3. Melanization of the exoskeleton of the narrow clayed crayfish, *Pontastacus leptodactylus*, a symptom of *Aphanomyces astaci* infection (own photo).

1.2.Control methods for pathogenic oomycetes in aquaculture

Despite these harmful effects, current methods to control oomycete infections in freshwater aquaculture systems are inadequate and unsustainable due to the lack of specific treatments and the fact that existing protocols use toxic chemicals that are harmful to human and animal health (Tedesco et al., 2019). Malachite green, for example, is highly effective in controlling *Saprolegnia* and *Aphanomyces* spp. (Alderman and Polglase, 1984; Willoughby and Roberts, 1992), but its use is not allowed in the EU (EC, 1990; EFSA, 2016) or the USA (Marking et al., 1994) due to its teratogenic and carcinogenic properties (Meyer and Jorgenson, 1983; Panandiker et al., 1992; Srivastava et al., 2004). However, the chemicals currently used, such as formalin (aqueous solution of formaldehyde), bronopol (2-bromo-2-nitro-1,3-propanediol), copper (II) sulphate, peracetic acid and hydrogen peroxide, are quite toxic as well, and thus unsuitable for extended usage. Formalin, for example, is dangerous to fish farm personnel who use it in therapeutic concentrations (Wooster et al., 2005), but also to consumers of the fish meat that contains its residues (Norliana et al., 2009). Bronopol, although considered a relatively weak pollutant, is easily hydrolytically and photolytically degraded in water, generating degradation products that are more toxic to aquatic biota than the parent compound and are likely to accumulate in the environment (Cui et al., 2011). Copper (II) sulphate can be toxic to zooplankton and alter its community composition (Jacob et al., 2016), while peracetic acid, a relatively new alternative, is toxic to aquatic biota at concentrations effective against *A. astaci* (Jussila et al., 2014). Finally, hydrogen peroxide has been reported to increase salmon mortality when applied at concentrations of 1.5 g/L or higher (Overton et al., 2018).

Therefore, there is an increasing need to replace harmful chemical agents used to control oomycetes in freshwater aquaculture with environmentally friendly treatments that should not only be effective against oomycete pathogens but also safe for humans, animals and the environment. In this context, studies over the last decades indicate that alcoholic extracts (Afzali and Wong, 2017; Borisutpeth et al., 2009; 2014; Campbell et al., 2001; Pagliarulo et

al., 2018; Tandel et al., 2021a) and essential oils (Campbell et al., 2001; Caruana et al., 2012; Gormez and Diler, 2014; Hoskonen et al., 2015; Khosravi et al., 2012; Madrid et al., 2015; Metin et al., 2015; Nardoni et al., 2019; Parikh et al., 2021; Pirbalouti et al., 2009; Saleh et al., 2015) of selected plants can inhibit oomycete pathogens *in vitro*. This is likely due to the abundance of bioactive compounds, i.e. plant specialized metabolites, which have numerous beneficial properties, such as antimicrobial, antiviral, antifungal and anti-oomycetic activity (Denaro et al., 2020; Loi et al., 2020; Montenegro et al., 2019). Furthermore, some studies also confirmed the activity of propolis preparations against oomycetes (Araújo et al., 2016; Campbell et al., 2001; Silva-Castro et al., 2018; Yusuf et al., 2005). The application of such preparations in aquaculture, either by dietary supplementation or by bathing eggs and animals in propolis/essential oil emulsions, has been shown to have positive effects on the immunity, growth and reproductive capacity of fish and crayfish, and the reduction of the oomycete infection rates (Abdelmagid et al., 2021; Fuat Gulhan and Selamoglu, 2016; Khosravi et al., 2012; Metin et al., 2015; Mişe Yonar et al., 2017; Özdemir et al., 2022; Sari and Ustuner-Aydal, 2018; Sönmez et al., 2015; Talas and Gulhan, 2009). Thus, essential oils and propolis are promising agents for the control of oomycete pathogens.

1.1.3. Essential oils

Essential oils are aromatic, volatile liquids isolated from plant material and rich in bioactive compounds. They are variable and complex mixtures of plant metabolites, consisting of terpene hydrocarbons, oxygenated terpenes and sesquiterpenes. As mentioned above, essential oils isolated from members of different plant families have been shown to inhibit pathogenic oomycetes *in vitro* (Campbell et al., 2001; Caruana et al., 2012; Gormez and Diler, 2014; Hoskonen et al., 2015; Khosravi et al., 2012; Madrid et al., 2015; Metin et al., 2015; Nardoni et al., 2019; Parikh et al., 2021; Pirbalouti et al., 2009; Saleh et al., 2015). Specifically, plants from the Lauraceae and Lamiaceae families, such as *Origanum onites*, *Laurus nobilis*, *Thymbra spicata* and *Cinnamomum zeylanicum*, have been reported to inhibit pathogenic freshwater oomycetes, mostly *Saprolegnia* spp. (Gormez and Diler, 2014; Khosravi et al., 2012; Metin et al., 2015; Nardoni et al., 2019; Özdemir et al., 2022; Parikh et al., 2021; Pirbalouti et al., 2009). In comparison, the inhibitory effect of essential oils (and plant extracts) against *Aphanomyces* spp. is much less studied (Pagliarulo et al., 2018; Parikh et al., 2021). So far, there are no reports on the inhibitory activity of essential oils against *A. astaci*, but essential oils of some plants, such as oregano and thyme from the Lamiaceae family, showed good inhibitory

activity against the phylogenetically related plant pathogen *Aphanomyces euteiches* (Parikh et al., 2021). Moreover, most studies have only tested the potential of essential oils to inhibit mycelial growth of oomycete pathogens (Campbell et al., 2001; Gormez and Diler, 2014; Metin et al., 2015; Nardoni et al., 2019; Parikh et al., 2021; Tampieri et al., 2003), while the knowledge on the inhibition of zoospores by essential oils is limited (Madrid et al., 2015; Saleh et al., 2015), although they are a major infectious phase of oomycete life cycle (Lone and Manohar, 2018) and could be inhibited at several levels: sporulation, zoospore motility and zoospore germination. Data on the specific components of essential oils that could form the basis for the observed inhibitory effects are also inadequate at present, but it has been shown that some molecules of plant origin, such as curcumin and cinnamaldehyde, can inhibit zoospore production and hyphal growth of *S. parasitica* and *S. australis in vitro* (Tandel et al., 2021b). These results were also confirmed by *in silico* predictions of their binding to *Saprolegnia* spp. key proteins.

Although essential oils are an environmentally acceptable alternative to the use of toxic chemicals for disease control, there are still several barriers to their large-scale application in aquaculture, such as high extraction costs, low yields and variability in the composition of essential oils extracted from plants from different locations and seasons (Russo et al., 2013). Therefore, the analyses of composition of essential oils and the identification of dominant compounds using appropriate methods (such as gas chromatography - mass spectrometry, GC-MS) are needed before each application. To avoid this, additional research is needed to standardise the quality of the stock solutions, for example by optimising the growing conditions and harvesting time of the plant material utilised for extraction (Pavela and Benelli, 2016). In addition, new essential oil isolation protocols are constantly being developed to reduce production costs. Essential oils are usually isolated by steam- or hydrodistillation, but novel low-cost and environmentally friendly production techniques, such as ultrasound or enzymatic degradation of the cell wall, are increasingly used as pretreatments to distillation. The aim is to either increase the yield of essential oils, improve their chemical composition (i.e. the amount of the dominant component of the essential oil of the selected plant) or both, while reducing the extraction time (Périno-Issartier et al., 2013). The advantages of using ultrasound in solid-liquid extraction are the intensification of mass transfer, improved penetration of the solvent into the plant tissue and capillary effects. It is believed that the collapse of cavitation bubbles near the cell walls causes cell disruption and at the same time good penetration of the solvent into the cells by the ultrasonic jet (Toma et al., 2001). Several studies have reported that the addition of

ultrasonic pretreatment prior to hydrodistillation reduces the extraction time (Assami et al., 2012; Lilia et al., 2018; Morsy, 2015; Seidi Damyeh et al., 2016), increases the yield (Kowalski et al., 2015; Kowalski and Wawrzykowski, 2009; Lilia et al., 2018; Morsy, 2015; Smigielski et al., 2014a) and improves the chemical composition of the oil (Lilia et al., 2018; Morsy, 2015; Périno-Issartier et al., 2013; Seidi Damyeh et al., 2016). In addition, enzyme-assisted extraction has been intensively studied in the last decade because the plant cell wall, as a resistant structure mainly composed of resistant polymers such as cellulose, xylan, lignin and pectin, can reduce the extraction efficiency. Enzymes such as cellulase, xylanase and pectinase are capable of degrading or disrupting cell wall components, thus enabling better release and more efficient extraction of bioactive compounds from plants and enhancing the bioactive content of essential oils and extracts (Balasubramaniam et al., 2019; Boulila et al., 2015; Hosni et al., 2013; Puri et al., 2012; Sowbhagya et al., 2010; 2011). However, while some authors reported that enzyme-assisted extraction increases the yield of essential oils and improves their chemical composition (Baby and Ranganathan, 2016; Boulila et al., 2015; Chandran et al., 2012; Chávez-González et al., 2016; Hosni et al., 2013; Smigielski et al., 2014b; Sowbhagya et al., 2010; 2011), others have failed to demonstrate such effects (Dimaki et al., 2017), suggesting that further research of the application of enzymes in extraction of plant essential oils is needed.

1.1.4. Propolis

Propolis is a product of honey bees rich in bioactive compounds that form the basis of its beneficial properties (Gülçin et al., 2010; Mašek et al., 2018; Mitsui et al., 2018; Veloz et al., 2016). It usually consists of 45 – 55% plant balsams and resins (flavonoids, including flavonols, flavones and flavanones, phenolic acids and esters), 8 – 35% wax (mainly beeswax), 5 – 10% essential oils and aromatic substances such as pinene, eudesmol, viridiflorol and tricosane), 5% fatty acids (mainly from wax), 5% pollen and 5% other organic compounds (ketones, lactones, quinones, steroids and sugars) and minerals, of which iron and zinc are the most abundant (de la Cruz-Cervantes et al., 2018). However, its chemical composition is highly variable and depends on the local vegetation and the time and method of collection (Bankova et al., 2014). So far, several types of propolis have been defined, depending on the geographical origin, the plant source and the predominant biologically active substances: Poplar, Birch, Green (Alecrim), Red (Clusia), Pacific and Canarian propolis, among many others (Bankova, 2005). Poplar-type propolis is most widespread in Europe, North America and the non-tropical regions of Asia, with *Populus* spp. being the dominant plant source and flavones (such as

chrysin and apigenin), flavanones (such as pinocembrin and galangin), cinnamic acids (such as ferulic, isoferulic, caffeic and *p*-coumaric acid) and their esters being the main biologically active substances. However, despite the large variability in chemical composition between the different botanical types of propolis, it was found that its biological properties are usually similar and significant, (Auamcharoen and Phankaew, 2016; Dias et al., 2012; Seidel et al., 2008).

Propolis shows strong inhibitory activity against a range of microbes (Petruzzi et al., 2020), from bacteria (Tukmechi et al., 2010) to fungi (Ota et al., 2001; Ramón-Sierra et al., 2019; Siqueira et al., 2015) and viruses (Yildirim et al., 2016), but its anti-oomycetic potential has not yet been sufficiently explored. Previous studies have demonstrated the inhibitory effect of propolis on mycelial growth of plant pathogenic oomycetes of the genus *Phytophthora* (Silva-Castro et al., 2018; Yusuf et al., 2005) and on mycelial growth and zoospores of animal pathogens of the genera *Aphanomyces* (*Aphanomyces invadans*) (Campbell et al., 2001) and *Pythium* (*Pythium insidiosum*) (Araújo et al., 2016). Additionally, some major propolis components, like pinocembrin, inhibited *Saprolegnia* spp. growth to some extent, but the effect was not strong enough to explain the potent inhibition by the propolis mixture, implying that synergistic effect of many minor components is at play (Montenegro et al., 2019). Finally, the effect of propolis on oomycete pathogens of freshwater animals, such as *A. astaci* and *S. parasitica*, has not been tested *in vitro* or *in vivo*.

1.3.Aims and hypothesis

The hypothesis of this dissertation is that essential oils of Mediterranean plants, bay laurel (*Laurus nobilis*), sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*), as well as fluid propolis formulations can inhibit mycelial growth and zoospores' germination and motility of oomycete pathogens important in freshwater aquaculture.

Aphanomyces astaci and *Saprolegnia parasitica* have been used as model pathogens because they cause serious diseases in animals important for aquaculture, namely crayfish and salmonid fish. Although there are studies indicating the anti-oomycetic potential of essential oils and propolis (Araújo et al., 2016; Campbell et al., 2001; Metin et al., 2015; Özdemir et al., 2022), the effects of bay laurel, sage and rosemary essential oils, and propolis on *A. astaci* and *S. parasitica* have mostly not been tested, especially the inhibitory effect of these preparations on zoospores, the main infectious stage of the oomycete life cycle.

The postulated hypothesis was tested by the following research objectives:

1. Determine the optimum conditions for the isolation of essential oils from Mediterranean wild plants, sage, bay laurel and rosemary, in order to increase the yield.

2. Determine the chemical composition of essential oils and fluid propolis formulations.

3. Determine the inhibitory potential of the essential oils and propolis samples against *S. parasitica* and *A. astaci* mycelial growth and zoospores' germination and motility.

2. ORIGINAL SCIENTIFIC PAPERS

2.1. Effect of enzymatic, ultrasound and reflux extraction pretreatments on the yield and chemical composition of essential oils

molecules

MDPI

Article

Effect of Enzymatic, Ultrasound, and Reflux Extraction Pretreatments on the Yield and Chemical Composition of Essential Oils

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Academic Editor: Petras Rimantas Venskutonis
Received: 8 September 2020; Accepted: 19 October 2020; Published: 20 October 2020

Abstract: The effect of different hydrodistillation pretreatments, namely, reflux extraction, reflux extraction with the addition of cell wall-degrading enzymes, and ultrasound, on the yield and chemical composition of essential oils of sage, bay laurel, and rosemary was examined. All pretreatments improved essential oil yield compared to no-pretreatment control (40–64% yield increase), while the oil quality remained mostly unchanged (as shown by statistical analysis of GC-MS results). However, enzyme-assisted reflux extraction pretreatment did not significantly outperform reflux extraction (no-enzyme control), suggesting that the observed yield increase was mostly a consequence of reflux extraction and enzymatic activity had only a minute effect. Thus, we show that ultrasound and reflux extraction pretreatments are beneficial in the production of essential oils of selected Mediterranean plants, but the application of enzymes has to be carefully re-evaluated.

Keywords: Clevenger hydrodistillation; *Salvia officinalis* L.; *Rosmarinus officinalis* L.; *Laurus nobilis* L.; cell wall-degrading enzymes; ultrasound pretreatment; reflux extraction pretreatment; GC-MS analysis

1. Introduction

Bioactive compounds are secondary plant metabolites with potential for many applications in human use. Due to the high content of bioactive compounds in essential oils [1–3] and their ability to stop or delay the aerobic oxidation of organic matter, their application in the food industry, cosmetic industry, phytotherapy, medicine, and many other fields is constantly growing [4–8].

Essential oils are usually isolated by steam- or hydrodistillation. Although these methods have been successfully used for years, novel extraction procedures are being developed in recent decades, with the aim to increase the yield of essential oils and/or improve their chemical composition, while shortening the extraction time [9]. To get closer to that ideal goal, novel production procedures, like ultrasound or enzymatic degradation of the cell wall, are being applied as pretreatments to distillation. Benefits of ultrasound application in solid–liquid extraction are the intensification of mass transfer, improved solvent penetration into the plant tissue, and capillary effects. The collapse of

cavitation bubbles near the cell walls is assumed to cause the cell disruption and at the same time a good penetration of the solvent into the cells through the ultrasonic jet [10]. Several studies have reported that the addition of ultrasonic pretreatment before hydrodistillation leads to a shortening of the extraction time [11–14], sometimes also accompanied by yield increase [12,13,15–17] and improvement in oil chemical composition [9,12–14].

Further, enzyme-assisted extraction has been intensively studied in the last decade, since the plant cell wall, as a structure composed mainly of resistant polymers like cellulose, xylan, lignin, and pectin, can reduce the extraction efficiency. Enzymes, like cellulase, xylanase, and pectinase, can degrade or disrupt cell wall components, thus enabling better release and more efficient extraction of bioactive compounds from plants and improving the bioactive content of essential oils and extracts [2,18–22]. However, while some authors reported that enzyme-assisted extraction enhanced essential oil yield and improved their chemical composition [2,19,21–26], others did not demonstrate such effect [1], indicating that further research of enzyme application in plant extractions is necessary.

We hypothesized that different hydrodistillation pretreatments, namely, ultrasound-assisted extraction, reflux extraction, and enzyme-assisted reflux extraction, can improve the yield of essential oils of selected Mediterranean wild plants: sage (*Salvia officinalis* L.), rosemary (*Rosmarinus officinalis* L.), and bay laurel (*Laurus nobilis* L.). Also, we hypothesized that these relatively mild pretreatments will not adversely affect the chemical composition of essential oils.

2. Results

2.1. Cellulase, Xylanase, and Pectinase Can Degrade Their Respective Substrates under the Reaction Conditions

Before applying cell wall-degrading enzymes (xylanase, pectinase, and cellulase) for the extraction of bioactive compounds from the plant material, their activity under the extraction conditions (1 h, 40 °C, in MiliQ water) was measured at a small scale by 3,5-dinitrosalicylic acid (DNSA) assay. The enzymes were mixed with their appropriate polymeric substrates in the MiliQ water and the reaction was incubated for 1 h at 40 °C. The results have shown that all enzymes were able to degrade their substrates: xylanase degraded xylan to 1.10 U/mL, pectinase pectin to 0.03 U/mL, and cellulase cellulose to 0.05 U/mL.

2.2. Optimization of Ultrasound Extraction Conditions

Optimal ultrasonic power and duration, chosen based on the overall maximum total phenol yield, were 30% of maximal ultrasonic power during 10 min (Table 1). In some cases, total phenol yield was slightly higher after 15 min ultrasound treatment (e.g. at 30% of maximal ultrasonic power for rosemary, and 90% for bay laurel). Still, since 15 min ultrasound treatment caused intensive solvent evaporation, the shorter time of 10 min was chosen as optimal.

Table 1. Optimization of ultrasound extraction parameters based on the total phenol yield.

	Time	Total Phenol Yield (mg/g)		
		30% of Max. Ultrasonic Power	60% of Max. Ultrasonic Power	90% of Max. Ultrasonic Power
Bay laurel	5 min	16.87 ± 0.18	8.86 ± 0.05	7.32 ± 0.14
	10 min	17.37 ± 0.53	8.27 ± 0.21	5.99 ± 0.00
	15 min	16.86 ± 0.18	8.30 ± 0.21	6.38 ± 0.15
Sage	5 min	29.46 ± 3.14	32.95 ± 2.6	32.20 ± 2.9
	10 min	35.71 ± 5.6	34.24 ± 7.2	36.83 ± 3.9
	15 min	31.25 ± 5.4	31.76 ± 3.4	31.82 ± 0.32
Rosemary	5 min	93.98 ± 1.41	46.35 ± 1.06	50.16 ± 2.75
	10 min	97.44 ± 2.12	45.27 ± 0.07	51.22 ± 0.78
	15 min	103.44 ± 2.12	43.08 ± 0.14	43.12 ± 0.14

2.3. Different Pretreatments Increased the Essential Oil Yield

All tested hydrodistillation pretreatments caused an increase in essential oil yield when compared to no-pretreatment control (HD): 56–64% for bay laurel, 40–60% for rosemary, and 45–55% for sage (Figure 1). However, only slight differences in essential oil yield were observed between different pretreatments for the same plant species. Importantly, none of the individual (HD-REX, HD-REC, HD-REP) or combined (HD-REPCX) enzymatic pretreatments significantly outperformed reflux extraction pretreatment (HD-RE). Since HD-RE served as a no-enzyme control, this indicates that enzymatic activity had only a minute effect on essential oil yield increase and that it should be mostly attributed to reflux extraction *per se*.

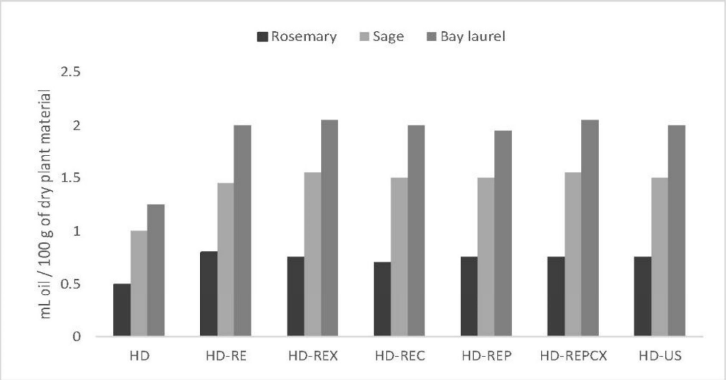

Figure 1. Effect of different hydrodistillation pretreatments on the extraction yield of rosemary, sage, and bay laurel essential oil. Hydrodistillation without pretreatment (negative control)—HD; hydrodistillation with reflux extraction pretreatment—HD-RE; hydrodistillation with reflux extraction pretreatment assisted with enzymes: xylanase—HD-REX; cellulase—HD-REC; pectinase—HD-REP; pectinase + cellulase + xylanase—HD-REPCX; hydrodistillation with ultrasonic pretreatment—HD-US.

When comparing different plants, Clevenger hydrodistillation of bay laurel leaves consistently resulted in the highest oil yield (from 1.95 mL oil/100 g dry plant for HD-REP to 2.05 mL oil/100 g dry plant for HD-REX and HD-REPCX), followed by sage (from 1.45 mL oil/100 g dry plant for HD-RE to 1.55 mL oil/100 g dry plant for HD-REX and HD-REPCX) and rosemary (from 0.7 mL oil/100 g dry plant for HD-REC to 0.8 mL oil/100 g dry plant for HD-RE).

2.4. Overall Chemical Composition of Essential Oils Was Not Significantly Affected by Different Hydrodistillation Pretreatments

The chemical composition of obtained essential oils was analyzed by GC-MS (Tables S1–S3, Figure 2) and NMR (Supplementary file 1, Figures S1–S19). The pretreatments did not significantly affect the quality of essential oils ($p < 0.05$), that is, the oil composition was comparable to the no-pretreatment control, as confirmed by Spearman's test performed using GC-MS data (Supplementary file 2, Figures S20–S22). GC-MS analysis showed that the essential oils of all plants were richest in monoterpenes: up to 74%, 71%, and 62% of total peak area in sage, bay laurel, and rosemary essential oils, respectively (Tables S1–S3, Figure 2). Sesquiterpenes were also well represented: up to 35%, 17%, and 56% of total peak area in sage, bay laurel, and rosemary essential oils, respectively. Dominant compounds (i.e., terpenes represented with more than 5% of total peak area) were confirmed by NMR (as described and represented in Supplementary file 1, Figures S1–S19 and Appendix 1): linalool, camphor, borneol, and berbenone in rosemary essential oil (Figure S19A), camphor, manool, α- and β-thujone, and veridiflorol in sage essential oil (Figure S19B), and α-terpenyl acetate, linalool, and methyleugenol in bay laurel essential oil (Figure S19C).

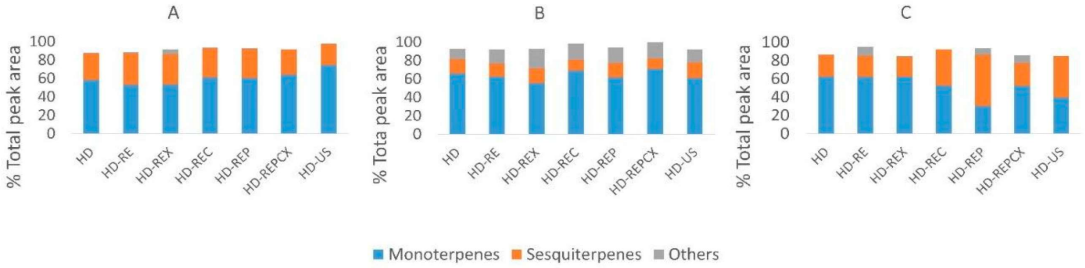

Figure 2. Overview of the chemical composition of sage (**A**), bay laurel (**B**), and rosemary (**C**) essential oils after different hydrodistillation pretreatments, as determined by GC-MS. Hydrodistillation without pretreatment (negative control)—HD; hydrodistillation with reflux extraction pretreatment—HD-RE; hydrodistillation with reflux extraction pretreatment assisted with enzymes: xylanase—HD-REX; cellulase—HD-REC; pectinase—HD-REP; pectinase + cellulase + xylanase—HD-REPCX; hydrodistillation with ultrasonic pretreatment—HD-US.

Seventy-one components were identified in the sage essential oils by GC-MS, representing up to 97% of total GC peak areas (Table S1). Sage essential oils were richest in oxygenated monoterpenes, such as α- and β–thujone (up to 23% and 10%, respectively), camphor (up to 17%), borneol (up to 8%), and 1,8-cineole (up to 8%). Additionally, some sesquiterpenes were present in significant quantities, such as manool and veridiflorol (up to 14% each). Among other compounds, phenylpropane derivates, eugenol (up to 3%), and methyleugenol (up to 2%) were detected with significant percentages, although much lower than monoterpene and sesquiterpene major compounds.

In the bay laurel essential oils, GC-MS allowed the identification of 84 components, covering up to 99% of the total GC profile (Table S2). Major detected monoterpenes were α-terpenyl acetate (up to 18%), 1,8-cineole (up to 27%), and linalool (up to 8%). Among sesquiterpenes, the most abundant were veridiflorol (up to 4%), trans-caryophyllene (up to 4%), bicyclogermacrene (up to 1.4%), and β-elemene (up to 1.6%). Other compounds detected in significant quantities were phenylpropane derivates, methyleugenol (up to 10%), and eugenol (up to 9%).

Finally, 60 components were detected in the rosemary essential oil, covering up to 95% of total GC peak areas (Table S3). Rosemary essential oil was richest in oxygenated monoterpenes, such as borneol (up to 24%), camphor (up to 18%), linalool (up to 6%), and 1,8–cineole (up to 9%). Major sesquiterpenes were berbenone (up to 22%) and t-muurolol (up to 6%). Other compounds, like eugenol (up to 6%) and methyleugenol (up to 4%), were also present in significant amounts.

3. Discussion

We have tested the effect of different pretreatments using selected Mediterranean plants: rosemary, sage, and bay laurel, and demonstrated that different hydrodistillation pretreatments can improve the yield of respective essential oils. At the same time, the pretreatments did not significantly affect the quality of oils and their composition was comparable to no-pretreatment controls. For the first time, we have demonstrated that reflux extraction (e.g., soaking of milled plant material at 40 °C for one hour) could significantly improve essential oil yield.

Rosemary and sage are aromatic, medicinal plants that have received particular attention in the Lamiaceae family due to their aromatic and chemical composition [2,27,28], while bay laurel is a valuable medicinal plant from the Lauraceae family, which has been widely used as a spice and flavoring agent [19]. Due to the numerous bioactive compounds, the essential oils of these plants show a wide range of biological activities such as antimicrobial, preservative, antioxidant, and antifungal, which makes them valuable in a range of applications, from medicinal to the food industry [29–33]. Considering these valuable properties, the improvement of current and the development of novel essential oil extraction procedures is a subject of many recent studies [28,34,35].

In this study, we have obtained similar volatile profiles of sage, rosemary, and bay laurel essential oils as reported elsewhere [2,3,19,35–39]. Also, the essential oil composition obtained after different

pretreatments was comparable to the essential oils obtained by direct hydrodistillation. Other studies had also shown that enzymatic and ultrasonic pretreatments did not affect the overall composition of the oil [11,15–17,21,22,26], although some authors reported that the quantities of individual major components varied significantly in relation to the extraction technique used [1,2,12–14,19,24]. For instance, Boulila et al. [19] reported that enzyme pretreatment of bay laurel leaves resulted in increased concentration of oxygenated monoterpenes, and explained such effect by a possible increase in oxidation after cell wall disruption or the presence of oxidases in the enzyme preparation. Also, cellulase pretreatment reportedly increased the amount of cis-verbenol and camphor and decreased the amounts of 1,8-cineol, α-pinene, fenchene, and terpinen-4-ol in rosemary essential oil [2]. We have also observed that the ratios of some components varied among different pretreatments. For example, in sage essential oil, the amount of almost all monoterpene hydrocarbons increased after HD-REPCX. On the other hand, HD-US resulted in a higher amount of major oxygenated monoterpenes (1,8–cineole, α-thujone, camphor). In bay laurel essential oil, all pretreatments, except HD-REX, resulted in an increase of 1,8-cineole, which is in line with the findings of Boulila et al. [19]. In rosemary essential oil, an increase of monoterpene hydrocarbons was noticeable after HD-US, while HD-RE resulted in an increase of 1,8-cineole and linalool, as well as phenylpropane derivatives eugenol and methyleugenol. However, such slight changes in the quantity of individual components observed after different pretreatments were not significant and did not change the overall oil quality, as confirmed by the statistical analysis of GC-MS results.

Among the different pretreatments applied in this study, the simplest was reflux extraction (HD-RE). The incubation of finely milled plant material for 1 h at 40 °C in MiliQ water before hydrodistillation resulted in up to 60% essential oil yield increase. It was previously shown that such soaking of the plant material in water [40] or acidic medium [1] before distillation may increase the quantity of oil by enhancing the leaching of ingredients from the already disrupted cells. Also, swelling and hydration of plant material, which enlarge the pores in cell walls and increase the turgor pressure in the still intact plant cells, might lead to the enhanced diffusion of the oil ingredients into the soaking medium. For example, soaking of agarwood in lactic acid for 168 h [41] and soaking of thyme leaves in distilled water overnight at 50 °C improved the essential oil yield [40]. Thus, soaking of plant material before hydrodistillation, as applied here for selected Mediterranean plants, presents a simple and cost-effective treatment that results in a significant increase in essential oil yield.

In addition, we have tested whether the application of cell wall-degrading enzymes prior to hydrodistillation has a positive effect on the extraction efficiency. We have followed previously described protocols [2,19] and applied separate and combined pretreatments with cellulase, pectinase, and xylanase to plant material. Prior to the extractions, we used a small-scale enzyme assay and confirmed the activity of all enzymes under the reaction conditions. However, the application of enzymes did not result in a significant increase in the yield of the essential oil above the reflux extraction pretreatments, suggesting that, in our case, the enzymatic activity had only a minor effect on the yield. On the contrary, the positive impact of enzyme application on the yield of essential oils was reported in multiple recent studies [2,19,21–23,25,26]. For instance, enzyme-assisted extraction pretreatment was used prior to hydrodistillation of bay laurel [19], rosemary, and thyme leaves [2], and reportedly resulted in an increase in essential oil yield (up to 109% for thyme leaves). However, these studies were lacking a no-enzyme control, and the observed yield increase was calculated by comparison with no-pretreatment control. In our case, the extraction results were compared to no-pretreatment control (HD) as well as to reflux extraction pretreatment (HD-RE), that served as a no-enzyme control since it was performed in the same conditions as the enzyme-assisted extraction pretreatments (MiliQ water at 40 °C for 1 h), only without addition of enzyme(s). In comparison to no-pretreatment control, an increase in essential oil yield was significant for all enzyme pretreatments individually, as well as for their combination, and there were no significant differences between each individual pretreatment. Unexpectedly, reflux extraction resulted in approximately the same essential oil increase as the enzyme-assisted pretreatments, leading to the conclusion that essential oil yield

increase can be more attributed to reflux extraction pretreatment *per se* (i.e., soaking of macerated plant material in warm water) than to enzymatic degradation of the cell wall. The positive effect of the soaking of plant material alone, as observed here, could not be properly assessed and compared with the effect of enzymes in the previous studies that were lacking no-enzyme controls [2,19,21–26]. Our results are congruent with the results of Dimaki and coworkers [1] who analyzed the effect of enzyme pretreatments on hydrodistillation and ultrasound-assisted extraction of *Sideritis* sp. essential oil. They have used both no-pretreatment and no-enzyme control (i.e., preincubation in acidic medium) and also found that enzymatic pretreatment was not superior to the mere soaking of plant material in an acidic buffer. Also, acidic and enzymatic pretreatments prior to the ultrasound-assisted extraction resulted in a similar increase in extraction yield in comparison to no-pretreatment control. In conclusion, our results and results of Dimaki et al. [1] point out that the application of enzymatic pretreatments with the aim to increase the yield of essential oils, although often reported, should be carefully re-evaluated.

Finally, we have demonstrated the beneficial effect of ultrasound pretreatment on the overall extraction efficiency. In comparison to the control experiment, ultrasound-assisted extraction pretreatment caused a 50% improvement in sage and rosemary and 60% in bay laurel essential oil yield, similar to the results of reflux-assisted extraction. This is comparable with the results of other studies [12,13,15–17]. For instance, 30 min-ultrasonic-maceration pretreatment prior to the steam distillation of milled *Thymus vulgaris* L., *Mentha piperita* L., and *Origanum majorana* L. leaves resulted in approximately 10% essential oil yield increase, compared to no-pretreatment control [15,16]. Ultrasound-assisted extraction as a pretreatment to hydrodistillation of grounded carrot seeds increased essential oil yield by approximately 33% [17]. Hydrodistillation of grounded *Elettaria cardamomum* L. seeds with ultrasonic pretreatment resulted in a 4.9% essential oil yield increase and extraction lasted less than an hour, compared to control, which lasted 6 h [13]. Even when there was no yield increase, the application of ultrasound resulted in a significant shortening of the essential oil isolation procedure [11,14]. In conclusion, we report a high increase in essential oil yield after ultrasound pretreatment when compared to other studies, accompanied by the shortening of the extraction time when compared to the reflux-assisted extraction pretreatment.

In conclusion, we have demonstrated the valuable effects of ultrasound and reflux extraction hydrodistillation pretreatments on the yield of essential oils of sage, rosemary, and bay laurel. The benefit of ultrasound is short extraction time, while reflux extraction is performed at mild temperature. Thus, both pretreatments are considered as gentle extraction procedures, resulting in unchanged overall oil quality, as demonstrated by essential oil composition analyzed here. Also, our results point out that when assessing the effect of enzymes as a hydrodistillation pretreatment, proper experiment controls are necessary. In our case, the soaking of plant material in the water at 40 °C was enough to disrupt the cells and additional enzyme activity was redundant.

4. Materials and Methods

4.1. Plant Material

Fresh leaves were collected from wild plants of rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), and bay laurel (*Laurus nobilis* L.) in the south Mediterranean region of Croatia in August 2018. The leaves were air-dried at room temperature (20 ± 2 °C) for one week. Dry leaves were packed in polyethylene bags and kept in a dark, dry, and cool place. Before being used for the extractions, the plant material was milled using a house blender (Tefal).

4.2. Chemicals

The following chemicals were used: 3,5-dinitrosalicylic acid (DNSA) (98%, Thermo Fisher Scientific, Maharahstra, India), sodium sulphite (Lach-ner, Brno, Chez Republic), sodium hydroxide (Lach-ner, Brno, Chez Republic), phenol (99 + %, Thermo Fisher Scientific, Maharahstra, India), potassium sodium tartrate (Sigma-Aldrich, Buchs, Switzerland), Folin–Ciocalteu reagent (Sigma-Aldrich, Buchs,

Switzerland), anhydrous sodium carbonate (Lach-ner, Brno, Chez Republic), gallic acid (anhydrous) for synthesis (Merck, Darmstadt, Germany), cellulase (from *Aspergillus niger*) (Sigma-Aldrich, Tokyo, Japan), pectinase (from *Aspergillus niger*) (Sigma-Aldrich, Buchs, Switzerland), xylanase (from *Theryomyces*, expressed in *Aspergillus oryzae*) (Sigma-Aldrich, Søborg, Denmark), beechwood xylan (Biosynth, Berkshir, England, UK), pectin from citrus peel (74.0%, Sigma-Aldrich, Buchs, Switzerland), sodium carboxymethyl cellulose (Sigma-Aldrich, Buchs, Switzerland), xylose (99%, Sigma-Aldrich, Buchs, Switzerland, D-(+)-glucose (99.5%, Sigma-Aldrich, Buchs, Switzerland), D-(+)-galacturonic acid monohydrate (97.0%, Sigma Aldrich, Buchs, Switzerland), C_9-C_{25} alkanes, deuterated chloroform for NMR spectroscopy ($CDCl_3$-d with 0.03% *v/v* TMS, 99.80%, Eurisotop, Saint-Aubin, France).

4.3. 3,5-Dinitrosalicylic Acid (DNSA) Assay for the Determination of Enzyme Activity

The determination of enzyme activity was done according to a DNSA colorimetric method as described in Ghose [42] and Miller [43] with modifications. Enzymes (cellulase, pectinase, and xylanase) were added to MiliQ water to a final concentration of 0.02 mg/mL. Next, 10 mg of the appropriate substrate (cellulose, pectin, and xylan, respectively) was added to 1 mL of enzyme solution and the reaction was incubated at 40 °C for 60 min in a thermoshaker (Biosan, TS-100) at 900 rpm. The sample was then cleared by centrifugation (5 min, 1568× *g*). Next, sample aliquots (600 µL final volume) were mixed with 600 µL of DNSA reagent containing 10.0 g/L DNSA, 0.5 g/L sodium sulfite, 10 g/L sodium hydroxide, and 2 mL/L phenol. The mixtures were incubated for 15 min at 95 °C before adding 200 µL of 40 g/L potassium sodium tartrate solution (1.4 mL final volume). The samples were chilled on ice for 5 min, and then the absorbance at 575 nm was measured (Cary Series UV-Vis Spectrophotometer, Agilent Technologies). Product concentrations were calculated from calibration curves generated with the corresponding reducing sugars (glucose, galacturonic acid, xylose, respectively). If needed, the original samples were appropriately diluted to produce the absorption values within the range of the calibration curves, and dilutions were taken into account in the calculation of the enzymatic activity. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar per minute under the specified assay conditions.

4.4. Determination of Total Phenols for Optimization of Ultrasonic Parameters

The total phenols of plant extracts were determined using the Folin–Ciocalteu method, according to the procedure of Singleton and Rossi [44]. Mass fraction of total phenols was expressed as mg of gallic acid equivalent (GAE) per g dry plant.

4.5. Extraction Procedures

Hydrodistillation (HD) was preceded by different pretreatments: hydrodistillation with reflux extraction pretreatment (HD-RE), hydrodistillation with reflux extraction pretreatment assisted with enzymes (pectinase, HD-REP; cellulase, HD-REC; xylanase, HD-REX; pectinase/cellulase/xylanase, HD-REPCX), and hydrodistillation with ultrasonic pretreatment (HD-U). Additionally, HD without pretreatment was performed as a negative control.

For each protocol, 20 g of milled plant material was mixed with 250 mL MiliQ water. For HD-RE, this mixture was subjected to reflux extraction at 40 °C for 1 h under stirring conditions. For enzyme-assisted pretreatments, we have followed previously described protocols [2,19] with slight modifications. Namely, the plant material in MiliQ water was subjected to reflux extraction at 40 °C for 1 h under stirring conditions in the presence of 10 mg pectinase, cellulase, or xylanase, that is, 0.5 mg of enzyme per g of dried plant material, or their combination (5 mg of each enzyme, i.e., 0.75 mg of total enzymes per g of dried plant material). For HD-U, the plant material in MiliQ water was treated with a 14 mm diameter ultrasonic probe (ultrasonic device UP200Ht, Hielscher, Teltow, Germany), at 30% of the maximal ultrasonic power for 10 min. Before the ultrasound extraction, optimal duration, and amplitude were determined (Table 1).

Next, the plant/water mixture was subjected to hydrodistillation using a Clevenger apparatus for 2.5 h. Every extraction was done once. The essential oil was stored in dark glass vials at 4 °C until further analyses.

4.6. Gas Chromatograph/Mass Spectrometer (GC-MS) Analysis

GC-MS analyses were carried out with an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph model 7890 A equipped with a mass selective detector (MSD) model 5975 C (Agilent Technologies, Palo Alto, CA, USA) and an HP-5MS 5% phenyl-methylpolysiloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness, Agilent Technologies, Palo Alto, CA, USA). In brief, the injector and detector temperatures were 250 °C and 300 °C, respectively; the column temperature was held at 70 °C for 2 min, and was then increased from 70 to 200 °C at 3 °C/min, and was finally held at 200 °C for 18 min; 1.0 μL of the sample (10 μL of the oil dissolved in 1 mL of pentane) was injected using split mode (split ratio 1:50). Helium was used as carrier gas (1.0 mL/min). The MSD (EI mode) was operated at 70 eV, the ion source temperature was 230 °C, and the scan range was set to 30–350 amu. Identification of volatile constituents was based on the comparison of their retention indices (RIs), determined relative to the retention times of a homologous series of n-alkanes (C_9–C_{25}), with those reported in the literature and their mass spectra with those of authentic compounds available in our laboratories or those listed in the NIST 08 and Wiley 9 mass spectral libraries (matches higher than 90%). Relative concentrations of components were calculated by the area normalization method without considering response factors.

4.7. Statistical Analyses

All variables were log-transformed (using base 10 logs) to improve the data distribution and homogeneity of variances. The data were tested for normality by comparing histograms of the sample data to a normal probability curve, after which the null hypothesis (that data were normally distributed) was rejected. To compare and plot the effects of different pretreatments on the chemical composition of essential oils for each plant (as determined by GC-MS), we applied Spearman's nonparametric measure of rank correlation using program R v. 3.2.0 [45]. The level of significance was set at $p \leq 0.05$.

Supplementary Materials: The following are available online at, Figure S1: Sage 1H NMR spectrum (600 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 32 *K* data points; 256 scans; 0.37 Hz/point; 1 s delay), Figure S2: Sage 1H-1H COSY NMR spectrum (600 MHz, CDCl3-d, 25 °C), Figure S3: Sage 1H-1H TOCSY NMR spectrum (600 MHz, CDCl3-d, 25 °C), Figure S4: Sage 1H-13C HMQC NMR spectrum (CDCl3-d, 25 °C). The 600 MHz 1H NMR spectrum is shown at the top edge and a 150 MHz 13C NMR spectrum at the left-hand edge, Figure S5: Sage 1H-13C HMBC NMR spectrum (CDCl3-d, 25 °C). The 600 MHz 1H NMR spectrum is shown at the top edge and a 150 MHz 13C NMR spectrum at the left-hand edge, Figure S6: Sage 13C APT NMR spectrum (150 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 64 *K* data points; 44506 scans; 0.60 Hz/point; 1 s delay), Figure S7: Bay laurel 1H NMR spectrum (600 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 32 *K* data points; 256 scans; 0.37 Hz/point; 1 s delay), Figure S8: Bay laurel 1H-1H COSY NMR spectrum (600 MHz, CDCl3-d, 25 °C), Figure S9: Bay laurel 1H-1H TOCSY NMR spectrum (600 MHz, CDCl3-d, 25 °C), Figure S10: Bay laurel 1H-13C HMQC NMR spectrum (CDCl3-d, 25 °C). The 600 MHz 1H NMR spectrum is shown at the top edge and a 150 MHz 13C NMR spectrum at the left-hand edge, Figure S11: Bay laurel 1H-13C HMBC NMR spectrum (CDCl3-d, 25 °C). The 600 MHz 1H NMR spectrum is shown at the top edge and a 150 MHz 13C NMR spectrum at the left-hand edge, Figure S12: Bay laurel 13C APT NMR spectrum (150 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 64 *K* data points; ca. 30000 scans; 0.60 Hz/point; 1 s delay), Figure S13: Rosemary 1H NMR spectrum (600 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 32 *K* data points; 128 scans; 0.37 Hz/point; 1 s delay), Figure S14: Rosemary 1H-1H COSY spectrum (600 MHz, CDCl3-d, 25 °C), Figure S15: Rosemary 1H-1H TOCSY spectrum (600 MHz, CDCl3-d, 25 °C), Figure S16: Rosemary 1H-13C HMQC spectrum (CDCl3-d, 25 °C). The 600 MHz 1H NMR spectrum is shown at the top edge and a 150 MHz 13C NMR spectrum at the left-hand edge, Figure S17: Rosemary 1H-13C HMBC spectrum (CDCl3-d, 25 °C). The 600 MHz 1H NMR spectrum is shown at the top edge and a 150 MHz 13C NMR spectrum at the left-hand edge, Figure S18: Rosemary 13C APT NMR spectrum (150 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 64 *K* data points; ca. 34000 scans; 0.60 Hz/point; 1 s delay), Figure S19: (A) Rosemary, (B) sage and (C) bay laurel essential oils 1H NMR spectra at 600 MHz in CDCl3-d. The enumeration scheme used for the assignment of the NMR spectra is shown for every compound. Figure S20: Scatter plot showing correlations between different pretreatments (on the diagonal) regarding the chemical composition of sage essential oils. Significant *p*-values based on Spearman's rank test are shown above the diagonal, while bivariate scatter plots are shown below the diagonal, Figure S21: Scatter plot showing correlations between different pretreatments (on the diagonal)

regarding the chemical composition of bay laurel essential oils. Significant *p*-values based on Spearman's rank test are shown above the diagonal, while bivariate scatter plots are shown below the diagonal, Figure S22: Scatter plot showing correlations between different pretreatments (on the diagonal) regarding the chemical composition of rosemary essential oils. Significant *p*-values based on Spearman's rank test are shown above the diagonal, while bivariate scatter plots are shown below the diagonal. Table S1. Chemical composition of sage essential oils isolated by hydrodistillation with and without different pretreatments, Table S2. Chemical composition of bay laurel essential oils isolated by hydrodistillation with and without different pretreatments, Table S3. Chemical composition of rosemary essential oils isolated by hydrodistillation with and without different pretreatments.

Author Contributions: Conceptualization, A.B., and M.D.; methodology, M.D., Z.M., I.J., M.A., T.R., S.R., and D.V.-T.; validation, A.M., M.D., and A.B.; formal analysis, D.G.; investigation, A.M., Z.M., I.J., M.A., T.R., S.R., and D.V.-T.; resources, A.B..; writing—original draft preparation, A.M.; writing—review and editing, A.B. and M.D.; visualization, A.M., D.G., and S.R.; supervision, A.B., and M.D.; project administration, A.B. and D.G.; funding acquisition, A.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the project "Interactions of freshwater pathogenic oomycetes and the environment" (InteractOomyc, UIP-2017-05-6267), Croatian Science Foundation.

Acknowledgments: The authors are thankful to Maja Macut and Gloria Hradec for technical assistance during the study and Professor Verica Dragović-Uzelac for her support and advice during this research.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Sample Availability: Samples of the compounds are not available from the authors.

2.2. Essential oils of sage, rosemary, and bay laurel inhibit the life stages of oomycete pathogens important in aquaculture

plants

MDPI

Article

Essential Oils of Sage, Rosemary, and Bay Laurel Inhibit the Life Stages of Oomycete Pathogens Important in Aquaculture

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Abstract: *Saprolegnia parasitica*, the causative agent of saprolegniosis in fish, and *Aphanomyces astaci*, the causative agent of crayfish plague, are oomycete pathogens that cause economic losses in aquaculture. Since toxic chemicals are currently used to control them, we aimed to investigate their inhibition by essential oils of sage, rosemary, and bay laurel as environmentally acceptable alternatives. Gas Chromatography–Mass Spectrometry (GC–MS) analysis showed that the essential oils tested were rich in bioactive volatiles, mainly monoterpenes. Mycelium and zoospores of *A. astaci* were more sensitive compared to those of *S. parasitica*, where only sage essential oil completely inhibited mycelial growth. EC_{50} values (i.e., concentrations of samples at which the growth was inhibited by 50%) for mycelial growth determined by the radial growth inhibition assay were 0.031–0.098 µL/mL for *A. astaci* and 0.040 µL/mL for *S. parasitica*. EC_{50} values determined by the zoospore germination inhibition assay were 0.007–0.049 µL/mL for *A. astaci* and 0.012–0.063 µL/mL for *S. parasitica*. The observed inhibition, most pronounced for sage essential oil, could be partly due to dominant constituents of the essential oils, such as camphor, but more likely resulted from a synergistic effect of multiple compounds. Our results may serve as a basis for in vivo experiments and the development of environmentally friendly methods to control oomycete pathogens in aquaculture.

Keywords: anti-oomycete activity; *Aphanomyces astaci*; EC_{50} values; Mediterranean wild plants; *Saprolegnia parasitica*

Citation: Miljanović, A.; Grbin, D.; Pavić, D.; Dent, M.; Jerković, I.; Marijanović, Z.; Bielen, A. Essential Oils of Sage, Rosemary, and Bay Laurel Inhibit the Life Stages of Oomycete Pathogens Important in Aquaculture. *Plants* **2021**, *10*, 1676. https://doi.org/10.3390/plants10081676

Academic Editor: Stefania Garzoli

Received: 29 July 2021
Accepted: 13 August 2021
Published: 15 August 2021

1. Introduction

Saprolegnia parasitica and *Aphanomyces astaci* (Oomycota) cause significant economic losses in freshwater aquaculture: *S. parasitica* affects salmonid hatcheries and farms [1], and *A. astaci* crayfish rearing facilities [2–4]. *Saprolegnia parasitica* is parasitic to salmonid fishes such as *Oncorhynchus mykiss* and *Salmo salar* and infects all developmental stages [5–7]. It can also infect crayfish species, especially when the animals are injured, and its pathogenicity towards *Astacus astacus*, *Pacifastacus leniusculus*, and *Procambarus clarkii* has been confirmed by infection experiments [8]. *Saprolegnia*-infected fish develop a cottony mycelium on the gills and injured skin, while the infected eggs usually die as a result of hyphal rupture of the chorionic membrane and subsequent osmotic shock [9]. *Aphanomyces astaci* is a causative agent of crayfish plague. Its hyphae penetrate the cuticle of crayfish and spread throughout the tissues, leading to the development of a fatal disease [10,11]. It is mainly known for its destructive effect on native European crayfish species, while North American crayfish are considered carriers of the pathogen but can still succumb to the disease under certain stressful conditions, such as in a high-density farming environment [12,13]. Therefore, *A. astaci* may pose a serious threat to the crayfish farming industry on a global

scale, both to native (e.g., *Astacus astacus*) and invasive (e.g., *Procambarus clarkii* and *Cherax quadricarinatus*) crayfish [2,4,14].

Diseases in freshwater aquaculture caused by oomycetes were previously treated successfully with malachite green, but its use is no longer allowed in the EU [15] and the USA [16] due to its teratogenic and carcinogenic properties [17–19]. Other chemicals currently used worldwide are also not sustainable solutions. In fact, formalin poses a health risk to fish farm workers who handle it [20] and to fish consumers, as it leaves residues in the fish [21]. Other agents such as bronopol, copper sulfate, and peracetic acid are less toxic but still pose a serious threat to the aquatic biota [22–24].

The toxicity of chemical agents used for oomycete control in fisheries prompts the search for environmentally friendly alternatives that should be effective against pathogenic oomycetes but also safe for operators, animals, and the environment. In this context, it has been increasingly shown that alcoholic extracts [25–30] and essential oils [31–39] of selected plants can inhibit pathogenic oomycetes in vitro, with essential oils generally being more potent than alcoholic extracts [38]. In particular, plants from the Lauraceae and Lamiaceae families, such as *Thymbra spicata* and *Cinnamomum zeylanicum*, have been reported to inhibit pathogenic freshwater oomycetes, mostly using the mycelium and zoospores of *Saprolegnia* spp. as models [32,34,36–38,40]. In comparison, the inhibitory activity of essential oils and their major components against *Aphanomyces* spp. is much less studied [28,40]. So far, there are no reports on the inhibitory activity of essential oils against *A. astaci*, but some essential oils showed good inhibitory activity against the phylogenetically related plant pathogen *A. euteiches* and the fish pathogen *A. invadans* [28,40].

The aim of this study was to test, for the first time, the inhibition of the oomycete pathogens *S. parasitica* and *A. astaci* by the essential oils of sage (*Salvia officinalis*), bay laurel (*Laurus nobilis*), and rosemary (*Rosmarinus officinalis*), which have previously shown potent antifungal and antimicrobial activity [41–43]. We analyzed the volatile composition of the essential oils and then tested the sensitivity of the alternative stages of the oomycete life cycle (i.e., mycelium and zoospores, as infectious stage) to essential oils.

2. Results

2.1. Chemical Composition of Essential Oils

The chemical composition of essential oils was analyzed by Gas Chromatography–Mass Spectrometry (GC–MS) (Figure 1, Table S1, Supplementary material). All essential oils were rich in volatile components, with the highest number of compounds identified in rosemary (65 compounds, 90% of total GC peak area), followed by bay laurel (53 compounds, 93% of total GC peak area), and eventually sage essential oil (35 compounds, 98% of total GC peak area). The major detected compounds in all three essential oils were monoterpenes, such as camphor (11.7%), α-pinene (10.8%), 1,8-cineole (7.3%), borneol (8.9%), and linalool (4.4%) in rosemary essential oil, then 1,8-cineole (26.8%), α-terphenyl acetate (13.2%), linalool (6.9%), sabinene (4.9%), and α-terpineol (4.1%) in bay laurel essential oil, and camphor (23.9%), α-thujone (20.3%), 1,8-cineole (12.5%), and camphene (5.2%) in sage essential oil. Some essential oils were also rich in sesquiterpenes, such as berbenone (6.1%), *trans*-caryophyllene (2.8%), and veridiflorol (2.6%) in rosemary essential oil, and veridiflorol (10.3%) and α-humulene (3.2%) in sage essential oil, while in bay laurel essential oil, a compound classified as a phenylpropane derivate, eugenol, was also identified in significant quantity (4.4% of total peak area).

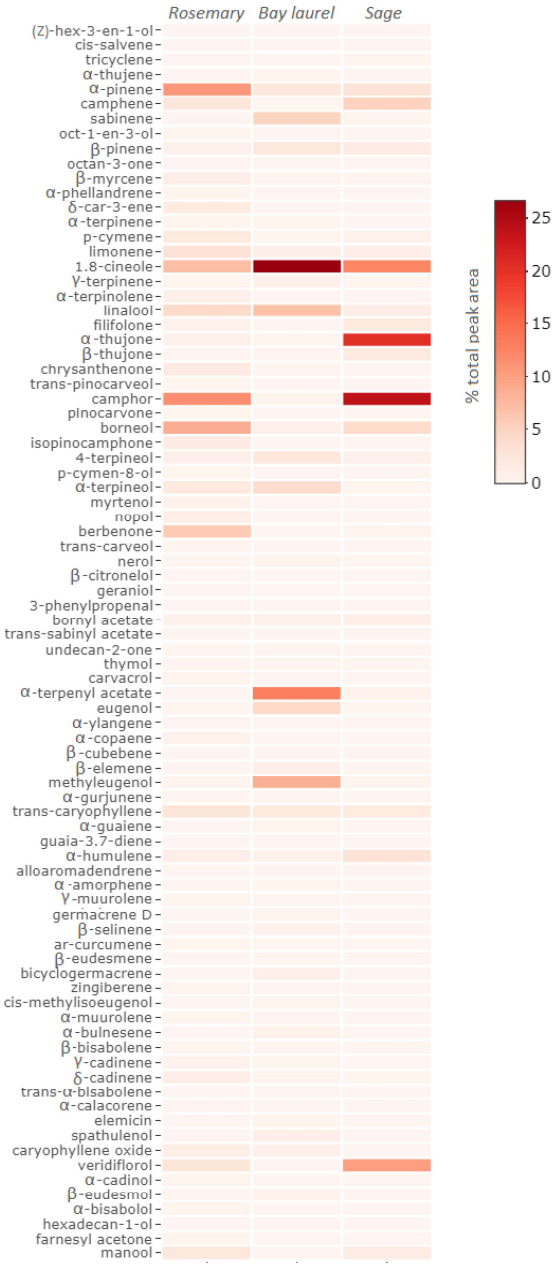

Figure 1. Heatmap of the volatile chemical composition of rosemary, bay laurel, and sage essential oils, as determined by Gas Chromatography–Mass Spectrometry (GC–MS).

2.2. Inhibition of Mycelial Growth

All essential oils tested inhibited mycelial growth of *A. astaci* (Figure 2, Table 1). Sage essential oil was the most effective, with an EC_{50} value (i.e., the concentration of the sample at which the mycelial growth was inhibited by 50%) two times lower than that of rosemary and three times lower than that of bay laurel (EC_{50} values were 0.03 µL/mL, 0.06 µL/mL, and 0.10 µL/mL, respectively). In the case of *S. parasitica*, only sage essential oil showed a

significant inhibitory effect on mycelial growth (EC_{50} = 0.04 µL/mL), while rosemary and bay laurel essential oils showed no such effect, and 100% inhibition could not be achieved at the concentrations tested, so EC_{50} values could not be calculated (Figure 2, Table 1). Thus, the mycelium of *A. astaci* was more sensitive to essential oils of selected Mediterranean plants than the mycelium of *S. parasitica*: the EC_{50} value for the effect of sage essential oil on the mycelium of *S. parasitica* was higher than the corresponding EC_{50} value for *A. astaci* (0.04 vs. 0.03 µL/mL), while the effect of rosemary and bay laurel essential oils was significantly more pronounced towards *A. astaci* (data for *S. parasitica* not shown). Finally, the mycelium of *S. parasitica* was six times more resistant to malachite green (the EC_{50} value of malachite green for *A. astaci* was 0.02 µg/mL, while for *S. parasitica* it was 0.12 µg/mL).

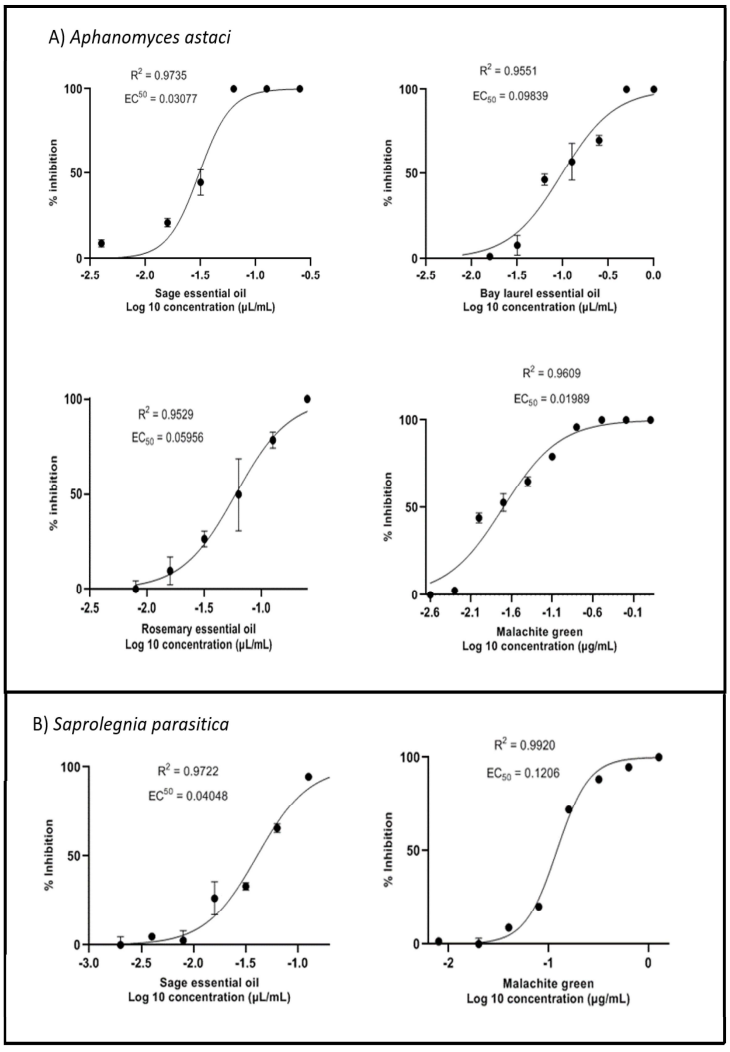

Figure 2. Mycelial growth inhibition curves of *A. astaci* and *S. parasitica* treated with sage, bay laurel, and rosemary essential oils. Non-linear regression curve fitting to estimate EC_{50} values (i.e., concentrations of samples at which mycelial growth was inhibited by 50%) for *S. parasitica* treated with rosemary and bay laurel essential oils could not be performed, since 100% inhibition was not achieved even with the highest tested concentrations. Mean values ± standard error (n = 3) are presented.

Table 1. EC_{50} values for inhibition of mycelial growth and zoospore germination of *A. astaci* and *S. parasitica* by rosemary, sage, and bay laurel essential oils.

	EC_{50} for Mycelium Growth (µL/mL)		**EC_{50} for Zoospore Germination (µL/mL)**	
	A. astaci	***S. parasitica***	***A. astaci***	***S. parasitica***
Rosemary essential oil	0.060	N.D. *	0.049	0.063
Sage essential oil	0.031	0.040	0.007	0.012
Bay laurel essential oil	0.098	N.D. *	0.015	0.013
	µg/mL		**µg/mL**	
Malachite green (pos. control)	0.020	0.120	0.020	0.032

* N.D.: EC_{50} values could not be determined since 100% inhibition was not achieved with the highest concentration tested.

2.3. Inhibition of Zoospore Germination

All essential oils inhibited the germination of zoospores of *A. astaci* and *S. parasitica*, with EC_{50} values ranging from 0.007 µL/mL to 0.063 µL/mL (Figure 3, Table 1). Among the essential oils tested, sage essential oil showed the strongest potency in inhibiting zoospore germination, followed by bay laurel essential oil and finally rosemary essential oil. In addition, the zoospores of *A. astaci* were slightly more sensitive to rosemary and sage essential oils (and malachite green) than the zoospores of *S. parasitica*, while the sensitivity of zoospores of both species to bay laurel essential oil was similar. When comparing the sensitivity of different life stages of pathogenic oomycetes, the zoospores were 4–6 times more sensitive than the mycelium to the effect of the tested samples. The only exception to this trend was the effect of rosemary essential oil and malachite green on the life stages of *A. astaci*, for which similar concentrations were required to inhibit zoospore germination and mycelial growth.

2.4. Correlation of the Observed Inhibitory Effects and Representation of Different Volatiles in the Essential Oils Studied

The multivariate partial least-squares regression (PLS-R2) technique was used to investigate the relationship between the observed inhibitory effects (EC_{50} values for mycelial growth and zoospore inhibition) and the representation of different volatiles in the essential oils of sage, rosemary, and bay laurel. The relationship between the predictor variables (different volatiles) and the response variables (EC_{50} values) is visually represented in the form of a correlation radar (Figure 4). The results showed that camphor, camphene, α-humulene, α-thujone, and veridiflorol were positively correlated with the inhibition of mycelial growth and moderately correlated with the inhibition of zoospore germination of both pathogens. In addition, β-pinene and 1,8-cineole were positively correlated with the inhibition of zoospore germination of *S. parasitica*.

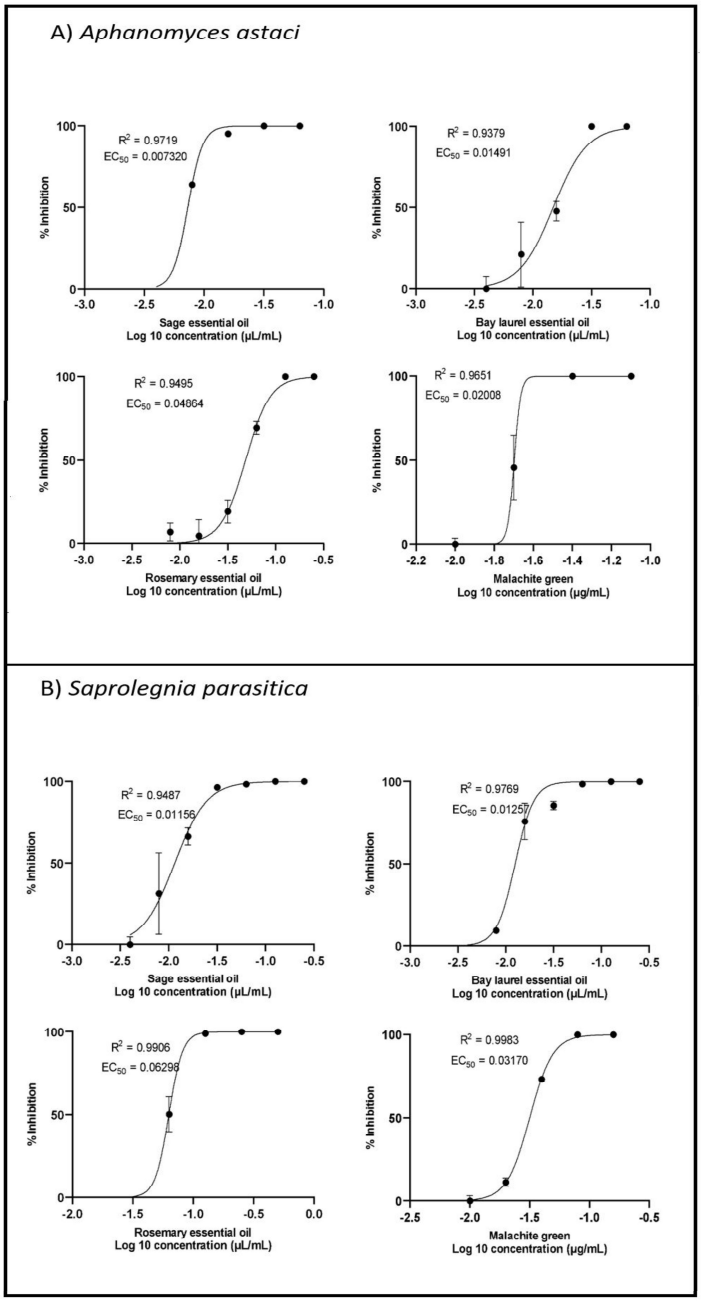

Figure 3. Zoospore germination inhibition curves of *A. astaci* and *S. parasitica* treated with sage, bay laurel, and rosemary essential oils. Mean values ± standard error (n = 3) are presented.

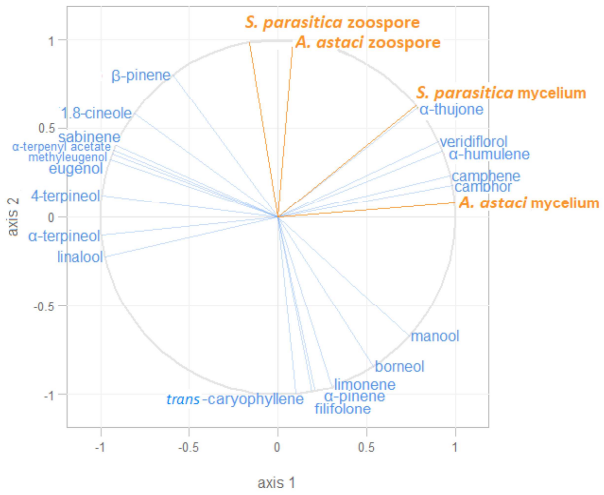

Figure 4. Partial least-squares regression (PLS-R2) radar of correlation. The orange lines represent the EC_{50} values for inhibition of mycelial growth and zoospore germination (response variables), while the blue lines represent volatile bioactive compounds present in the essential oils (predictors). Variables placed on the same side of the square within the circle are positively correlated, while those on the opposite side are negatively correlated.

3. Discussion

We demonstrated for the first time the inhibitory potential of sage, bay laurel, and rosemary essential oils, as natural substances rich in volatile bioactive constituents, against mycelial growth and zoospore germination of two oomycete pathogens important in aquaculture, *S. parasitica* and *A. astaci*.

The essential oils tested showed significant inhibition of mycelial growth of *A. astaci* and, in the case of sage essential oil, inhibition of mycelial growth of *S. parasitica*. This is the first study to report the inhibitory effect of sage essential oil on the mycelium of *S. parasitica*, although the existing literature indicates the strong potential of plants from the Lamiaceae and Lauraceae families to inhibit the mycelial growth of *S. parasitica* [32,36,37,44]. However, it should be noted that previously reported inhibitory concentrations were up to three orders of magnitude higher than those determined in this study (i.e., 0.1–100 µL/mL compared to ~0.05 µL/mL). This may be partially explained by methodological differences, as the inhibitory concentrations for the disk diffusion assay (usually reported in the literature) are generally higher than the inhibitory concentrations for the agar dilution assay used here [45]. It is also possible that sage essential oil has stronger bioactive properties than previously tested plants from the Lamiaceae and Lauraceae families, such as oregano (*Origanum onites*), thyme (*Thymbra spicata*), savory (*Satureja cuneifolia*), and cinnamon (*Cinnamomum verum*) [32,36,44,46]. Moreover, this is the first report of the inhibitory effect of essential oils against *A. astaci*. Nevertheless, some authors reported a good inhibitory effect of tea tree oil against the mycelial growth of the fish pathogen *A. invadans* [28] and of 38 essential oils, including rosemary essential oil and essential oils from some other Lauraceae and Lamiaceae plants, against the mycelial growth of the plant pathogen *A. euteiches* [40].

Most of the existing studies focused on the inhibition of mycelial growth, while there are very few reports on the inhibition of zoospores, which are the infective stage [35,39]. Our study shows a good inhibitory activity of the tested essential oils against zoospore germination of *A. astaci* and *S. parasitica*, with EC_{50} values ranging from 0.007 to 0.063 µL/mL and sage essential oil being the most potent. Similarly, the essential oils of *Mentha longifolia* and *Thymus daenensis* (Lamiaceae) completely inhibited the germination of *S. parasitica* zoospores, but at much higher concentrations of 2.5 and 5 µL/mL, respectively [39]. This

discrepancy can be partly explained by different protocols used but nevertheless indicates the promising properties of the essential oils tested here, especially sage.

We observed differences between species in sensitivity to the effects of the essential oils tested. The mycelium of *A. astaci* was much more sensitive than the mycelium *of S. parasitica*, and the same trend was observed for zoospores, although less pronounced. The higher sensitivity of mycelium and zoospores of *Aphanomyces* spp. compared to those of *Saprolegnia* spp. has been reported previously. Namely, an absolute ethanol extract of *Cassia fistula* (Fabaceae) inhibited mycelial growth of *S. parasitica* and *S. diclina* at 2000 µg/mL, compared to 500 µg/mL required for the inhibition of *A. invadans*, and similar values were obtained for zoospore germination [26].

The inhibitory effect of the essential oils tested differed markedly between oomycete life cycle stages, with zoospores being 1.2–6.5 times more sensitive than mycelium. For example, essential oils of bay laurel and rosemary showed strong inhibitory activity against zoospore germination of *S. parasitica* but had only a weak effect on mycelial growth. Previous studies comparing the sensitivity of mycelium and zoospores of pathogenic oomycetes to various compounds showed different results [26,27,35,47,48]. For example, the EC_{50} values of propamocarb hydrochloride for the inhibition of mycelial growth were two or more orders of magnitude higher than the EC_{50} values for the germination of zoospores of different isolates of *Phytophthora nicotianae* [47]. In another study, the concentrations of an absolute ethanol extract of *Cassia fistula* required to inhibit zoospore germination of *S. parasitica*, *S. diclina*, and *A. invadans* were similar to the concentrations required to inhibit mycelial growth [26]. Finally, the concentrations of *Laureliopsis philippiana* essential oil from bark and leaf required to inhibit zoospore formation of *S. parasitica* and *S. australis* were equal to or higher than the concentrations required to inhibit mycelial growth [35]. All of these studies, including the results presented here, suggest that some compounds are more potent zoospore inhibitors (such as the essential oils of sage, rosemary, and bay laurel), while others preferentially target the mycelium, or both life stages. This likely reflects differences in the mode of action of different compounds on zoospores and mycelium, as well as different detoxification mechanisms in different life stages of oomycetes, but further studies, including transcriptomic and proteomic analyses, are needed to clarify this.

The observed inhibitory activities of the essential oils could be attributed to their rich content of bioactive volatiles (mainly monoterpenes, such as camphor, and sesquiterpenes) and the synergistic activities of numerous minor compounds. The chemical composition of the tested essential oils was similar to those previously reported by our group [49] and in other studies [50–54]. Sage essential oil showed the strongest inhibitory potential on both pathogens and both life cycle stages. As indicated by PLS-R2 analysis, its anti-oomycete activity could be attributed to some of its major constituents, camphor, α-thujone, veridiflorol, camphene, and α-humulene, which were absent or present at low levels in other essential oils. In addition, β-pinene and 1,8-cineole (present in significant amounts in both sage and bay laurel essential oils) were positively correlated with the inhibition of *S. parasitica* zoospore germination, explaining the similar EC_{50} values of these two essential oils for *S. parasitica* zoospore germination (EC_{50} 0.012 and 0.013 µL/mL, respectively). Some of these compounds were previously reported to exhibit good anti-oomycete [44,55] and antifungal [56–59] activity. For example, camphor (up to 38.06 µg/mL) progressively slowed down the mycelial growth of *S. parasitica* and *S. delica*, while thujone and β-pinene (500 and 1000 µg/mL, respectively) inhibited the mycelial growth of *S. parasitica* [44,55]. Moreover, α-thujone and camphor have potent antifungal activity against *Fusarium graminearum*, *F. culmorum*, and *Schizosaccharomyces pombe*, which is mainly explained by the induction of oxidative stress and subsequent apoptotic cell death, but also by a decrease in genomic stability and epigenetic changes [56–59]. Thus, the high camphor content in sage essential oil probably contributed significantly to the observed inhibitory effects. The mechanism underlying the inhibition of oomycetes by camphor remains to be investigated but could be due to oxidative stress-mediated apoptosis, similar to the data obtained for fungal

cells. However, it should be noted that it has previously been shown that the synergistic effect of many compounds present in essential oils is stronger than that of any single compound [35,44], and this was probably the case here.

Based on the obtained results, we propose that the essential oils of wild Mediterranean plants, especially sage, could be used as an ecologically acceptable method to control *A. astaci* and *S. parasitica* in aquaculture. This will require in vivo experiments to test the applicability of these essential oils either by dietary supplementation or by bathing the eggs and animals in essential oil suspensions. Previous studies suggest that the application of some essential oils and plant extracts may be useful in controlling *Saprolegnia* and *Aphanomyces* spp. infections in eggs and/or adults. For example, repeated incubation of *S. parasitica*-infected rainbow trout eggs with some essential oils, such as those of *Zataria multiflora* and *Satureja cuneifolia* at concentrations of 5 ppm or higher, resulted in an increase in hatching rate [34,36]. In addition, dietary supplementation with various essential oils, including sage essential oil and plant extracts, improved the immune response, fatty acid utilization, and growth performance of rainbow trout [60,61] and even conferred resistance to *A. invadans* infection in Indian major carp (*Labeo rohita*) [62]. In addition, immersion of infected fish in water containing 1% aqueous leaf extract of *Azadirachta indica* for 5 min daily for 24 days resulted in gradual healing of induced lesions in *Chana striata* [63]. However, some of the current constraints to large-scale application of essential oils, such as high extraction costs and variations in the composition of essential oils obtained from plants from different locations and seasons, have yet to be addressed. Production costs could be reduced by including inexpensive pretreatments prior to hydrodistillation that improve essential oils yields [49], while the quality of stock solutions could be standardized by optimizing growing conditions and harvest timing and by using genetic engineering [64,65]. Therefore, further experiments are needed as an extension of the results presented here to open the prospect of developing an ecologically acceptable control of *S. parasitica* and *A. astaci* infections through the application of essential oils. In particular, the possible toxicity of the effective concentrations of essential oils to fish/crayfish, as well as the possible rejection of the essential oil-supplemented feed due to their strong taste and odor [66], must be excluded.

4. Conclusions

The essential oils of Mediterranean wild plants are rich in bioactive volatiles, and in this study we demonstrated their potent activity against the pathogens *A. astaci* and *S. parasitica*. Thus, our results open a perspective for an environmentally friendly and sustainable control of diseases caused by oomycetes in salmonid and crayfish aquaculture through the application of essential oils. The inhibitory effect was particularly pronounced for sage essential oil, and some of its most abundant components, such as camphor, may be the major inhibitory molecules, although a synergistic effect of many minor components is also likely. Following our results, further studies are needed to develop protocols for the administration of essential oils as egg/animal baths or feed supplements and to standardize the production of essential oils on a large scale.

5. Materials and Methods

5.1. Microorganisms

Two oomycete pathogens of freshwater animals used were: *Aphanomyces astaci* (Schikora, 1906) strain B, PsI genotype (isolate PEC 8), and *Saprolegnia parasitica* Coker isolate CBS 223.65. *Aphanomyces astaci* was provided by F. Grandjean (University of Poitiers, Poitiers, France) and belonged to genotype PsI, which has been shown to exhibit marked virulence, particularly against native European crayfish species such as the noble crayfish *Astacus astacus* [67,68]. *Saprolegnia parasitica* CBS 223.65 is a reference strain isolated in the Netherlands from northern pike (*Esox lucius*) and was provided by R. Galuppi (University of Bologna, Bologna, Italy). Microorganisms were maintained in the laboratory at 18 °C on PG1 solid medium supplemented with ampicillin and oxolinic acid [69].

5.2. Plant Material and Essential Oil Isolation

Fresh leaves were collected from wild plants of rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), and bay laurel (*Laurus nobilis*) in the south Mediterranean region of Croatia in August 2020 and air-dried at room temperature (20 ± 2 °C) for one week. Dry leaves were packed in polyethylene bags and kept in a dark, dry, and cool place. Before being used for the extractions, all leaves were separated from branches and stems, and the bay laurel leaves were cut into four pieces each. The plant material was then ground using a household blender (Tefal) for 20 s into fine powder. The essential oils were isolated by hydrodistillation with reflux extraction pretreatment from 20 g of ground plant material mixed with 250 mL of purified water according to a previously developed protocol [49], yielding 0.2, 0.5, and 0.3 mL of oil/g of dry plant material of rosemary, sage, and bay laurel, respectively. For subsequent testing of anti-oomycete activity, the essential oils were diluted 1:9 in 96% ethanol. These 100 µL/mL stock solutions were then further diluted while performing the inhibition assays described in Section 5.4.

5.3. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

GC–MS analyses were carried out with an Agilent Technologies (Palo Alto, CA, USA) gas chromatograph model 7890 A equipped with a mass-selective detector (MSD) model 5975 C (Agilent Technologies, Palo Alto, CA, USA) and an HP-5MS 5% phenyl-methylpolysiloxane capillary column (30 m × 0.25 mm, 0.25 µm film thickness). In brief, the injector and detector temperatures were 250 °C and 300 °C, respectively; the column temperature was held at 70 °C for 2 min, was then increased from 70 to 200 °C at 3 °C/min, and was finally held at 200 °C for 18 min; 1.0 µL of the sample was injected using split mode (split ratio 1:50). Helium was used as a carrier gas (1.0 mL/min). The MSD (EI mode) was operated at 70 eV, and the scan range was set to 30–350 amu. Identification of volatile constituents was based on the comparison of their retention indices (RIs), determined relative to the retention times of a homologous series of *n*-alkanes (C_9–C_{25}), with those reported in the literature, and their mass spectra with those of authentic compounds available in our laboratories or those listed in NIST 17 (D-Gaithersburg, MD, USA) and Wiley W9N08 (Wiley, New York, NY, USA). Relative concentrations of components were calculated by the area normalization method without considering response factors.

5.4. Testing of Anti-Oomycete Activity of Essential Oils

5.4.1. Inhibition of Mycelial Growth

The inhibitory effect of sage, bay laurel, and rosemary essential oils on mycelial growth of *S. parasitica* and *A. astaci* was tested using a radial growth inhibition assay followed by the determination of EC_{50} values (i.e., concentrations of samples at which mycelial growth is inhibited by 50%), as previously described [48], with some modifications. Malachite green, a known oomycete inhibitor [70], was used as a positive control (stock solution was 512 µg/mL in distilled water). Briefly, 100 µL of each sample was dissolved in 10 mL of molten PG1 medium and poured into 550 mm radius Petri dishes. In control plates, solvents were added in place of the samples, i.e., ethanol was used as a negative control for essential oils, and distilled water for malachite green. Up to eight twofold dilutions were tested for each sample, with initial concentrations of 1 µL/mL for essential oils and 1.28 µg/mL for malachite green. Each plate was inoculated by placing a 5 mm agar plug containing mycelium (taken from the edge of an actively growing mycelial mat) in the center and incubated at 18 °C. There was no statistically significant difference in the growth of the test species in the presence of ethanol or distilled water (*t*-test; $p > 0.05$). Three biological replicates were performed for each oomycete species, sample, and concentration. The assay was terminated after the mycelium in the negative control plates reached the end of the Petri dish, i.e., after five days for *A. astaci* and after two days for *S. parasitica*. Next, two perpendicular measurements of mycelial radius were taken for each plate and averaged, and the agar plug size was subtracted to obtain the final measurements of radial

growth. Measurements from the sample plates were subtracted from the negative control measurements and converted to percent inhibition.

5.4.2. Inhibition of Zoospore Germination

Sporulation of *A. astaci* and *S. parasitica* was induced by washing a grown mycelium incubated at 18 °C with sterile stream water, as described in detail in previous studies [67,71]. Zoospores were counted in the Thoma chamber using a light microscope (Zeiss Primo Star, Carl Zeiss, Oberkochen, Germany) at 100× magnification. The final concentration of zoospores was approximately 100,000 zoospores/mL for *A. astaci* and 80,000 zoospores/mL for *S. parasitica*.

To assess the effect of essential oils on the germination of zoospores of *A. astaci* and *S. parasitica*, we used previously described protocols with some modifications [48,71,72]. Up to six twofold dilutions of the test samples were used, with starting concentrations of 0.125 µL/mL for essential oils and 2.54 µg/mL for malachite green. To induce germination of *A. astaci* zoospores, 2 mL of stream water with the addition of $CaCl_2$ (11.1 g/L) was mixed with 20 µL of the essential oil samples and 2 mL of water with zoospores and incubated in 12-well plates at room temperature for 16 h. In the case of *S. parasitica*, zoospores were vortexed for 45 s, then 2 mL of water with zoospore suspension was mixed with 2 mL of fresh PG1 liquid medium and 20 µL of essential oil samples and incubated for 1 h at 18 °C. After incubation, samples were photographed using an inverted microscope (Carl Zeiss, Oberkochen, Germany) at 200× magnification. Three replicates were made for each species, sample, and dilution. The percentage of germinating spores was determined by counting at least 200 spores in three to four randomly selected objective fields. A spore with a germ tube of at least one cyst diameter in length was counted as germinated. The germination percentage in the wells of negative controls to which solvents were added instead of samples was 33.4% and 97.5% for *A. astaci* and *S. parasitica*, respectively, in agreement with previously published data [71–73]. The germination percentages obtained for the different samples were converted to percent germination inhibition values.

5.5. Statistical Analysis

The heatmap of the volatile chemical composition of the essential oils of rosemary, bay laurel, and sage obtained by GC–MS was generated using the heatmaply package in R v. 3.2.0 [74] and the default methods for calculating the distance matrix ("euclidean").

To estimate EC_{50} values for inhibition of mycelial growth and zoospore germination (i.e., concentrations of samples at which mycelial growth/zoospore germination was 50% inhibited), compound concentrations were log-transformed, data normalized, and nonlinear regression with curve fitting (by least squares) was performed using GraphPad Prism version 9.

To investigate the effects of different volatiles on the inhibitory potential of sage, rosemary, and bay laurel essential oils against *A. astaci* and *S. parasitica*, the partial least-squares regression (PLS-R2) approach was used. The response variables were the EC_{50} values for mycelial growth and zoospore germination, while the volatile bioactive compounds detected in the essential oils with at least 2% of the total GC–MS peak area served as predictors. PLS-R2 analysis was performed using the package "plsdepot" [75] in the program R v. 3.2.0 [74].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10081676/s1, Table S1: Volatile composition of essential oils determined by GC–MS.

Author Contributions: Conceptualization, A.B.; methodology, A.M., M.D., D.G., D.P., I.J., Z.M. and A.B.; validation, A.M., A.B. and I.J.; formal analysis, D.G.; investigation, A.M., D.P., M.D., Z.M. and I.J.; resources, A.B.; writing—original draft preparation, A.M.; writing—review and editing, A.B.; visualization, A.M. and D.G.; supervision, A.B.; project administration, A.B. and D.G.; funding acquisition, A.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the project "Interactions of freshwater pathogenic oomycetes and the environment" (InteractOomyc, UIP-2017-05-6267), Croatian Science Foundation.

Acknowledgments: The authors are thankful to F. Grandjean and R. Galuppi for providing the *A. astaci* and *S. parasitica* isolates and to Ivana Čakarić and Ana Kozina for help in the laboratory work.

Conflicts of Interest: The authors declare no conflict of interest.

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2.3. Bioactive compounds in fluid propolis preparations inhibit different life stages of pathogenic oomycetes *Aphanomyces astaci* and *Saprolegnia parasitica*

Aquaculture 552 (2022) 737982

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Bioactive compounds in fluid propolis preparations inhibit different life stages of pathogenic oomycetes *Aphanomyces astaci* and *Saprolegnia parasitica*

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ARTICLE INFO

Keywords:
Antioomycetic potential
Molecular docking
Mycelial growth
Propolis
Zoospore germination
Zoospore motility

ABSTRACT

The pathogenic oomycetes *Saprolegnia parasitica*, causative agent of saprolegniosis in salmonid fish, and *Aphanomyces astaci*, causative agent of crayfish plague, have a negative impact on freshwater aquaculture. As they are controlled worldwide with chemicals that are harmful to humans and the environment, there is a growing need to replace them with ecologically acceptable alternatives. Propolis has documented antimicrobial activity as well as positive effects on the immune response, growth performance and/or reproductive capacity of fish and crayfish. Therefore, we aimed to investigate the possible inhibition of *S. parasitica* and *A. astaci* by fluid propolis formulations. Chemical analysis showed that the propolis formulations were rich in volatile and non-volatile phenolic bioactive components (chrysin was the dominant flavone and pinocembrin the dominant flavanone). Overall, the fluid propolis formulations showed good *in vitro* inhibition of mycelial growth and zoospores of both pathogens, but we found differences in sensitivity depending on species and life cycle stage. Mycelial growth was more sensitive for *A. astaci*, with EC_{50} values (the samples concentrations causing 50% inhibition) up to 8.59 μg/mL, compared to 206.60 μg/mL for *S. parasitica*. Zoospore motility was more affected in *S. parasitica*, where MIC (minimum inhibitory concentration) values were up to 61.88 μg/mL, compared to 154.68 μg/mL for *A. astaci*. Zoospore germination of both pathogens was similarly sensitive to the fluid propolis preparations, with EC_{50} values of up to 19.52 μg/mL for *A. astaci* and up to 23.62 μg/mL for *S. parasitica*. In addition, molecular docking was used to analyze the binding of selected propolis components to oomycete proteins suggested to play a role in pathogenesis. Apigenin, chrysin and pinocembrin were predicted to bind strongly to the endochitinase of *A. astaci*, which is mainly expressed in the mycelium, and to the thrombospondin of *S. parasitica*, mainly expressed in the cysts, which is consistent with the results of the *in vitro* inhibition experiments. Overall, our results suggest that propolis could be used in salmonid and crayfish aquaculture not only as an immunostimulant but also as an antioomycetic agent.

1. Introduction

Oomycetes are fungal-like microorganisms that include many pathogens (Beakes et al., 2012; Diéguez-Uribeondo et al., 2009) that cause devastating diseases in freshwater aquaculture and natural environment (Iberahim et al., 2018; Phillips et al., 2008). In particular, the number of disease outbreaks caused by oomycetes in aquaculture is increasing due to massive production that is stressful for the animals and thus favours disease development (Van den Berg et al., 2013). Species of the genus *Saprolegnia*, such as *Saprolegnia parasitica*, cause saprolegniosis in

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https://doi.org/10.1016/j.aquaculture.2022.737982
Received 14 November 2021; Received in revised form 13 January 2022; Accepted 26 January 2022
Available online 31 January 2022

numerous freshwater fish species, affecting eggs, juveniles and adults. Saprolegniosis causes significant economic losses in salmonid hatcheries and farms worldwide (Van den Berg et al., 2013). In addition, *Aphanomyces astaci*, the causative agent of crayfish plague, is an important oomycete pathogen for crayfish aquaculture. Currently, all freshwater crayfish are considered to be susceptible to *A. astaci*, albeit with varying sensitivity depending on the species and individual immunity, and crayfish plague is listed among notifiable animal diseases by the World Organisation for Animal Health (OIE, 2019). Although the negative effects of *A. astaci* in natural ecosystems are much better understood, the damage caused by this pathogen in astaciculture should be of concern, especially in the production of native European crayfish species, which are highly susceptible to infection by *A. astaci* (Becking et al., 2015; Harlioğlu, 2008; Holdich, 1993; Souty-Grosset and Reynolds, 2009).

Despite these harmful effects, current methods to control oomycete infections in freshwater farming facilities are inadequate and unsustainable due to the lack of specific treatments and the fact that existing protocols use toxic chemicals that are harmful to human and/or animal health (Tedesco et al., 2019). For example, although this compound is highly effective in controlling *Saprolegnia* and *Aphanomyces* species (Alderman and Jane, 1984; Willoughby and Roberts, 1992), the use of malachite green is not approved in the EU (EC, 1990; EFSA, 2016) or the USA (Marking et al., 1994) due to its teratogenic and carcinogenic properties (Meyer and Jorgenson, 1983; Panandiker et al., 1992; Srivastava et al., 2004). However, the chemicals currently used, such as formalin, bronopol and copper sulphate, also have significant toxicity and are therefore not sustainable in the long term. Formalin, for example, is dangerous to fish farm personnel who apply it in therapeutic concentrations (Wooster et al., 2005), but also to consumers of the fish meat that contains its residues (Norliana et al., 2009). Bronopol, copper sulphate and peracetic acid, although considered less potent pollutants, have been shown to have toxic effects on aquatic biota (Cui et al., 2011; Jacob et al., 2016; Jussila et al., 2011).

Therefore, there is a growing need to replace harmful chemical agents used to control oomycetes in freshwater aquaculture with environmentally friendly treatments. In this context, propolis, a honey bee product, is rich in bioactive compounds that form the basis of its beneficial properties (Gülçin et al., 2010; Mašek et al., 2018; Mitsui et al., 2018; Veloz et al., 2016). It can improve the immune response and enhance the reproductive performance of host animals such as fish and crayfish (Abdelmagid et al., 2021; Fuat Gulhan and Selamoglu, 2016; Mişe Yonar et al., 2017; Talas and Fuat Gulhan, 2009). At the same time, propolis shows strong inhibitory activity against a range of microbes (Petruzzi et al., 2020), from bacteria (Tukmechi et al., 2010) to fungi (Ota et al., 2001; Ramón-Sierra et al., 2019; Siqueira et al., 2015) and viruses (Yildirim et al., 2016), but its antioomycetic potential has not yet been sufficiently explored. Previous studies have demonstrated the inhibitory effect of propolis on mycelial growth of plant pathogenic oomycetes of the genus *Phytophthora* (Silva-Castro et al., 2018; Yusuf et al., 2005) and on mycelial growth and zoospores of animal pathogens of the genera *Aphanomyces* (*A. invadans*) (Campbell et al., 2001) and *Pythium* (*P. insidiosum*) (Araújo et al., 2016).

Our aim was therefore to test for the first time the effect of two propolis formulations on oomycete pathogens *A. astaci* and *S. parasitica*: P1 – a fluid formulation of pure propolis, and P2 - a fluid propolis formulation with addition of sage and peppermint, since we have previously demonstrated an inhibitory effect of the plants from Lamiaceae family towards these pathogens (Miljanović et al., 2021). We analyzed the phenolic and volatile composition of fluid propolis preparations and then demonstrated the *in vitro* sensitivity of alternative stages of the oomycete life cycle, mycelium and zoospores (as infectious stage) to fluid propolis preparations. In addition, we tested the binding of selected propolis components to target proteins of *A. astaci* and *S. parasitica* by molecular docking. This indispensable *in silico* tool for drug development has recently been used to predict potentially inhibitory molecules of oomycete virulence effector proteins (Kumar et al., 2020; Bhat et al., 2020; Shah et al., 2021; Tandel et al., 2021).

2. Materials and methods

2.1. Fluid propolis formulations

Two different fluid propolis formulations (PIP Ltd., Croatia) were used: P1 with 200,000 μg of propolis dry mass/mL, and P2 with 250,000 μg of propolis dry mass/mL with the addition of sage (*Salvia officinalis*) and peppermint (*Mentha piperita*) extracts (250,000 and 190,000 μg/mL, respectively). Propolis was dissolved in 96% ethanol. Malachite green oxalate (Kemika, Croatia) served as a positive control.

2.2. Microorganisms

Two oomycete pathogens of freshwater animals were used: *Aphanomyces astaci* Schikora, 1906, strain B, Psl – genotype (isolate PEC 8) (provided by F. Grandjean, University of Poitiers, France), and *Saprolegnia parasitica* Coker, CBS 223.65 (provided by R. Galuppi, University of Bologna, Italy). Microorganisms were maintained in the laboratory at 18 °C on PG1 solid medium supplemented with ampicillin and oxolinic acid (Unestam, 1965).

2.3. Chemical analyses of fluid propolis formulations by mass spectrometry

The phenolic composition of fluid propolis formulations was determined by UPLC-MS^2. The UPLC analyses were performed on an Agilent 1290 RRLC instrument (Agilent Technologies, CA, USA) coupled to a binary gradient pump and thermostated column compartment. For chromatographic separation Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 μm) (Agilent, CA, USA) was used. The gradient conditions were reported previously (Elez Garofulić et al., 2018). Mass spectrometry experiments were performed using a triple quadrupole mass spectrometer (QQQ 6430, Agilent, CA, USA). The mass spectrometer was used in the electrospray (ESI)-positive and negative mode and operated with the following source parameters: capillary voltage, +4000/−3500 V, nitrogen drying gas temperature maintained at 300 °C with a flow rate of 11 L/h and nebulizer pressure was set at 40 psi. Data were collected in the dynamic multiple reactions monitoring (dMRM) mode and Masshunter software was used for data acquisition and analysis. All measurements were performed in duplicate. An external standard calibration methodology was applied and calibration curves were obtained by injection of eight known concentrations of the authentic phenolic standards, i.e. kaempferol-3-rutinoside, quercetin-3-glucoside, galangin, naringenin, pinocembrin, ferulic acid, caffeic acid, *p*-coumaric acid, gallic acid, cinnamic acid and vanillic acid (Sigma-Aldrich, Germany), apigenin, luteolin (Extra synthese, France) and rutin (Acros Organics, Thermo Fisher Scientific, Belgium), prepared by consecutive dilutions from a stock methanol solution. Identification of the phenolic compounds was carried out by comparing retention times and mass spectra with those of authentic standards. In case of unavailability of standards, the identification was carried out by comparing the obtained mass fragments with the previously reported mass fragmentation patterns (Pellati et al., 2011; Ristivojević et al., 2015a). Quantitative data for all phenolic compounds were obtained by using calibration curves of authentic standards and if authentic standard was not available, concentration estimates were made using the calibration curve of standards from the same phenolic group.

The composition of volatile compounds in propolis formulations was analyzed by gas chromatography mass spectrometry (GC–MS), preceded by headspace Solid-Phase Microextraction (HS-SPME), following the previously published protocol (Jerković et al., 2016). Shortly, HS-SPME was performed with a manual SPME holder using two fibers covered with Polydimethylsiloxane/Divinylbenzene (PDMS/DVB), Carboxen/PDMS (CAR/PDMS) and DVB/CAR/PDMS obtained from Supelco Co.,

USA. For HS-SPME, fluid propolis formulations (1 mL) were placed in 10 mL glass vials and hermetically sealed. The vials were maintained at 60 °C during the equilibration (15 min) and extraction (45 min). Thereafter, the SPME fiber was withdrawn and inserted into the GC–MS injector (250 °C) for 6 min for thermal desorption. HS-SPME was done in duplicate and the results are presented as mean values. GC–MS analyses were done on an Agilent Technologies (USA) gas chromatograph model 7890A equipped with a mass spectrometer (MSD) model 5977E (USA) and HP-5MS capillary column (5% phenyl-methylpolysiloxane, Agilent J and W). The oven temperature was set at 70 °C for 2 min, then increased from 70 to 200 °C (3 °C/min) and held at 200 °C for 15 min; the carrier gas was helium (1.0 mL/min). The compounds identification was based on the comparison of their retention indices (RI), determined relatively to the retention times of n-alkanes (C_9–C_{25}), with those reported in the literature (El-Sayed, 2012) and those from Wiley 9 (Wiley, NY, USA) and NIST 17 (d-Gaithersburg) mass spectral libraries. The percentage composition of the samples was computed from the GC peak areas using the normalization method (without correction factors).

2.4. Analyses of antioomycetic activity of fluid propolis formulations

The inhibitory effect of fluid propolis formulations, chrysin and pinocembrin (as dominant compounds in fluid propolis formulations) and malachite green, a known inhibitor of oomycetes (Zahran and Noga, 2010), on mycelial growth of *S. parasitica* and *A. astaci* was tested. Propolis and pinocembrin were dissolved in ethanol, malachite green in distilled water, and chrysin in 0.1 M NaOH. Within all *in vitro* assays, three biological replicates (from independently grown cultures) were performed for each oomycete species and each concentration of each sample.

2.4.1. Inhibition of mycelial growth

For the disk diffusion assay (Fig. 1a), the samples were tested at initial concentrations of 200,000 μg/mL for P1, 250,000 μg/mL for P2, 2048 μg/mL for chrysin, 512 μg/mL for pinocembrin and malachite green and diluted 5×, 10× and 15×. Five microliters of each sample were added to sterile 6 mm filter disks (Whatman paper no. 1). After drying, four filter disks containing the same sample at different concentrations were placed at regular intervals around the edges of the PG1 plate, together with a negative control disk containing the appropriate solvent. Each plate was then inoculated by placing a 5 mm agar plug with mycelium (taken from the leading edge of an actively growing mycelial mat) in the center and incubated at 18 °C (Innova® 42 incubator, Eppendorf, Germany). Assay was stopped and the radial growth was measured after nine days (*A. astaci*) and four days (*S. parasitica*) (since this was the time it took mycelium to reach the edge of the plate in negative control experiments). The growth of the mycelium in the presence of the test samples (*i.e.* mycelial radius, marked with white arrows in Fig. 1a) was compared to the growth in the presence of the respective solvents and the inhibitory effect of each sample/concentration was calculated as the percentage inhibition of mycelial growth compared to the solvent control. There was no statistically significant difference in growth between the test species in the presence of ethanol, distilled water or 0.1 M NaOH (*t*-test; $p > 0.05$).

Radial growth inhibition was further assessed by dissolving 25 μL of the samples in 10 mL of PG1 medium and inoculating such plates with agar plugs overgrown with mycelium, as described in detail in Miljanović et al. (2021) and shown in Fig. 1b. For each sample, up to 10 twofold dilutions were tested in 50 mm radius Petri dishes, with initial concentrations of 2500.0 μg/mL for fluid propolis preparations, 5.12 μg/mL for chrysin and 1.28 μg/mL for pinocembrin and malachite green. The control plates with solvents instead of samples were also analyzed and there was no statistically significant difference in the growth of the test species in the presence of different solvents (*t*-test; $p > 0.05$). Details on the measurements and calculations of the inhibition percentages can be found in Miljanović et al. (2021).

2.4.2. Inhibition of zoospore germination and motility

Sporulation of *A. astaci* and *S. parasitica* was induced by washing a grown mycelium with sterile stream water at 18 °C (Diéguez-Uribeondo et al., 1994; Makkonen et al., 2012). Zoospores were counted in the Thoma chamber using a light microscope (Zeiss Primo Star, China) at 100× magnification. The final concentration of zoospores was about 100,000 zoospores/mL for *A. astaci* and 80,000 zoospores/mL for *S. parasitica*. The effect of fluid propolis formulations and selected pure compounds on *A. astaci* and *S. parasitica* zoospore germination and motility was then investigated. Up to six twofold dilutions were tested for each sample, with initial concentrations of 1244.00 μg/mL for fluid propolis preparations, 10.18 μg/mL for chrysin and 2.54 μg/mL for pinocembrin and malachite green.

Zoospore germination was tested in 12-well plates using the previously described protocols with modifications (Diéguez-Uribeondo et al., 1994; Lawrence et al., 2018; Sensson and Unestam, 1975). To induce germination of *A. astaci* zoospores, 2 mL of stream water with the addition of $CaCl_2$ (11.1 g/L) was mixed with 20 μL of the samples (or solvent for negative controls) and 2 mL of water with zoospores and

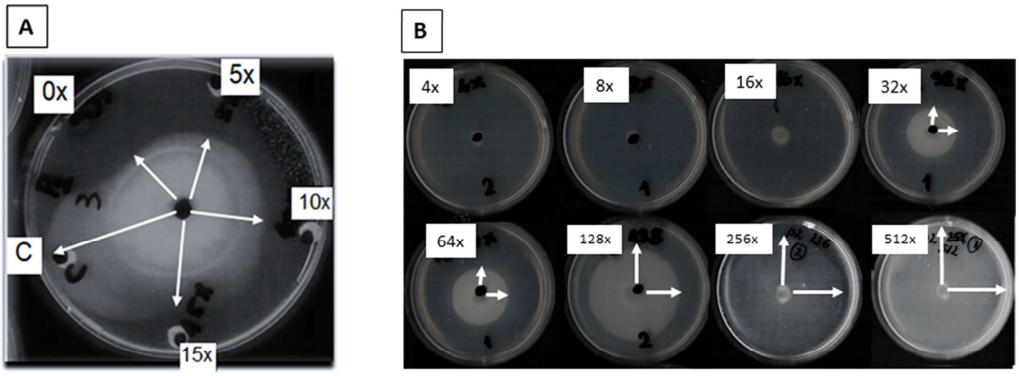

Fig. 1. *In vitro* testing of the inhibitory effect of P2 fluid propolis formulation towards the mycelial growth of *Aphanomyces astaci*. Mycelium radius, measured for each sample, is marked with white arrows.
(A) Disk diffusion assay. C – control (ethanol); 0× - undiluted P2 sample (250,000 μg/mL), followed by 5×, 10× and 15× diluted P2.
(B) Radial growth inhibition assay. The initial P2 concentration in the plate was 625 μg/mL, and 4× (100% inhibition), 8× (100% inhibition), 16× (100% inhibition), 32×, 64×, 128×, 256×, and 512× (no inhibition) dilutions were tested.

incubated for 16 h at room temperature, followed by photography with an inverted microscope (Carl Zeiss, Germany) at 200× magnification. In the case of *S. parasitica*, zoospores were vortexed for 45 s and then 2 mL of water containing zoospore suspension was mixed with 2 mL of fresh PG1 liquid medium and 20 μL of the samples. After 1 h incubation at 18 °C, the germinating cysts were photographed with an inverted microscope. Inhibition of zoospore germination was assessed as previously described (Miljanović et al., 2021).

Zoospore motility assays were performed in 12-well plates as previously described (Hu et al., 2007; Lawrence et al., 2018). Briefly, two milliliters of zoospore suspension (diluted to approximately 10,000 zoospores/mL) were added to triplicate wells containing 20 μL of the samples at different concentrations, while an equal volume of appropriate solvent was added to the control wells. Zoospores were observed with the inverted microscope and the minimal inhibitory concentration (MIC) for zoospore motility was defined as the lowest concentration of the test samples that completely inhibited motility after 5 min at 18 °C.

2.5. Molecular docking of phytochemicals present in propolis with differentially expressing proteins of A. astaci and S. parasitica

To predict the possible molecular mechanisms of oomycete inhibition by propolis, molecular docking of selected propolis components (chrysin, apigenin, pinocembrin and cinnamic acid) with selected oomycete proteins was performed. The target proteins of *A. astaci* and *S. parasitica* were selected based on the available literature: endochitinase (Hochwimmer et al., 2009) and trypsin proteinase (Bangyeekhun et al., 2001) from the mycelium of *A. astaci*, the lamin-like protein, thrombospondin and the host targeting protein-1 (htp-1) expressed in *S. parasitica* cysts, and V-type proton ATPase from *S. parasitica* mycelium (Andersson and Cerenius, 2002; Bangyeekhun et al., 2001; Hayek et al., 2014; Srivastava et al., 2018; Wawra et al., 2012; Van West et al., 2010).

The homology modelling of the V-type proton ATPase, thrombospondin and lamin-like proteins of *S. parasitica* and trypsin proteinase and endochitinase proteins of *A. astaci* were performed using the multiple threading approach of the I-Tasser server (Yang and Zhang, 2015). In the case of *S. parasitica* host targeting protein-1, a template was searched by using the fold-based method pDOMTHREADER as described in our previous study (Tandel et al., 2021). The better-scored template identified by pDOMTHREADER was taken for homology modelling using Modeller 9.18 standalone version (Šali and Tom, 1993). The modelled 3D structures were refined using ModRefiner (https://zhanglab.ccmb.med.umich.edu/ModRefiner/). The SAVES server was used to check the quality of the modelled tertiary structures (SAVESv6.0 - Structure Validation Server (ucla.edu)). The phytochemicals were retrieved from Pubchem in SDF format: chrysin (Pubchem CID: 5281607), apigenin (Pubchem CID: 5280443), pinocembrin (Pubchem CID: 68071) and cinnamic acid (Pubchem CID: 444539). The binding sites of each protein were predicted by the COACH meta server (Yang et al., 2013). The search grid parameters of *S. parasitica* and *A. astaci* proteins are listed in Table 2.

The AutoDockVina software was used to simulate the ligand into the active site of the protein to calculate the binding energy of the ligand-receptor complexes (Trott and Olson, 2009). The exhaustiveness was set to twenty, and the software predicted nine docking poses for each ligand-receptor complex. The desirable binding pose between protein and ligand, with more negative docking score, were taken to study hydrogen bonding and hydrophobic interactions (Bhat et al., 2021). The three-dimensional (3D) structures and docking were visualized in PyMOL, and two dimensional (2D) were visualized in LigPlot 2.1 (Laskowski and Swindells, 2011).

2.6. Statistical analyses

The heatmaps of the chemical composition of fluid propolis formulations were generated in R v. 3.2.0 (R Development Core Team, 2017) using the heatmaply package and the default methods for calculating the distance matrix ("euclidean"). The percentage of the total peak area of each compound was used for GC–MS data, and the concentrations in μg/mL for UPLC-MS^2 data.

In order to examine the differences in the inhibition of mycelial growth between P1 and P2 fluid propolis formulations, differences between concentrations within P1 and P2, and differences between oomycete species (data followed a normal data distribution according to Shapiro–Wilk's test), independent *t*-test was performed. The significance cut-off value was set at $p = 0.05$.

To estimate EC_{50} values for inhibition of mycelial growth and zoospore germination (*i.e.*, concentrations of samples at which mycelial growth/zoospore germination was 50% inhibited), compound concentrations were log-transformed, data normalized, and nonlinear regression with curve fitting (by least squares) was performed using GraphPad Prism version 9.

3. Results

3.1. Fluid propolis formulations are rich in phenolic and volatile compounds

The polyphenolic and volatile compounds of the ethanolic propolis formulations were analyzed by UPLC-MS^2 and GC–MS, respectively. A total of 23 components were identified and quantified in both samples by UPLC-MS^2 (Fig. 2A, Supplementary Table S1). Chrysin was the most abundant flavone in both fluid propolis formulations (734 μg/mL in P1 and 838 μg/mL in P2), followed by apigenin (183 and 120 μg/mL, respectively) and luteolin (30 and 19 μg/mL, respectively). Other major constituents were: pinocembrin, as the dominant flavanone (35 and 16 μg/mL, respectively); cinnamic acid (30 and 58 μg/mL, respectively), isoferulic acid (25 and 50 μg/mL, respectively), ferulic acid (23 and 40 μg/mL, respectively) and *p*-coumaric acid (27 and 43 μg/mL, respectively) belonging to the group of phenolic acids and derivatives; galangin as the dominant flavonol (7 μg/mL in both samples) and vanillin (39 and 22 μg/mL, respectively), among other compounds. Regarding the profile of volatile components, 19 compounds were identified in P1 and 56 in P2, as shown in Fig. 2B and Supplementary Table S2. The major components in P1 were *cis*-calamenene (7%) among sesquiterpene hydrocarbons, α-eudesmol (5%) and β-eudesmol (5%) among oxygenated sesquiterpenes, and ethyl dodecanoate (19%) and benzoic acid (13%) among other compounds. In P2 the main compounds were *cis*-calamenene (8%) and δ-cadinene (5%) among sesquiterpene hydrocarbons as well as β-thujone (7%), α-thujone (5%) and 1,8-cineole (5%) among oxygenated monoterpenes and ethyl dodecanoate (8%) among other compounds.

3.2. Fluid propolis formulations inhibits mycelial growth of Aphanomyces astaci and Saprolegnia parasitica

Overall, the results of the disk diffusion assay (Fig. 3) and determined EC_{50} values (Supplementary Fig. S1, Table 1) showed that P1 and P2 inhibited the mycelial growth of *A. astaci* and *S. parasitica*.

The maximum inhibition of mycelial growth obtained by the application of undiluted fluid propolis formulations in the disk diffusion assay was 48% and 33% for P1 (200,000 μg propolis dry weight/mL) for *A. astaci* and *S. parasitica*, respectively, while for P2 (250,000 μg propolis dry weight/mL) it was 40% and 37% for *A. astaci* and *S. parasitica*, respectively (Fig. 3). In comparison, 512 μg/mL of malachite green was required to achieve similar inhibition percentages for *A. astaci* and *S. parasitica* mycelia (53% and 29%, respectively). Tested propolis constituents, chrysin (up to 2048 μg/mL) and pinocembrin (up to 512 μg/mL), showed no inhibition (data not shown), although the applied concentrations were higher than in undiluted propolis stock solutions. No significant overall difference in the inhibition was observed between P1 and P2 (*t*-test, $p > 0.05$) or between oomycete species (*t*-test, $p >$

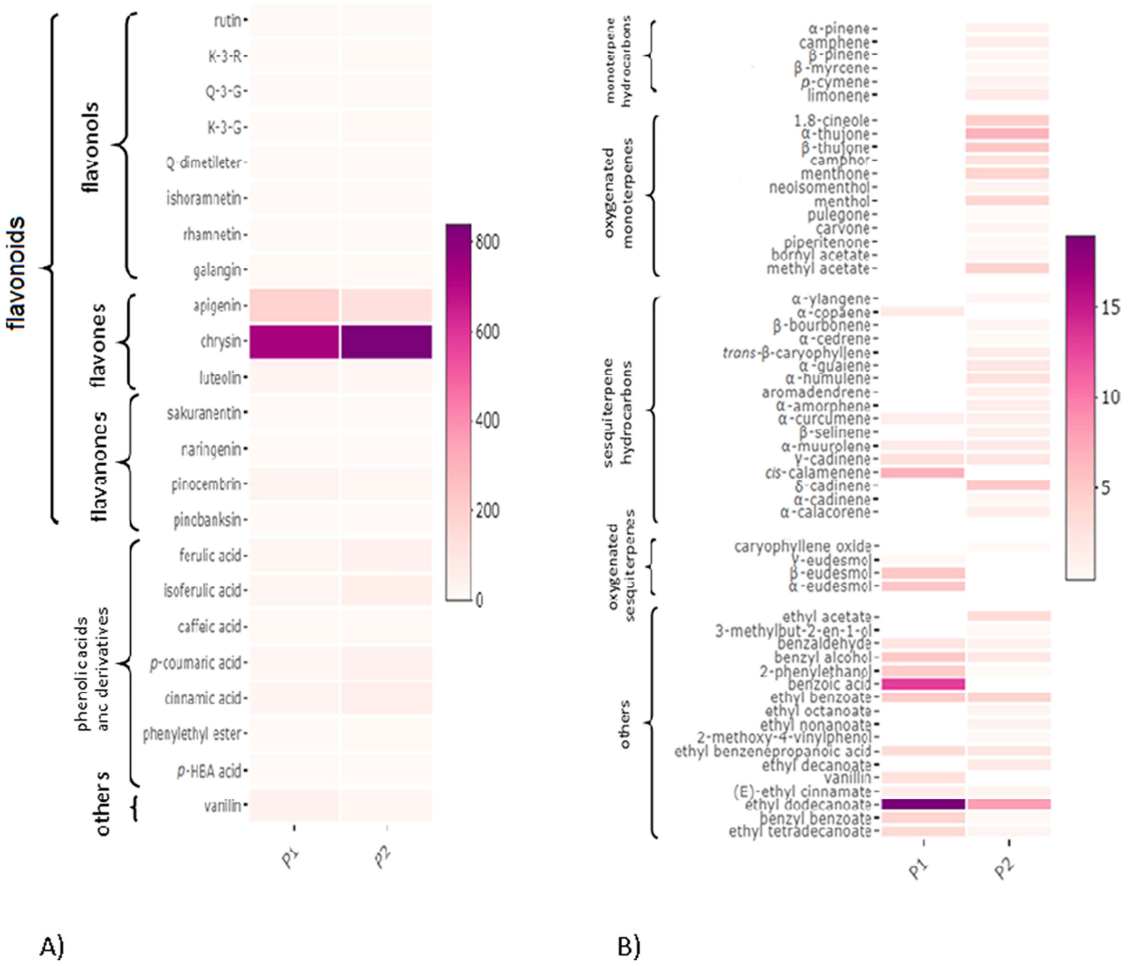

Fig. 2. Heatmaps of the (A) polyphenol (μg/mL) and (B) volatile (% of the total peak area) chemical composition of fluid propolis formulation P1 and P2, as determined by UPLC-MS^2 and GC–MS, respectively.

0.05), although *A. astaci* was significantly more sensitive to 40,000 μg/mL P1 and to malachite green in concentrations of 102 μg/mL and 512 μg/mL (*t*-test, $p < 0.05$, Fig. 3).

Further, within species EC_{50} values for P1 and P2 were also similar (206.20 and 206.60 μg/mL for *S. parasitica*, and 5.58 and 8.59 μg/mL for *A. astaci*; Table 1, Supplementary Fig. S1), although 2–3 orders of magnitude higher than those for malachite green, a potent oomycete inhibitor but highly toxic to humans (Zahran and Noga, 2010). Chrysin, one of the dominant propolis constituents, impaired mycelial growth of *A. astaci* with an EC_{50} value of 2.59 μg/mL (corresponding to chrysin concentration in approximately 300-fold diluted fluid propolis formulations), while its effect on mycelial growth of *S. parasitica* was somewhat weaker and the EC_{50} value could not be determined as the highest concentration tested (5.12 μg/mL) caused only partial growth inhibition (data not shown). Pinocembrin showed no inhibitory potential at the concentrations tested (up to 1.28 μg/mL), corresponding to approximately 30-fold and 10-fold dilutions of P1 and P2, respectively (data not shown). When comparing two pathogens, the mycelium of *A. astaci* was significantly more sensitive to the propolis formulations compared to *S. parasitica*, as evidenced by EC_{50} values that were two orders of magnitude lower (Table 1, Supplementary Fig. S1).

3.3. Fluid propolis formulations inhibit the germination and motility of *Aphanomyces astaci* and *Saprolegnia parasitica* zoospores

Both fluid propolis formulations showed similar efficacy in inhibiting the germination of *A. astaci* and *S. parasitica* zoospores, with EC_{50} values of 15.06 and 23.62 μg/mL for P1 and 19.52 and 19.01 μg/mL for P2, respectively (Table 1, Supplementary Fig. S2). The results of the motility assay showed that the effect of fluid propolis formulations on *S. parasitica* zoospore motility was similar to the effect on germination (MIC values of 61.88 and 38.67 μg/mL for P1 and P2, respectively), while the motility of *A. astaci* zoospores was less affected (MIC values of 123.76 and 154.68 μg/mL, Table 1). Malachite green was a stronger inhibitor than propolis formulations, with EC_{50} and MIC values several hundred times lower, but the same trend was observed, *i.e.* zoospore germination was equally sensitive in both species, while zoospore motility was less affected in *A. astaci*.

The propolis components chrysin and pinocembrin showed limited inhibition of oomycete zoospores, although chrysin caused somewhat more pronounced inhibition effect. It caused a 50% inhibition of *S. parasitica* zoospore germination at 3.10 μg/mL (= EC_{50} value), which corresponds to its concentration in about 250-fold diluted fluid propolis

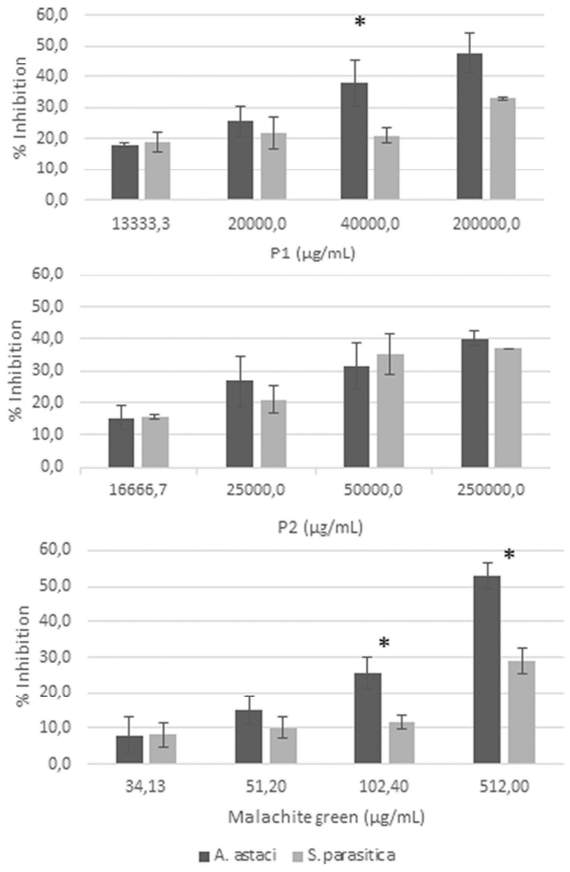

Fig. 3. Inhibition of mycelial growth of *Saprolegnia parasitica* and *Aphanomyces astaci*, as determined by disk diffusion assay. Significant differences ($p < 0.05$) between *S. parasitica* and *A. astaci* mycelial growth at the same concentration are marked by asterisk.

formulations. In comparison, tested chrysin concentrations up to 10.18 μg/mL caused only partial inhibition of *A. astaci* zoospore germination (Supplementary Fig. S3), and therefore EC_{50} value was not calculated. Furthermore, MIC for chrysin-induced inhibition of *A. astaci* zoospore motility was 10.14 μg/mL (corresponding to its concentration in 70–80-fold diluted fluid propolis formulations), while *S. parasitica* zoospore motility was less affected (chrysin concentrations up to 20.28 μg/mL caused only partial inhibition of zoospore motility, data not shown). Finally, pinocembrin caused partial inhibition (34.73%) of *S. parasitica* zoospore germination only at the highest concentration tested (2.54 μg/mL, which corresponds to its's concentration in about 10-fold diluted fluid propolis formulations), while no inhibition was observed for zoospore germination of *A. astaci* (Supplementary Fig. S4), or zoospore motility of both pathogens (data not shown).

Importantly, the concentrations of the tested samples required for the inhibition of *S. parasitica* zoospore motility and germination were lower than those required for mycelial growth, which contrasts with *A. astaci*, where the concentration of the tested components required for inhibition of zoospore germination and motility was higher than the inhibition of mycelial growth.

3.4. Propolis constituents bind the selected oomycete proteins

The phytochemicals from propolis analyzed by molecular docking were chrysin and apigenin (the two most abundant flavones), pinocembrin (the dominant flavanone) and cinnamic acid (the dominant phenolic acid). Ligands stimulated into the active sites of the structural and virulent proteins of *S. parasitica* and *A. astaci* examined by molecular docking showed the presence of hydrogen bonding and hydrophobic interactions, following Lipinski's "rule of five" (Lipinski, 2004). The detailed interactions between the active sites of the oomycete proteins and the ligands are shown in Table 2 and in Supplementary Figs. S5 - S10.

Among the selected proteins, the phytochemicals contained in propolis were found to interact strongly with endochitinase and trypsin proteinase, which are expressed in the mycelium of *A. astaci*. Of the studied phytochemicals, apigenin was predicted to bind most strongly with endochitinase through two hydrogen bonds and eight hydrophobic interactions, obeying Lipinski's "rule of five" with the lowest binding energy of −7.9 Kcal/mol. Similarly, it also showed good interaction with the *A. astaci* trypsin proteinase through one hydrogen bond and nine hydrophobic interactions, obeying Lipinski's "rule of five" with a binding energy of −6.8 Kcal/mol. Strong interactions with *A. astaci* proteins were also predicted for the other ligands, especially for chrysin and pinocembrin with endochitinase (lowest binding energy of −7.5 and − 7.4 Kcal/mol, respectively). The thrombospondin and lamin-like proteins expressing in the cyst stage of *S. parasitica* also bind strongly to the phytochemicals of propolis. Particularly strong binding was predicted between *S. parasitica* thrombospondin and chrysin, pinocembrin and apigenin, *i.e.* two hydrogen bonds; 11, 6 and 8 hydrophobic interactions; binding energy of −7.9, −7.9 and −7.8 Kcal/mol, respectively. In addition, *S. parasitica* htp-1 with a pronounced expression at the cyst stage also showed good binding affinity with the phytochemicals present in propolis, especially apigenin, through four hydrogen bonds and a binding energy of 5.6 Kcal/mol. In comparison, the binding of the ligands to the V-type proton ATPase expressed in the mycelium of *S. parasitica* was weaker, with a predicted binding energy of ≥ −5.1 Kcal/mol.

4. Discussion

We have demonstrated for the first time the inhibitory potential of fluid propolis formulations rich in bioactive components against the life stages of two oomycete pathogens important for aquaculture,

Table 1
EC_{50} values for radial mycelial growth inhibition and zoospore germination, and minimal inhibitory concentrations (MICs) required to cause complete inhibition of zoospore motility. The values are given in μg/mL. Malachite green (MG) was used as positive control.

Sample	EC_{50} for mycelium growth		EC_{50} for zoospore germination		MIC for zoospore motility	
	A. astaci	*S. parasitica*	*A. astaci*	*S. parasitica*	*A. astaci*	*S. parasitica*
P1	5.58	206.20	15.06	23.62	123.76	61.88
P2	8.59	206.60	19.52	19.01	154.68	38.67
chrysin	2.59	>5.12*	>10.18*	3.10	10.14	>20.28*
pinocembrin	>1.28*	>1.28*	>2.54*	>2.54*	>5.07*	>5.07*
MG	0.02	0.12	0.02	0.03	0.64	0.08

* Highest concentration tested, that didn't cause complete inhibition, thus EC_{50}/MIC values weren't calculated.

Table 2
Detailed interactions between the *Aphanomyces astaci* and *Saprolegnia parasitica* proteins and phytochemicals from propolis.

Oomycete stage	Protein with GenBank Accession no.	Ligands	Residues involved in hydrophobic interactions	Residues of involved in hydrogen bonding and bond length (Å)	Total no. of hydrogen bonds	Docking energy (kcal/mol)	Search grid dimensions (Å)
A. astaci mycelium	Endochitinase (ACJ04636)	Chrysin	Leu23, Phe398, Lys22, Thr447, Asn369, Lys366, Val19, Glu19, Thr471	Pro456: 2.76	1	−7.5	center_x = 79.7398776602 center_y = 82.7063876532 center_z = 77.9239 size_x = 78.1709570826 size_y = 55.9133064671 size_z = 84.9799964905
		Cinnamic acid	Leu23, Ly22, Glu19, Lys366, Gly450 Pro456, Phe398, Thr447	Nil	Nil	−5.5	
		Pinocembrin	Thr447, Glu19, Lys22, Lys366, Thr471,Asn369, Phe398, Leu23	Nil	Nil	−7.4	
		Apigenin	Pro469, Asn369, Thr471, Glu19, Leu23, Thr447, Lys366, Pro456	Lys22: 2.95 Phe398: 2.7	2	−7.9	
	Trypsin proteinase (AAK39097)	Chrysin	Thr64, Asn204, Ser207, Phe65, Trp153	Gly205: 3.10 Ser226: 3.24 His81: 2.94	3	−6.4	center_x = 58.6721495884 center_y = 58.8418602856 center_z = 58.8154 size_x = 49.9973424167 size_y = 42.270371935 size_z = 42.8355037689
		Cinnamic acid	Ile131, Arg51, Phe47, His95, Thr94	Gly48:3.23 Gly93:2.90	2	−5.5	
		Pinocembrin	Cys82, Phe65, His81, Trp227, Ser202, Asn204, Val225, Ser207	Cys203: 3.04 Gly228:3.11, 2.71	3	−6.4	
		Apigenin	Phe 65, Ser226, Ser207, Asn204, Gly228, Lys230, Cys203, Val225, Gly205	His81: 3.05	1	−6.8	
	Lamin-like protein (XP_012208344)	Chrysin	Trp199, Leu196, Arg200, Ile311	Arg201: 3.16	2	−6.1	center_x = 101.669048536 center_y = 102.09141859 center_z = 110.8364 size_x = 148.513622974 size_y = 138.355158107 size_z = 71.1174911499
		Cinnamic acid	Lys276, Arg293, Lys297, Asp300, Tyr271,Glu270	Arg296: 2.93	1	−4.9	
		Pinocembrin	Tyr87, Lys27, Val26,Tyr22, Leu15	Asn88: 3.08 Lys84: 3.13	2	−6.5	
		Apigenin	Arg206, Glu252, Asp314, Leu256	Asp249: 2.87 Ser253: 3.21	2	−6.2	
S. parasitica cysts	Thrombospondin (XP_012209965	Chrysin	Trp469, Pro488, Ser517, Cys519, Cys472, Gln474, Arg530, Trp513, Gly514, Val486, Asn512	Leu525: 3.15 Asp520: 2.95	2	−7.9	center_x = 158.709077723 center_y = 136.794980583 center_z = 154.5554 size_x = 267.545351691 size_y = 166.990332479 size_z = 52.8454025269
		Cinnamic acid	Leu3, Leu5, Leu903, Ala905, Val911, Ser4, Val917	Val913: 2.93	1	−5.1	
		Pinocembrin	Pro488, Trp469, Glu518, Trp513, Ser517, Val486	Asp520: 2.81 Cys519: 3.34		−7.9	
		Apigenin	Arg530, Trp513, Val486, Ala491, Trp469, Pro488, Cys472,Cys519	Asn512: 2.85 Asp520: 2.02	2	−7.8	
	Host targeting protein-1 (htp-1) (ADB84848)	Chrysin	Lys108, Phe105, Ala59, Val64, His61	His60: 2.97, 2.80	2	−5.6	center_x = 7.76622751916 center_y = 43.4584462771 center_z = 12.813 size_x = 54.2809449022 size_y = 48.953391029 size_z = 117.805700302
		Cinnamic acid	Gln86, Ser72, Asn120, His122	Gln73: 3.13, 3.09 Glu83: 2.92	3	−4.5	
		Pinocembrin	Lys106, Phe105, Leu107, His61, Ala59, Val64	His60: 2.81, 3.19	2	−5.6	
		Apigenin	Thr171, Gly180, Pro178, Pro184, Ala183, Ala182	Asp190: 2.75 Thr185: 2.94, 3.11 Thr181: 3.25	4	−5.6	
S. parasitica mycelium	V type proton ATPase (XP_012207285)	Chrysin	Ala246, Phe485, Tyr492, Pro490, Leu488	Asp489: 3.07, 3.17	2	−5.1	center_x = 218.4131 center_y = 199.2273 center_z = 119.2418 size_x = 82.7397949219 size_y = 0.514002990723 size_z = 95.2704933167
		Cinnamic acid	Tyr492, Phe485, Leu554, Ala246	Nil	Nil	−3.7	
		Pinocembrin	Ala246, Leu488, Tyr4982, Leu554, Pro490	Asp489: 3.17, 3.16	2	−4.9	
		Apigenin	Pro490, Leu488	Ala246: 3.13 Tyr492: 3.34 Asp489: 3.15	3	−5.0	

S. parasitica and *A. astaci*.

The fluid propolis formulations tested inhibited mycelial growth of both oomycete species, but *S. parasitica* (EC_{50} ~ 200 μg/mL) was markedly more resistant than *A. astaci* (EC_{50} ~ 6–9 μg/mL). In comparison, 100 μg/mL propolis completely inhibited mycelial growth of the plant pathogenic oomycetes *Phytophthora plurivora* and *P.* ×*alni* and partially inhibited mycelial growth of *P. cambivora* (Silva-Castro et al., 2018), while the minimum fungicidal concentration (MFC) of propolis amended on plates with growing mycelium of *P. capsici*, *P. infestans* and *P. parasitica* was 3 μg/mL (Yusuf et al., 2005). Furthermore, 2500 μg/mL of propolis was required to completely inhibit mycelial growth of *A. invadans*, the causative agent of epizootic ulcerative syndrome in fish, while for the mammalian pathogen *Pythium insidiosum* MFC was 1000 μg/mL (Araújo et al., 2016; Campbell et al., 2001). Thus, a wide range of propolis concentrations was reported to inhibit mycelial growth of oomycetes. This is likely due in part to the high variability in propolis composition depending on the plant origin (Bankova, 2005), but also points to the interspecies differences in sensitivity of oomycete pathogens to propolis reported both in this study and in the literature (Araújo et al., 2016; Campbell et al., 2001; Silva-Castro et al., 2018; Yusuf et al., 2005).

Motile zoospores are an important stage in the asexual part of the oomycete life cycle, able to travel long distances and germinate, and in the case of pathogenic oomycetes, they represent infectious agents (Diéguez-Uribeondo et al., 1994; Lawrence et al., 2018). They are therefore an important target of antioomycetic strategies (Lawrence et al., 2018; Madrid et al., 2015; Miljanović et al., 2021; Saleh and Soltani Islami, 2015). However, we are the first to report the inhibitory activity of the propolis formulations against *A. astaci* and *S. parasitica* zoospores. Fluid propolis formulations showed good inhibitory potential against germination and motility of zoospores of both species, with EC_{50} values for germination ranging from 15.06 to 23.62 μg/mL and MIC values for motility ranging from 38.67 to 154.68 μg/mL. Similarly, the minimum propolis concentration required to inhibit zoospore motility of *A. invadans* was 10 μg/mL (Campbell et al., 2001). The slightly lower inhibitory concentration is probably partly a consequence of the different species and experimental design: Campbell et al. (2001) used a longer exposure duration (1 h compared to the 5 min used here).

As mentioned above, our results show the differences in sensitivity to fluid propolis formulations between different oomycete species and life cycle stages. The mycelium of *A. astaci* was up to 37 times more sensitive to P1 and P2 than that of *S. parasitica*. Such higher sensitivity of *Aphanomyces* spp. mycelium to plant secondary metabolites compared to *Saprolegnia* spp. was also reported by Borisutpeth et al. (2014). The concentration of absolute ethanolic extract of *Cassia fistula* required to inhibit mycelial growth of *S. parasitica* and *S. diclina* was 4× higher compared to *A. invadans* (Borisutpeth et al., 2014). On the other hand, zoospore motility of *S. parasitica* was significantly more impaired by fluid propolis formulations, as evidenced by up to 8 times lower MIC values for *S. parasitica* compared to *A. astaci*. Finally, EC_{50} values for zoospore germination were similar for both pathogens. When comparing the effects of P1 and P2 on the different life stages of the same pathogen species, the zoospores of *S. parasitica* were generally more susceptible to treatment than the viability of the mycelium, while the opposite pattern was observed for *A. astaci*. Similar to mycelial resistance of *S. parasitica*, up to 250-fold higher concentrations of propolis and malachite green were required for the inhibition of mycelial growth of *A. invadans* than for zoospore production and motility (Campbell et al., 2001). All this probably reflects differences in the mode of action of propolis formulation components against different oomycete species and life cycle stages as well as yet unexplored differences in their detoxification mechanisms.

The observed inhibitory activities of fluid propolis formulations could be attributed to their rich content of bioactive volatile and phenolic components. The fluid propolis formulations differed significantly in the number and abundance of volatile compounds identified due to the presence of sage and peppermint in P2. As previously reported, sage and peppermint are rich in oxygenated monoterpenes such as 1,8-cineole and α- and β-thujone (Hawrył et al., 2015; Miljanović et al., 2020), so these components identified in P2 are probably derived from the aforementioned plants. Recently, we have demonstrated the inhibition of *A. astaci* and *S. parasitica* by the essential oils of some Mediterranean wild plants (mainly sage and bay laurel) and speculated that some of their major components such as camphor, α-thujone, camphene, α-humulene, β-pinene and 1,8-cineole are among the molecules responsible for the observed inhibitory effect (Miljanović et al., 2021). All these phytochemicals were found in P2. However, P1 and P2 showed similar inhibitory activity, suggesting that the addition of sage and pepper mint in the P2 did not contribute significantly to its inhibitory effect (probably the concentration of active biomolecules was too low) and that propolis extract *per se* was the dominant inhibitor.

In addition, the composition of polyphenols with dominant flavonoid components such as the flavones chrysin and apigenin, the flavanone pinocembrin and the flavonol galangin, as well as derivatives of hydroxycinnamic acid (ferulic acid, isoferulic acid, caffeic acid and *p*-coumaric acid) was typical for poplar-type propolis (Ristivojević et al., 2015b). We selected chrysin as the dominant flavone and pinocembrin as the dominant flavanone in fluid propolis formulations and tested their antioomycetic activity *in vitro*. We used realistic concentrations that could be applied in aquaculture by diluting raw propolis formulations at least 100-fold. We also predicted *in silico* the presence of hydrogen bonds and hydrophobic interactions between chrysin, apigenin, cinnamic acid and pinocembrin (as possible inhibitors) and selected oomycete virulence effector proteins.

Overall, pinocembrin did not exert significant toxicity to the life stages *A. astaci* and *S. parasitica* at the concentrations tested, although it was previously shown to inhibit mycelial growth and zoospore germination of *Saprolegnia* spp. at ≥100-fold higher concentrations (Montenegro et al., 2019). In comparison, the toxicity of chrysin to mycelial growth and zoospore motility of *A. astaci* and zoospore germination of *S. parasitica* was significant at concentrations approximately corresponding to 100-fold or more diluted fluid propolis formulations (1–10 μg/mL), which is consistent with previous report of the fungicidal effect of chrysin against *Candida albicans* and *Fusarium oxysporum* (Favre-Godal et al., 2013).

In addition, our analyses predict strong binding of apigenin, chrysin and pinocembrin to the active site of *A. astaci* endochitinase, an enzyme mainly expressed in the mycelium of *A. astaci* and thought to play a role in pathogenesis by degrading the chitin layer of the crayfish cuticle during the infection process (Andersson and Cerenius, 2002). This is consistent with the observed strong inhibitory effect of fluid propolis formulations on the mycelium of *A. astaci*. As for the target proteins of *S. parasitica*, the most efficient binding of the propolis components apigenin, chrysin and pinocembrin was predicted to be with thrombospondin. It was hypothesized that thrombospondin of *S. parasitica* may play a role in adhesion to fish cells during the initial phase of the infection process, and it was found to be highly expressed in cysts (Srivastava et al., 2018). In comparison, the binding of the analyzed phytochemicals to the V-type proton ATPase of *S. parasitica* (Srivastava et al., 2018), which is expressed in mycelium, is much weaker, consistent with the higher resistance of *S. parasitica* mycelium to zoospores/cysts observed *in vitro*. However, it should be noted that the predicted binding of chrysin and pinocembrin with the analyzed set of oomycete proteins was similar, although the *in vitro* results show that chrysin has a stronger effect. This suggests that other proteins that bind chrysin more strongly than pinocembrin also play a role in propolis-mediated oomycete inhibition.

Finally, the inhibition caused by fluid propolis formulations was effective at a dilution of 100 to 4000 times. This suggests that the observed antioomycetic effect cannot be explained only by the activity of chrysin as the dominant component, but is probably due to the synergistic effect of various compounds present in propolis at low

concentrations (Ristivojević et al., 2015b). Moreover, the biological properties of propolis are always similar and significant despite the large variability in chemical composition between different botanical types of propolis (Auamcharoen and Phankaew, 2016; Dias et al., 2012; Seidel et al., 2008). Based on the results obtained, we therefore suggest that propolis extracts could be used as an ecologically acceptable method to control *A. astaci* and *S. parasitica* in aquaculture. This will require *in vivo* experiments involving the application of propolis extracts in aquaculture, either by dietary supplementation (Abdelmagid et al., 2021; Mişe Yonar et al., 2017) or by bathing eggs and animals in propolis suspensions (Fuat Gulhan and Selamoglu, 2016; Talas and Fuat Gulhan, 2009) or both. So far, there are no reports on antioomycetic activity of propolis *in vivo*, but some studies show a positive effect of propolis application on host innate immunity and reproductive efficiency. For example, the crayfish diet containing 4% propolis improved the reproductive efficiency of the animals by increasing the number of pleopodal eggs and reducing oxidative stress under controlled hatchery conditions (Mişe Yonar et al., 2017). Nile tilapia feed enriched with propolis or propolis nanoparticles (10 g/kg) mitigated the effects of glyphosate-induced oxidative stress and immunosuppression (Abdelmagid et al., 2021). Furthermore, immersion of rainbow trout (*Oncorhynchus mykiss*) in 0.01 g/L propolis suspension resulted in positive changes in blood biochemical, electrolytic and haematological parameters (Fuat Gulhan and Selamoglu, 2016; Talas and Fuat Gulhan, 2009).

5. Conclusions

Overall, our results show that the tested fluid propolis formulations are rich in bioactive components and exhibit strong activity against the oomycete pathogens *A. astaci* and *S. parasitica*. This suggests that propolis could be used in salmonid and crayfish aquaculture not only as an immunostimulant but also as an environmentally friendly and sustainable antioomycetic agent. However, further studies are needed, mainly the testing of the possible protective effect of propolis on oomycete infections, to develop the protocols for administration of propolis as egg/animal baths or feed supplements.

Funding

This study was funded by the Croatian Science Foundation. Experimental costs were funded by the project "Interactions of freshwater pathogenic oomycetes and the environment" (InteractOomyc, UIP-2017-05-6267) and A.M. was funded by the Young Researchers' Career Development Project no. DOK-2018-01-8751.

CRediT authorship contribution statement

Anđela Miljanović: Methodology, Investigation, Writing – original draft, Visualization. **Raja Aadil Hussain Bhat:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Ritesh Shantilal Tandel:** Methodology, Writing – review & editing. **Dora Pavić:** Methodology, Investigation. **Dorotea Grbin:** Formal analysis, Visualization, Project administration. **Maja Dent:** Methodology. **Zvonimir Marijanović:** Methodology, Investigation. **Igor Jerković:** Methodology, Investigation. **Sandra Pedisić:** Methodology, Investigation. **Ivana Maguire:** Conceptualization, Writing – review & editing. **Ana Bielen:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are thankful to Ivana Čakarić and Karla Vasari for the help with inhibition assays, and to Igor Slivac and Sunčica Bosak and their teams for the help with the inverted microscope.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.737982.

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3. DISCUSSION

In this doctoral thesis, it was shown for the first time that the essential oils of selected Mediterranean plants, bay laurel, sage and rosemary, as well as fluid propolis formulations can inhibit mycelial growth and zoospores of *Aphanomyces astaci* and *Saprolegnia parasitica*, oomycete pathogens important in freshwater aquaculture. In addition, the conditions for essential oil isolation were optimised to achieve a significant increase in yield, facilitating the potential large-scale application of essential oils for disease control in aquaculture.

3.1. Different hydrodistillation pretreatments can improve the yield of essential oils while their chemical composition remains largely unchanged

Rosemary and sage are aromatic medicinal plants within the Lamiaceae family that have received special attention due to their aromatic and chemical composition (Hamrouni-Sellami et al., 2013; Hosni et al., 2013), while bay laurel is a valuable medicinal plant from the Lauraceae family that is widely used as a spice and flavouring agent (Boulila et al., 2015). Due to the numerous bioactive compounds, the essential oils of these plants exhibit a wide range of biological activities, such as antimicrobial, preservative, antioxidant and antifungal, making them valuable in a number of applications, from medicine to food industry (Ali et al., 2014; Fidan et al., 2019; Soares et al., 2015). Recently, the potential of using these plants in aquaculture has also become increasingly apparent. Plant extracts are used as feed additives that can improve fish growth and/or immune response and even reduce disease- or toxin-related mortality (Metin et al., 2020; Naiel et al., 2019; Salomón et al., 2020; Turan et al., 2016; Zoral et al., 2017). For instance, when bay laurel essential oil solution was applied as a bath for the fertilised eggs of rainbow trout, it reduced the negative effects of *S. parasitica* infection (Özdemir et al., 2022).

We investigated the effects of different hydrodistillation pretreatments on the yield and chemical composition of essential oils isolated from the selected Mediterranean plants rosemary, sage and bay laurel. Different pretreatments [hydrodistillation with reflux extraction pretreatment, HD-RE, hydrodistillation with reflux extraction pretreatment assisted with enzymes (pectinase, HD-REP; cellulase, HD-REC; xylanase, HD-REX; pectinase/cellulase/xylanase, HD-REPCX), hydrodistillation with ultrasound pretreatment, HD-US] significantly improved the yield of essential oils compared to the no-pretreatment

control (HD). At the same time, the pretreatments did not affect the quality of the oils and their composition was comparable to the no-pretreatment controls, as shown by the statistical analysis of GC-MS results. The obtained volatile profiles of the essential oils of sage, rosemary and bay laurel corresponded to those described in the literature: sage essential oil was dominated by oxygenated monoterpenes such as α-thujone and camphor and sesquiterpenes such as manool and veridiflorol; the main components of bay laurel essential oil were the monoterpenes α-terpenyl acetate and 1,8-cineole; and rosemary essential oil was richest in oxygenated monoterpenes such as borneol and camphor and in berbenone among the sesquiterpenes (Boulila et al., 2015; Hatipoglu et al., 2016; Hosni et al., 2013; Olmedo et al., 2015; Russo et al., 2013). The composition of the essential oils after the different pretreatments was also comparable to the composition of the essential oils obtained by direct hydrodistillation. Other studies had also mostly shown that enzymatic and ultrasound pretreatments had no effect on the overall composition of the oil (Assami et al., 2012; Kowalski and Wawrzykowski, 2009; Smigielski et al., 2014a; 2014b; Sowbhagya et al., 2010, 2011), although some authors reported that the quantities of individual major components varied significantly depending on the extraction technique used (Boulila et al., 2015; Chandran et al., 2012; Dimaki et al., 2017; Hosni et al., 2013; Lilia et al., 2018; Morsy, 2015; Seidi Damyeh et al., 2016). We also observed that the ratios of some components varied between the different pretreatments, but these changes were not significant and did not alter the overall quality of the oil.

Among the various pretreatments used in our study, reflux extraction (HD-RE) was the simplest. Incubation of finely ground plant material for 1 hour at 40 °C in purified water prior to hydrodistillation increased the essential oil yield by up to 60%. It has been previously shown that such soaking of plant material in water (Awada et al., 2012) or acidic medium (Dimaki et al., 2017) prior to distillation can increase the quantity of the isolated oil by promoting leaching of the constituents from the already disrupted cells. Swelling and hydration of the plant material, which enlarges the pores in the cell walls and increases the turgor pressure in the still intact plant cells, could also lead to better diffusion of the oil constituents into the soaking medium. For example, soaking agarwood in lactic acid for 168 hours (Nor Fazila and Ku Halim, 2012) and soaking thyme leaves in distilled water overnight at 50 °C improved the yield of essential oil (Awada et al., 2012). Thus, soaking the plant material prior to hydrodistillation, as applied here for selected Mediterranean plants, is a simple and cost-effective treatment that leads to a significant increase in essential oil yield.

We also tested whether the application of cell wall-degrading enzymes prior to hydrodistillation had a positive effect on extraction efficiency. We used this procedure as disruption of the cell walls could potentially facilitate the release of the essential oils from the plant cells. We followed previously described protocols (Boulila et al., 2015; Hosni et al., 2013) and applied separate and combined pretreatments with cellulase, pectinase and xylanase to the plant material. Prior to the extractions, we performed a small-scale enzyme assay and confirmed the activity of all enzymes under the reaction conditions. However, the application of the enzymes did not increase the essential oil yield beyond the reflux extraction pretreatments, suggesting that their activity was superfluous in our case. Contrary to our results, several studies have reported positive effects of cell-wall disrupting enzymes' application on essential oil yield (Baby and Ranganathan, 2016; Boulila et al., 2015; Chandran et al., 2012; Chávez-González et al., 2016; Hosni et al., 2013; Smigielski et al., 2014b; Sowbhagya et al., 2010; 2011). For example, enzyme-assisted extraction pretreatment was used prior to hydrodistillation of bay laurel (Boulila et al., 2015), rosemary and thyme leaves (Hosni et al., 2013) and reportedly resulted in an increase in essential oil yield (up to 109% for thyme leaves). However, all the studies listed above lacked a no-enzyme control and the observed yield increase was calculated in comparison to a no-pretreatment control. In our case, the extraction results were compared with both the no-pretreatment control (HD) and the reflux extraction pretreatment (HD-RE), which served as a no-enzyme control since it was carried out under the same conditions as the enzyme-assisted extraction pretreatments (purified water at 40 °C for 1 hour), only without the addition of enzyme(s). Compared to the no-pretreatment control, the increase in essential oil yield was significant for all enzyme pretreatments individually, as well as for their combination, but there were no significant differences between the individual pretreatments (i.e. pretreatment with only one of the enzymes or all of them). Unexpectedly, reflux extraction resulted in approximately the same increase in essential oil yield as the enzyme-assisted pretreatments. This suggests that the increase in essential oil yield was due to the reflux extraction pretreatment itself (i.e. soaking the macerated plant material in warm water) and not to the enzymatic degradation of the cell wall. The beneficial effect of soaking the plant material alone observed here could not be adequately assessed and compared with the effect of enzymes in the earlier studies that were lacking no-enzyme controls (Baby and Ranganathan, 2016; Boulila et al., 2015; Chandran et al., 2012; Chávez-González et al., 2016; Hosni et al., 2013; Smigielski et al., 2014b; Sowbhagya et al., 2010; 2011). Besides our work, there are only two studies (Costa et al., 2021; Dimaki et al., 2017) that used the appropriate controls (i.e., both no-pretreatment and no-enzyme control) and both report results consistent with this study. Costa et al. (2021)

observed only a tiny increase in essential oil yield from *Croton argyrophyllus* leaves when they were pretreated with a multienzyme extract. Dimaki et al. (2017) found no significant difference in the essential oil yield of *Sideritis* sp. after applying enzymatic pretreatment to hydrodistillation or ultrasound-assisted essential oil extraction. The enzymatic pre-treatment resulted in approximately the same yield of essential oil as merely soaking the plant material in acidic buffer, similar to our results. All this suggests that the use of enzymatic pretreatments with the aim of increasing essential oil yields, although commonly reported as beneficial, should be carefully re-evaluated. This is particularly important considering the high cost of applying enzymes on a large scale. In summary, enzyme-assisted hydrodistillation pretreatments should be avoided unless a significant improvement in yield over a no-enzyme control can be demonstrated.

Finally, we showed that the yield of essential oil increased by 50 – 60% after ultrasound pretreatment (HD-US: 30% of the maximum ultrasound power for 10 min) compared to the no-pretreatment control (HD). This was similar to the results of reflux-assisted extraction pretreatment (HD-RE) and better than or comparable to the results of other studies that applied ultrasound as hydrodistillation pretreatment and reported yield increases between 5 and 35% (Kowalski et al., 2015; Kowalski and Wawrzykowski, 2009; Lilia et al., 2018; Morsy, 2015; Smigielski et al., 2014a). Even when the yield remained the same, the use of ultrasound pretreatment significantly shortened the essential oil isolation procedure (Assami et al., 2012; Seidi Damyeh et al., 2016).

In summary, we obtained a high, 50 – 60%, increase in essential oil yield after reflux extraction (HD-RE) and ultrasound hydrodistillation pretreatment (HD-US). The HD-RE was the most cost-effective method, while HD-US resulted in the most significant reduction in total extraction time. Recently, it has been calculated that the application of bay laurel essential oil at the concentration required to suppress saprolegniosis in fertilised rainbow trout eggs is cheaper than the application of a therapeutic concentration of formalin, but more expensive than the application of hydrogen peroxide (Özdemir et al., 2022). Further reducing the cost of essential oil production, possibly by adding hydrodistillation pretreatments to current essential oil isolation protocols, would therefore enable the large-scale application of essential oils in aquaculture, reduce the use of harmful chemical treatments and thus facilitate the switch towards environmentally friendly practices. Another research direction we are currently pursuing as an extension of this doctoral thesis is the application of hydrodistillation by-products for disease control in aquaculture. Indeed, the production of essential oils generates a

considerable amount of understudied and underexploited by-products, which are sometimes even considered waste. These by-products include hydrolates, water residues and solid residues, all of which have been shown to be rich in bioactive compounds (Abdel-Hameed et al., 2015; Bajer et al., 2017; Boulila et al., 2015; Maciąg and Kalemba, 2015; Rajeswara Rao et al., 2002; 2003; Sánchez-Vioque et al., 2015; Santana-Méridas et al., 2014; Smail et al., 2011; Veličković et al., 2008; Wollinger et al., 2016). Our unpublished results point to the valuable chemical composition of the by-products of rosemary, sage and bay laurel essential oil production with the potential for application in aquaculture industry.

3.2. Fluid propolis formulations and essential oils of Mediterranean wild plants inhibit mycelium and zoospores of pathogenic freshwater oomycetes

We have tested the inhibitory activity of essential oils (rosemary, laurel bay, sage), fluid propolis formulations [P1 – 200,000 mg of propolis dry mass/L, and P2 – 250,000 mg of propolis dry mass/L with the addition of sage (*Salvia officinalis*) and peppermint (*Mentha piperita*) extracts (250,000 and 190,000 mg/L, respectively)], propolis main components chrysin and pinocembrin, and malachite green (as positive control) towards *S. parasitica* and *A. astaci*. All tested compounds showed inhibitory potential against the life stages of pathogenic oomycetes, although with differences in efficacy depending on the compound, oomycete species and oomycete life cycle stage. The overview of EC_{50} (i.e. sample concentration causing 50% inhibition) and MIC (i.e. minimum inhibitory concentrations) values determined for different compounds in the Miljanović et al. (2021) and Miljanović et al. (2022) is presented in Table 1.

Table 1. EC_{50} values for radial mycelial growth inhibition and zoospore germination, and MICs for zoospore motility. P1 –fluid formulation of pure propolis, and P2 - fluid propolis formulation with addition of sage and peppermint. N.A. – not analyzed.

	EC_{50} for mycelium growth		EC_{50} for zoospore germination		MIC for zoospore motility	
	µL/L		µL/L		µL/L	
Sample	*A. astaci*	*S. parasitica*	*A. astaci*	*S. parasitica*	*A. astaci*	*S. parasitica*
Rosemary essential oil	59.6	>1000*	48.6	63.0	N.A.	N.A.
Sage essential oil	30.8	40.5	7.3	11.6	N.A.	N.A.
Bay laurel essential oil	98.4	>1000*	14.9	12.6	N.A.	N.A.
	mg/L		mg/L		mg/L	
P1	5.58	206.20	15.06	23.62	123.76	61.88
P2	8.59	206.60	19.51	19.01	154.68	38.67
Chrysin	2.59	>5.12*	>10.18*	3.10	10.14	>20.28*
Pinocembrin	>1.28*	>1.28*	>2.54*	>2.54*	>5.07*	>5.07*
Malachite green (pos. control)	0.020	0.120	0.020	0.032	0.64	0.08

*The highest concentration tested that did not cause 100% inhibition. Thus, EC_{50} value could not be determined.

Overall, the results of the inhibition experiments showed that the mycelium of *A. astaci* was the most sensitive to fluid propolis formulations, while sage essential oil was the most effective inhibitor of *S. parasitica* mycelium (Table 1). The mycelium of *S. parasitica* was markedly more resistant to the effect of fluid propolis formulations than the mycelium of *A. astaci* (EC_{50} ~200 mg/L versus 6 – 9 mg/L). Similarly, the essential oils tested showed significant inhibition of mycelial growth of *A. astaci* (EC_{50} = 30.8 – 98.4 µL/L), while in the case of *S. parasitica*, mycelial growth was significantly inhibited only by sage essential oil (EC_{50} = 40.5 µL/L). Other studies have also shown inhibitory effects of essential oils and propolis on oomycetes, including *S. parasitica*, some *Aphanomyces* spp. and some more distantly related oomycetes such as the plant pathogen *Phytophthora* spp. The range of inhibitory concentrations of propolis samples against mycelial growth of such diverse oomycete species considered was wide, ranging from 3 to 2500 mg/L (Araújo et al., 2016; Campbell et al., 2001; Silva-Castro et al., 2018; Yusuf et al., 2005). In addition, previously reported inhibitory concentrations of essential oils from plants of the Lamiaceae and Lauraceae families, including bay laurel, against mycelium of *S. parasitica* ranged from 100 to 100 000 µL/L (Gormez and Diler, 2014; Metin et al., 2015; Nardoni et al., 2019; Özdemir et al., 2022; Tampieri et al., 2003). This wide range of concentrations of fluid propolis formulations and

essential oils required to inhibit mycelial growth of oomycetes may be due to variability in the chemical composition of the samples tested depending on differences in plant and geographical origin (Bankova, 2005; Campbell et al., 2001) and differences in the methodological approaches used (Klancnik et al., 2009), but also point to the interspecies differences in the sensitivity of oomycete pathogens to propolis and essential oils reported both in this study and in the literature (Araújo et al., 2016; Campbell et al., 2001; Gormez and Diler, 2014; Metin et al., 2015; Nardoni et al., 2019; Silva-Castro et al., 2018; Yusuf et al., 2005).

Motile zoospores are an important stage in the asexual part of the life cycle of oomycetes. They are able to travel long distances and then germinate on a suitable substrate (Diéguez-Uribeondo et al., 1994a; Lawrence et al., 2017). In the case of pathogenic oomycetes, they represent infectious agents and are therefore an important target for anti-oomycetic strategies (Lawrence et al., 2017; Madrid et al., 2015; Saleh et al., 2015). We are the first to report the inhibitory activity of fluid propolis formulations and essential oils of sage, bay laurel and rosemary against the zoospores of *A. astaci* and *S. parasitica*. All samples tested showed good inhibitory potential against the germination of zoospores of both species, with EC_{50} values of ~20 mg/L for propolis and between 7.3 and 63.0 µL/L for essential oils. The effect of the propolis formulations on zoospore motility was also tested, and the MIC values ranged from 40 to 150 mg/L. Similary to our results, the minimum propolis concentration required to inhibit zoospore motility of *A. invadans* was 10 mg/L (Campbell et al., 2001). Furthermore, the essential oils of *Mentha longifolia* and *Thymus daenensis* (Lamiaceae) completely inhibited the germination of zoospores of *S. parasitica*, albeit at much higher concentrations than those tested in this study (2500 and 5000 µL/L, respectively) (Saleh et al., 2015), indicating the promising properties of the essential oils tested in this doctoral thesis, particularly sage.

We found differences between sensitivity of the tested species to the effects of the samples tested. Namely, for all the tested samples, the mycelium of *A. astaci* was more sensitive than the mycelium of *S. parasitica*. On the other hand, in both pathogens similar sensitivity of the germination of zoospores was observed, while *S. parasitica* zoospores were up to four times more sensitive to the application of the essential oils and propolis than the zoospores of *A. astaci* in terms of zoospore motility. The higher sensitivity of the mycelium of *Aphanomyces* spp. compared to *Saprolegnia* spp. has been reported previously: an absolute ethanol extract of *Cassia fistula* (Fabaceae) inhibited mycelial growth of *S. parasitica* and *S. diclina* at 2000 mg/L, compared to 500 mg/L required to inhibit *A. invadans* (Borisutpeth et al., 2014).

When comparing the effects of the tested natural products on the different life stages of the same pathogen species, the zoospores were generally more susceptible to the same treatment than the mycelium, with the exception of the inhibitory effect of propolis on *A. astaci*, where the EC_{50} values for inhibition of mycelial growth were up to three times lower than the EC_{50} values for germination of zoospores and up to 20 times lower than the MIC values for motility of zoospores. On the other hand, the mycelium of *S. parasitica* was up to 11 times more resistant than the zoospores for all essential oils and propolis tested. For example, the essential oil of bay laurel and rosemary showed a strong inhibitory effect on the germination of *S. parasitica* zoospores, but their effect on the growth of the mycelium was much weaker. Previous studies comparing the sensitivity of mycelium and zoospores of pathogenic oomycetes to various compounds showed different results (Borisutpeth et al., 2009; 2014; Hu et al., 2007; Lawrence et al., 2017; Madrid et al., 2015). For some combinations of oomycete species and anti-oomycete agents, the mycelium was more resistant than the zoospores: e.g. the effect of propamocarb hydrochloride on *Phytophthora nicotianae* (Hu et al., 2007) and the effect of propolis and malachite green on *A. invadans* (Campbell et al., 2001). Sometimes zoospores were more resistant, such as the effect of *Laureliopsis philippiana* essential oil on *S. parasitica* and *S. australis* (Madrid et al., 2015), and sometimes similar sensitivity was observed for both zoospores and mycelium, such as the effect of *C. fistula* extract on *S. parasitica*, *S. diclina* and *A. invadans* (Borisutpeth et al., 2014). All these studies, including the results presented here, suggest that some compounds are more potent zoospore inhibitors (such as the essential oil sage, rosemary and bay laurel and fluid propolis formulations on *S. parasitica*), while others preferentially target the mycelium (such as the fluid propolis formulations on *A. astaci*). This probably reflects differences in the mode of action of the different compounds on zoospores and mycelium, as well as different detoxification mechanisms at different life stages and in different oomycete species. Further studies, including transcriptome and proteome analyses, are needed to clarify this.

The observed inhibitory effects could be attributed to the rich bioactive compound of the essential oils and fluid propolis formulations, as shown by the results of GC-MS and UPLC/MS-MS analyses. The chemical composition of the essential oils tested was consistent with previous reports in which α-thujone, camphor and borneol were among the dominant components in sage essential oil, 1-8-cineole, linalool and sabinene in bay laurel essential oil, and camphor and carvacrol in rosemary essential oil (Boulila et al., 2015; Hatipoglu et al., 2016; Hosni et al., 2013; Olmedo et al., 2015; Russo et al., 2013). The composition of the fluid

propolis formulations was typical of poplar-type propolis (Ristojević et al., 2015), with the dominant flavonoid components being the flavones chrysin and apigenin, the flavanone pinocembrin and the flavonol galangin, and derivatives of hydroxycinnamic acid (ferulic acid, isoferulic acid, caffeic acid and p-coumaric acid). In addition, the P2 propolis formulation utilized in our studies was enriched by the addition of sage and peppermint extracts and therefore contained a higher number and abundance of the volatile compounds identified. As previously reported and also shown in this study, sage and peppermint are rich in oxygenated monoterpenes such as 1,8-cineole and α- and β-thujone (Hawrył et al., 2015; Vosoughi et al., 2018), so these components identified in P2 are likely derived from these plants.

The results of the PLS analysis indicate that the anti-oomycetic activity of sage essential oil as the strongest inhibitor among the essential oils tested could be attributed to some of its major constituents, camphor, α-thujone, veridiflorol, camphene and α-humulene, which were either absent or present only in low amounts in the other essential oils. In addition, β-pinene and 1,8-cineole (present in significant amounts in sage and bay laurel essential oils, and in low amounts in rosemary essential oil) were positively correlated with inhibition of germination of *S. parasitica* zoospores. Some of these compounds were previously reported to have good anti-oomycetic (Tampieri et al., 2003; Tedesco et al., 2020) and antifungal activity (Agus et al., 2019; 2020; Gazdağlı et al., 2018; Teker et al., 2021). For example, camphor at concentrations up to 38 mg/L progressively slowed mycelial growth of *S. parasitica* and *S. delica*, while thujone and β-pinene (in concentrations between 500 and 1000 mg/L, respectively) inhibited mycelial growth of *S. parasitica* (Tampieri et al., 2003; Tedesco et al., 2020). Moreover, α-thujone and camphor were found to have a strong inhibitory effect on the fungi *Fusarium graminearum*, *Fusarium culmorum* and *Schizosaccharomyces pombe*, mainly explained by the induction of oxidative stress and subsequent apoptotic cell death, but also by a decrease in genomic stability and epigenetic changes (Agus et al., 2019; 2019; Gazdağlı et al., 2018; Teker et al., 2021). Thus, the high camphor content in sage essential oil likely contributed significantly to the observed inhibitory effects. The mechanism underlying the inhibition of oomycetes by camphor remains to be investigated, but could be due to oxidative stress-mediated apoptosis, similar to that observed in fungi. All these phytochemicals were also found in P2, but not in P1. However, both propolis preparations, i.e. P1 and P2, showed similar inhibitory effects, suggesting that the addition of plant extracts in P2 did not contribute significantly to its inhibitory effect. The concentration of active biomolecules from sage and pepper mint was probably too low, so the propolis formulation as such was the dominant inhibitor.

We also tested the anti-oomycetic activity of the major propolis constituents. We chose chrysin as the dominant flavone and pinocembrin as the dominant flavanone and used realistic concentrations that can be applied in aquaculture by diluting the crude propolis formulations 100-fold or more. Overall, pinocembrin did not exert significant toxicity to any of the life stages of *A. astaci* and *S. parasitica* at the concentrations tested, although it has previously been shown to inhibit mycelial growth and zoospore germination of *Saprolegnia* spp., albeit at ≥ 100-fold higher concentrations (Montenegro et al., 2019). In comparison, the toxicity of chrysin to mycelial growth and zoospore motility of *A. astaci* and zoospore germination of *S. parasitica* was significant at concentrations approximately corresponding to 100-fold or more times diluted fluid propolis formulations (1 – 10 mg/L), consistent with previous reports of the fungicidal activity of chrysin against *Candida albicans* and *Fusarium oxysporum* (Favre-Godal et al., 2013).

We have also applied molecular docking to predict the binding of selected propolis components chrysin, apigenin, cinnamic acid and pinocembrin to various oomycete proteins suggested to play a role in pathogenesis. In agreement with the observed strong inhibitory effect of fluid propolis formulations on the mycelium of *A. astaci*, we predicted strong binding of apigenin, chrysin and pinocembrin to the active site of *A. astaci* endochitinase, an enzyme mainly expressed in the mycelium and thought to play a role in pathogenesis by degrading the chitin layer of the crayfish cuticle during the infection process (Andersson and Cerenius, 2002). Regarding the target proteins of *S. parasitica*, the most efficient binding of the propolis components apigenin, chrysin and pinocembrin was predicted to be with thrombospondin. It was hypothesised that thrombospondin of *S. parasitica* may play a role in adhesion to fish cells during the initial phase of the infection process, and it was found to be highly expressed in cysts (Srivastava et al., 2018). In comparison, the predicted binding of the analysed phytochemicals to the V-type proton ATPase of *S. parasitica* (Srivastava et al., 2018), which is expressed in mycelium, was much weaker, consistent with the higher resistance of *S. parasitica* mycelium observed *in vitro* compared to zoospores/cysts. However, it should be noted that the predicted binding of chrysin and pinocembrin to the analysed group of oomycete proteins was similar, although the results of *in vitro* experiments show that chrysin has a stronger effect. This suggests that other proteins that bind chrysin more strongly than pinocembrin may play a role in propolis-mediated oomycete inhibition.

All this suggests that the observed anti-oomycetic effect of the tested fluid propolis formulations and essential oils cannot be explained only by the activity of the dominant

components, such as chrysin or camphor, but is probably due to the synergistic action of different components present in low concentrations (Madrid et al., 2015; Ristojević et al., 2015; Tampieri et al., 2003). Based on the results obtained, we suggest that propolis extracts and essential oils from Mediterranean wild plants could be used as an ecologically acceptable method to control *A. astaci* and *S. parasitica* in aquaculture. Essential oils and propolis extracts have already been tested for the possibility of use in aquaculture, either through dietary supplementation (Abdelmagid et al., 2021; Metin et al., 2020; Mişe Yonar et al., 2017; Sari and Ustuner-Aydal, 2018; Sönmez et al., 2015;) or by bathing the eggs and animals in essential oil or propolis suspensions (Fuat Gulhan and Selamoglu, 2016; Khosravi et al., 2012; Metin et al., 2015; Özdemir et al., 2022; Talas and Gulhan, 2009; Zoral et al., 2017) or both. Studies have shown that the application of propolis and plant extracts has a positive effect on animal immunity, reproductive performance and growth. For example, crayfish feed containing 4% propolis improved the reproductive performance of the animals by increasing the number of pleopodal eggs and reducing oxidative stress under controlled hatchery conditions (Mişe Yonar et al., 2017). Nile tilapia (*Oreochromis niloticus*) feed enriched with propolis or propolis nanoparticles (10 g/kg) mitigated the effects of glyphosate-induced oxidative stress and immunosuppression (Abdelmagid et al., 2021). Immersion of rainbow trout (*O. mykiss*) in 0.01 g/L propolis suspension resulted in positive changes in blood biochemical, electrolytic and haematological parameters (Fuat Gulhan and Selamoglu, 2016; Talas and Gulhan, 2009). It has also been shown that bay laurel and sage leaf extracts used as feed additives can act as growth promoters in aquaculture (Salomón et al., 2020; Turan et al., 2016). Feeds enriched with rosemary and sage plant extracts have also been effective in reducing the harmful effects of pathogens, both bacteria such as *Aeromonas sobria* and *Streptococcus iniae* (Abutbul et al., 2004; Metin et al., 2020) and parasites such as the monogenean *Dactylogyrus minutus* (Zoral et al., 2017). Most importantly, the application of 500 ppm bay laurel essential oil as a bath for fertilised eggs of rainbow trout has been shown to reduce the negative effects of infection with *S. parasitica* (Özdemir et al., 2022).

The results of this dissertation open many new research directions. For instance, as sage was the most potent inhibitor of *S. parasitica*, it's potential protective effect against *S. parasitica* infection could be tested by immersion of trout eggs in sage essential oil suspensions and monitoring the relevant endpoints such as hatching rate and larval survival rate. Also, the effect of dietary addition of propolis on mortality caused by *A. astaci* in native European

crayfish of aquaculture importance, such as *Pontastacus leptodactylus* and *Astacus astacus*, could be tested.

4. CONCLUSIONS

Overall, the results of this doctoral thesis represent a starting point for further *in vivo* testing of the application of essential oils and propolis formulations in freshwater aquaculture and open the perspective for the development of environmentally acceptable alternatives for the control of diseases caused by oomycetes. The following main conclusions arise from the results obtained:

Considering the high cost of essential oil production compared to the price of chemical treatments currently used to control oomycete diseases in aquaculture, we optimised the isolation of essential oil from bay laurel, sage and rosemary leaves to increase the yield. Ultrasonic hydrodistillation pretreatment and reflux extraction pretreatment proved to be environmentally friendly methods that increased essential oil yields by up to 60%. At the same time, the results of GC-MS analyses showed that the overall quality of the oil remained largely unchanged. In contrast, the use of enzyme-assisted pretreatments did not increase the essential oil yield compared to the reflux extraction pretreatments, which served as no-enzyme controls, i.e. soaking the plant material in water at 40 °C was sufficient to disrupt the cells and additional enzyme activity was redundant.

In general, all tested samples showed inhibitory potential against zoospore motility and germination and mycelial growth of both pathogens, although with differences in efficacy depending on the substance, oomycete species and oomycete life cycle stage. The fluid propolis formulations most potently inhibited mycelial growth of *A. astaci*, while the sage essential oil most strongly inhibited zoospore germination and mycelial growth of *S. parasitica* and zoospore germination of *A. astaci*. The observed inhibitory effects could be attributed to the abundant bioactive content of the natural products, as shown by the results of GC-MS and UPLC/MS-MS analyses. Although our results point to several molecules as the basis of the inhibitory effect, such as camphor in the sage essential oil and chrysin in the fluid propolis formulations, the observed anti-oomycetic effect cannot be explained only by the activity of the dominant components, but is probably due to the synergistic effect of different components present in low concentrations.

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6. SUPPLEMENTARY DATA

Supplementary file 1 of the manuscript:

Effect of Enzymatic, Ultrasound, and Reflux Extraction Pretreatments on the Yield and Chemical Composition of Essential Oils

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Academic Editor: Petras Rimantas Venskutonis
Received: 8 September 2020; Accepted: 19 October 2020; Published: 20 October 2020

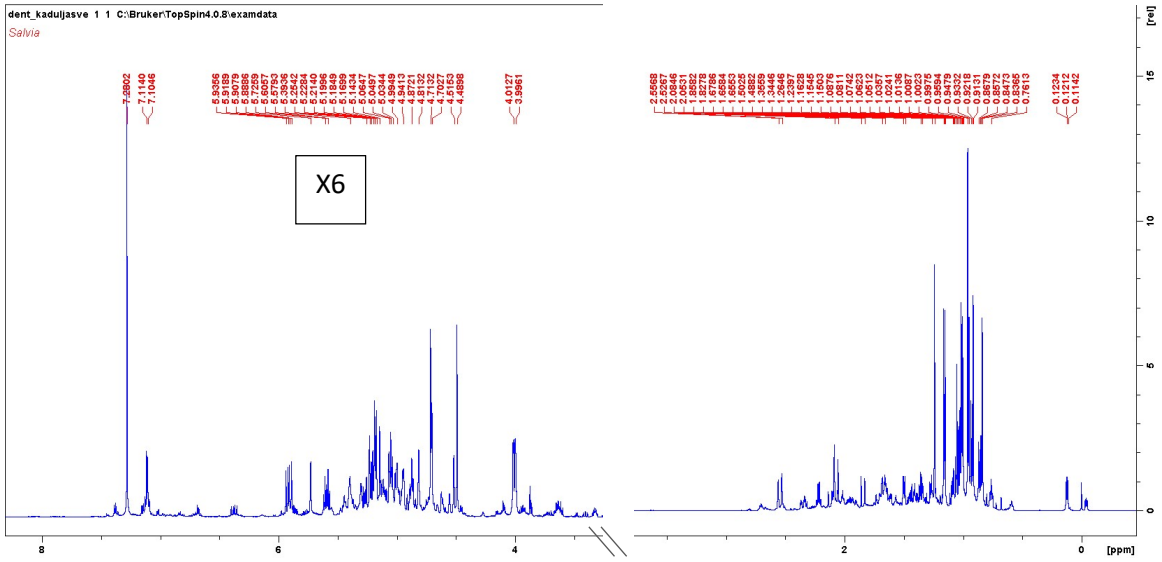

Figure S1. Sage ^{1}H NMR spectrum (600 MHz; 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 32 *K* data points; 256 scans; 0.37 Hz/point; 1 s delay).

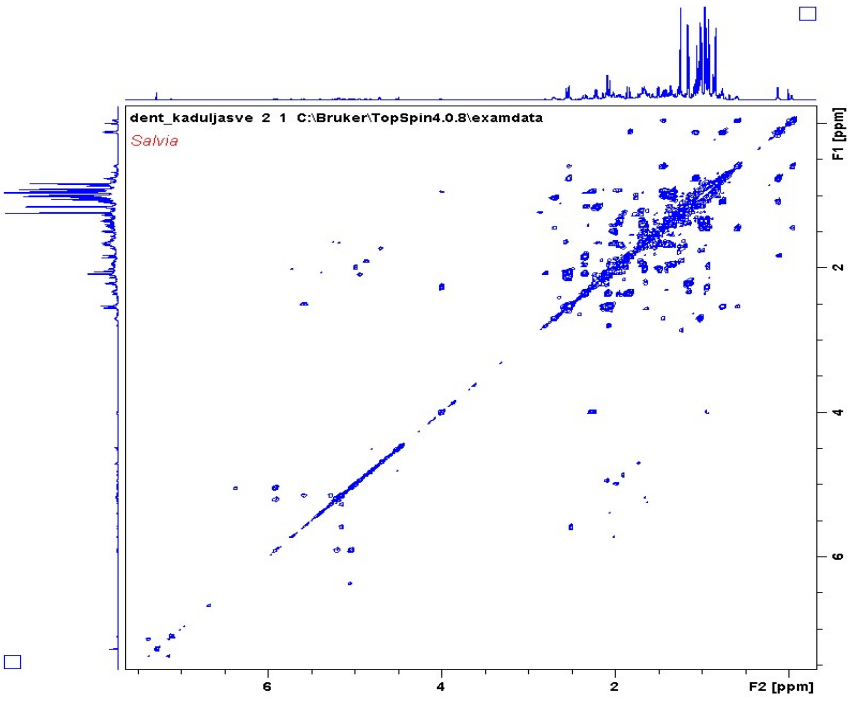

Figure S2. Sage ^{1}H-^{1}H COSY NMR spectrum (600 MHz, $CDCl_3$-*d*, 25 °C).

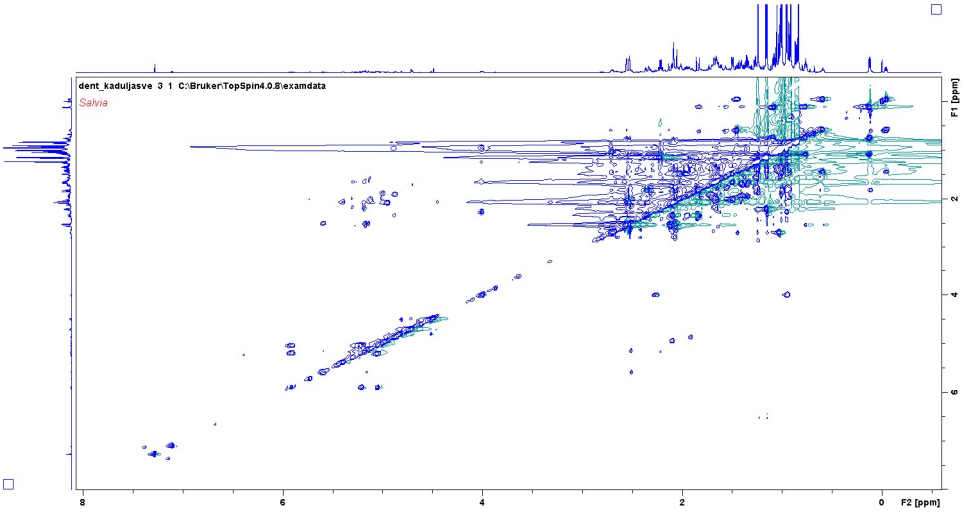

Figure S3. Sage ^{1}H-^{1}H TOCSY NMR spectrum (600 MHz, $CDCl_3$-*d*, 25 °C).

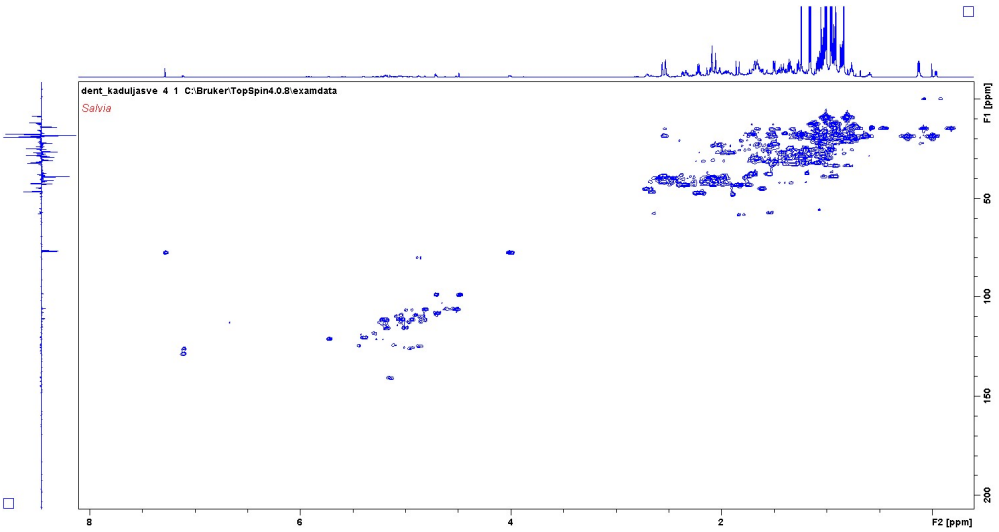

Figure S4. Sage ^{1}H-^{13}C HMQC NMR spectrum ($CDCl_3$-*d*, 25 °C). The 600 MHz ^{1}H NMR spectrum is shown at the top edge, and a 150 MHz ^{13}C NMR spectrum at the left-hand edge.

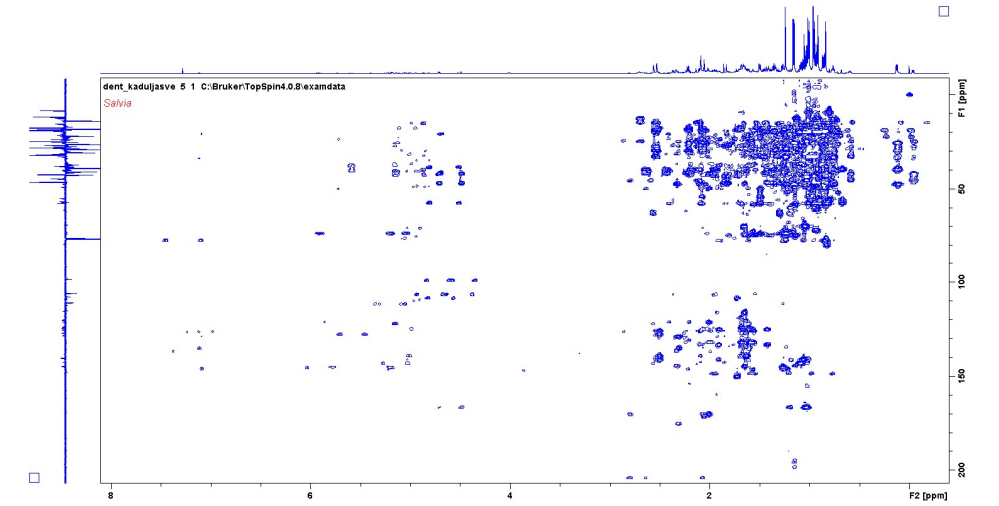

Figure S5. Sage ^{1}H-^{13}C HMBC NMR spectrum ($CDCl_3$-*d*, 25 °C). The 600 MHz ^{1}H NMR spectrum is shown at the top edge, and a 150 MHz ^{13}C NMR spectrum at the left-hand edge.

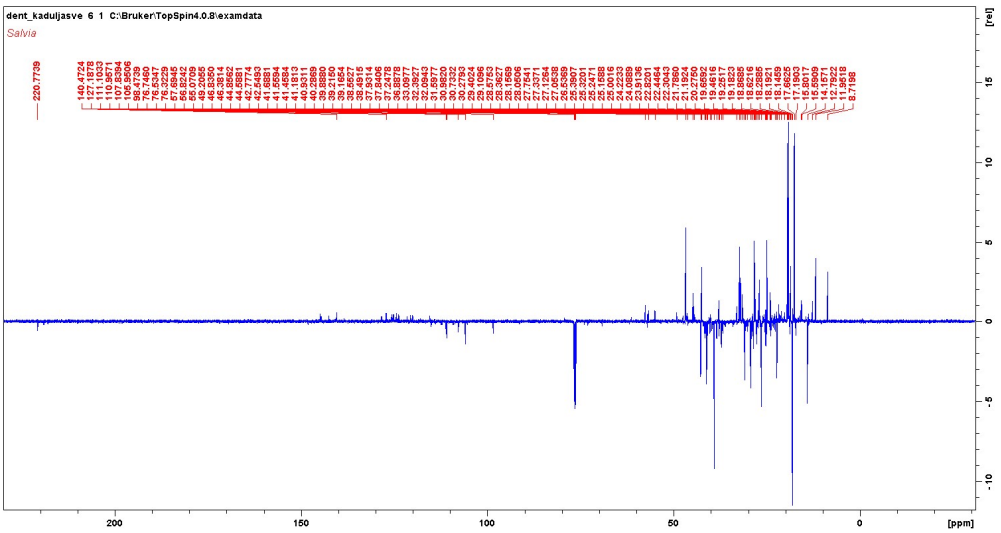

Figure S6. Sage ^{13}C APT NMR spectrum (150 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 64 *K* data points; 44506 scans; 0.60 Hz/point; 1 s delay).

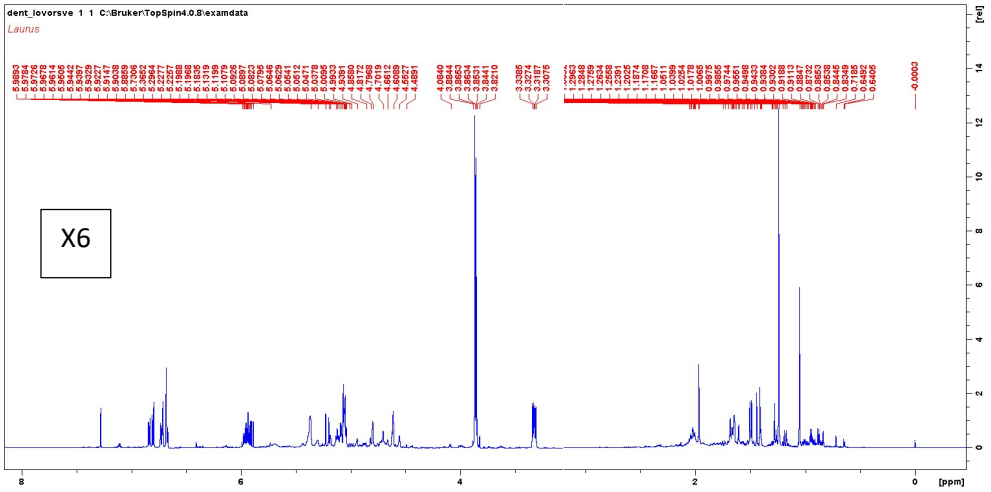

Figure S7. Bay laurel ^1H NMR spectrum (600 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 32 *K* data points; 256 scans; 0.37 Hz/point; 1 s delay).

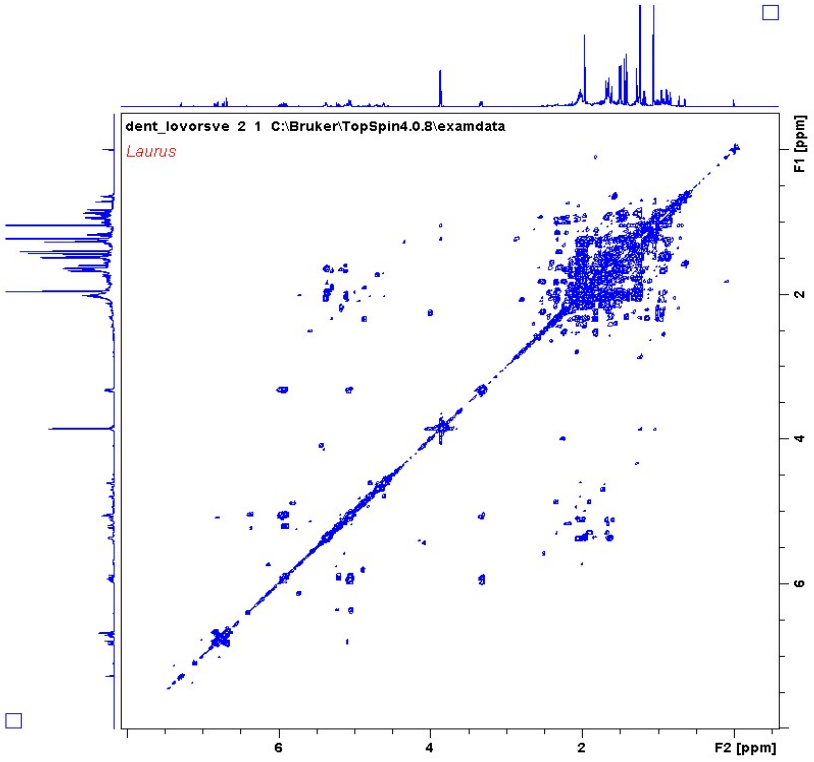

Figure S8. Bay laurel ^1H-^1H COSY NMR spectrum (600 MHz, $CDCl_3$-*d*, 25 °C).

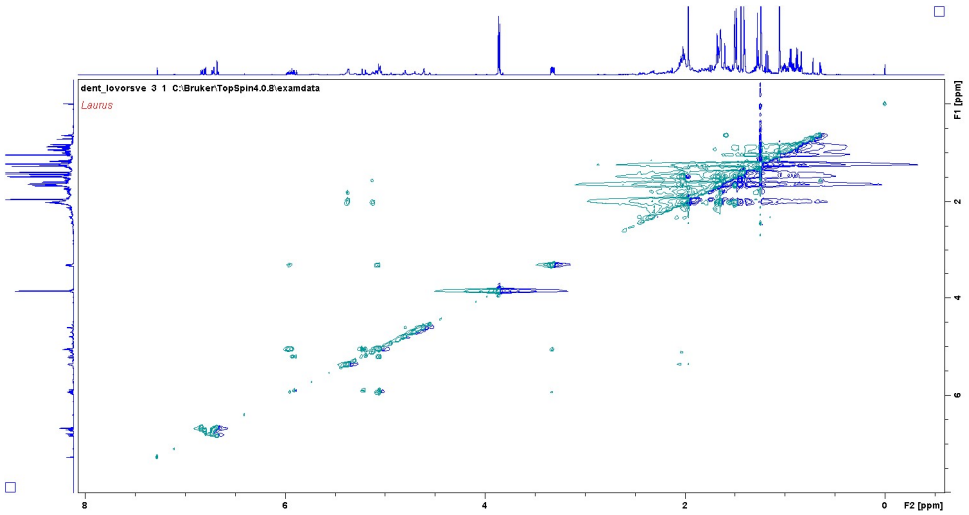

Figure S9. Bay laurel ^{1}H-^{1}H TOCSY NMR spectrum (600 MHz, $CDCl_3$-*d*, 25 °C). .

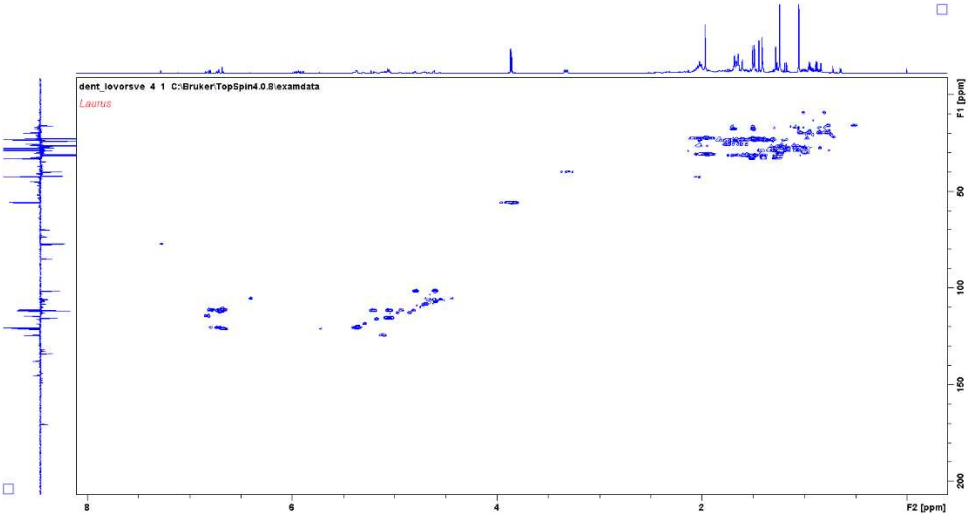

Figure S10. Bay laurel ^{1}H-^{13}C HMQC NMR spectrum ($CDCl_3$-*d*, 25 °C). The 600 MHz ^{1}H NMR spectrum is shown at the top edge and a 150 MHz ^{13}C NMR spectrum at the left-hand edge.

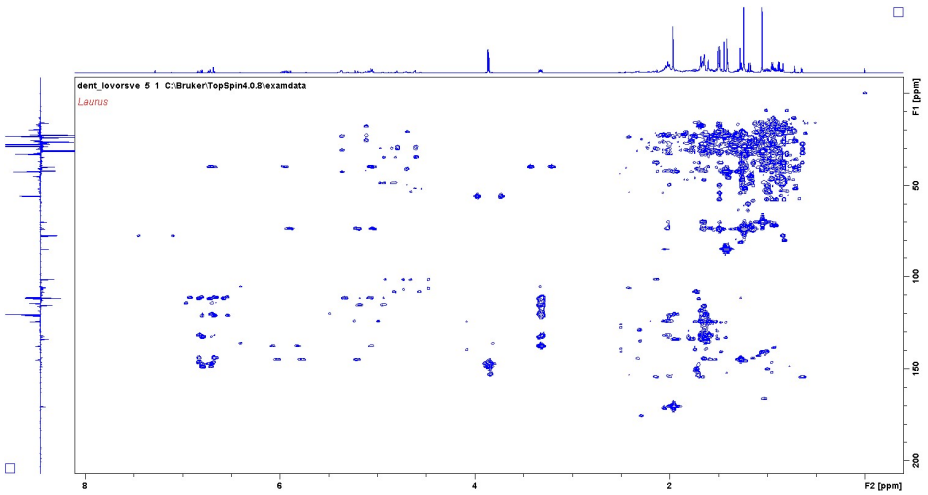

Figure S11. Bay laurel ^{1}H-^{13}C HMBC NMR spectrum ($CDCl_3$-*d*, 25 °C). The 600 MHz ^{1}H NMR spectrum is shown at the top edge and a 150 MHz ^{13}C NMR spectrum at the left-hand edge.

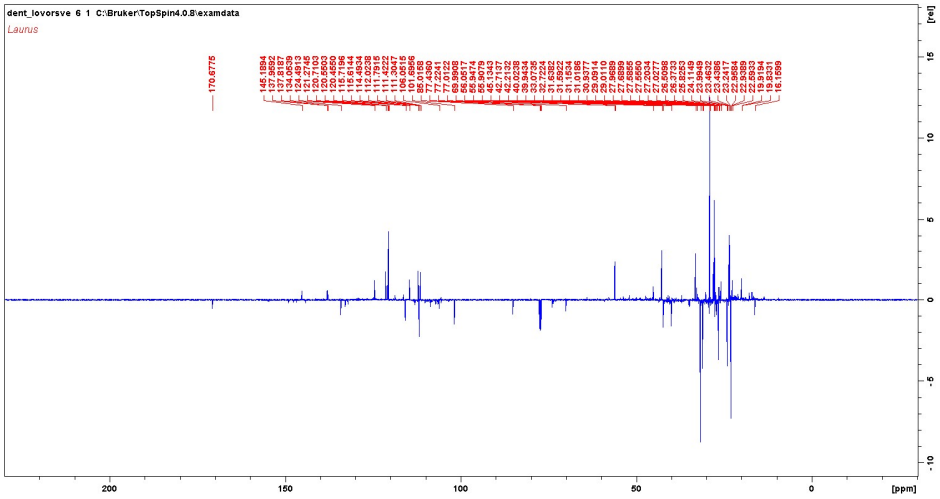

Figure S12. Bay laurel ^{13}C APT NMR spectrum (150 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 64 *K* data points; *ca.* 30000 scans; 0.60 Hz/point; 1 s delay).

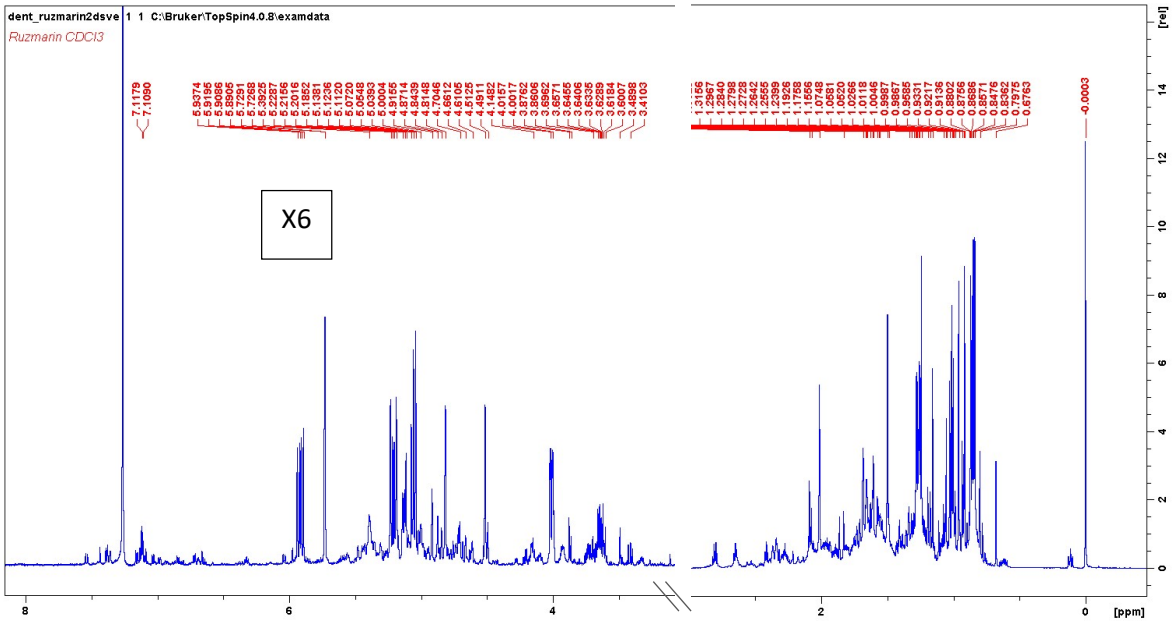

Figure S13. Rosemary ^{1}H NMR spectrum (600 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 32 *K* data points; 128 scans; 0.37 Hz/point; 1 s delay).

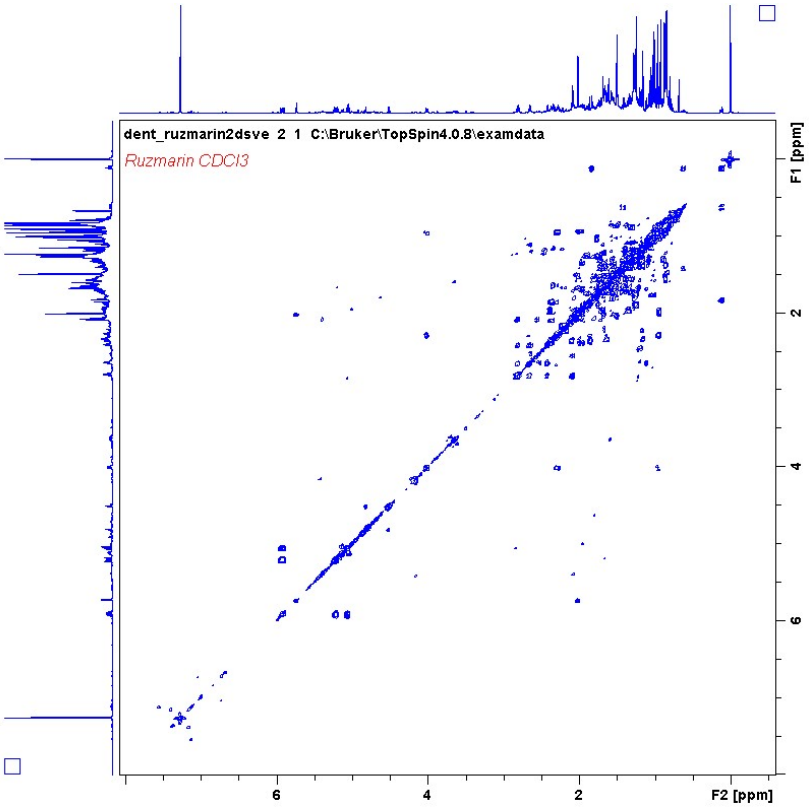

Figure S14. Rosemary ^{1}H-^{1}H COSY spectrum (600 MHz, $CDCl_3$-*d*, 25 °C).

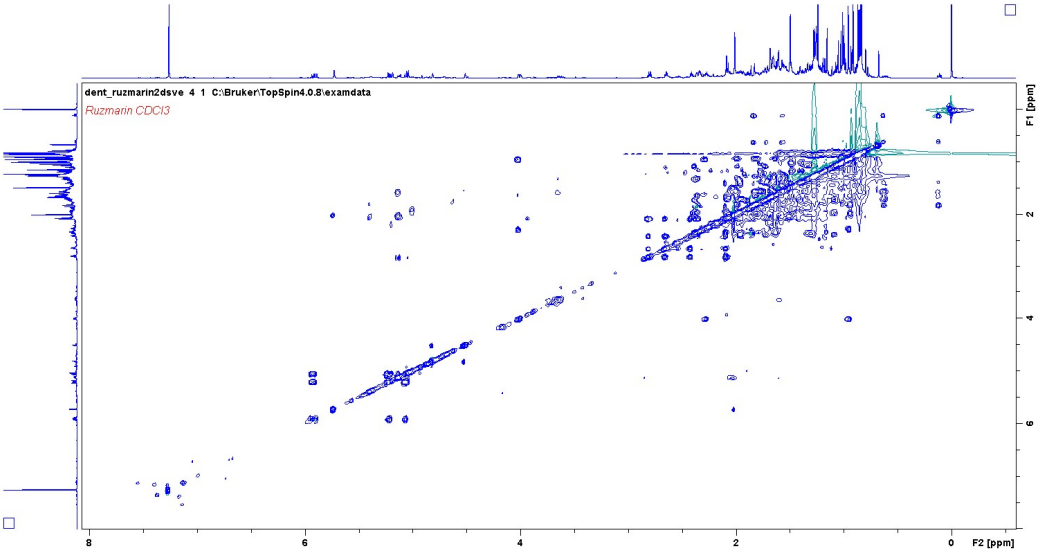

Figure S15. Rosemary ^{1}H-^{1}H TOCSY spectrum (600 MHz, $CDCl_3$-*d*, 25 °C). .

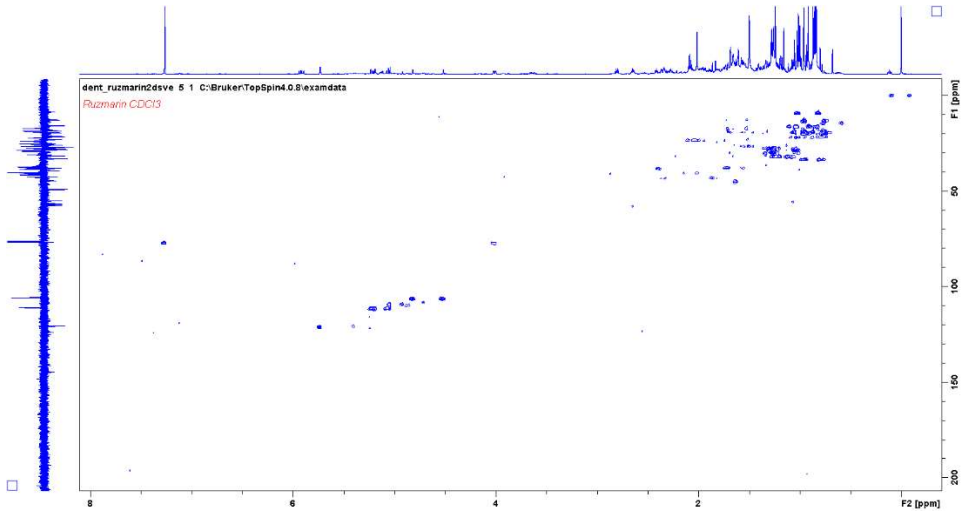

Figure S16. Rosemary ^{1}H-^{13}C HMQC spectrum ($CDCl_3$-*d*, 25 °C). The 600 MHz ^{1}H NMR spectrum is shown at the top edge and a 150 MHz ^{13}C NMR spectrum at the left-hand edge.

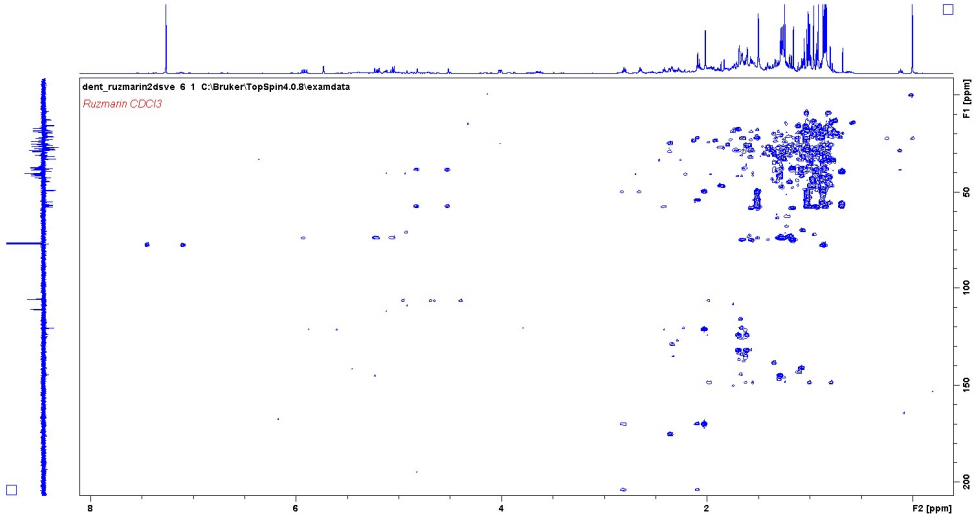

Figure S17. Rosemary ^{1}H-^{13}C HMBC spectrum ($CDCl_3$-*d*, 25 °C). The 600 MHz ^{1}H NMR spectrum is shown at the top edge and a 150 MHz ^{13}C NMR spectrum at the left-hand edge.

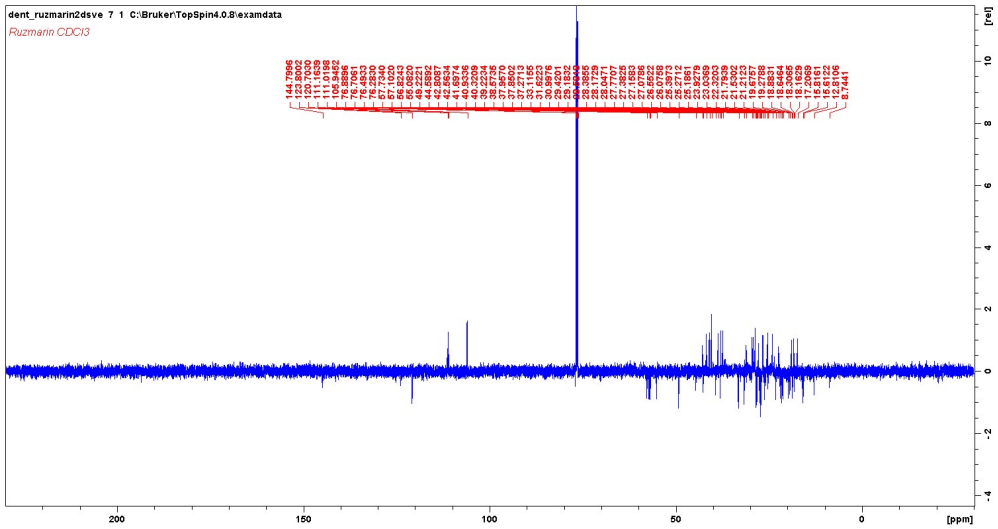

Figure S18. Rosemary ^{13}C APT NMR spectrum (150 MHz, 0.5 mL $CDCl_3$; 5 mm sample tube; 25 °C; 64 *K* data points; *ca.* 34000 scans; 0.60 Hz/point; 1 s delay).

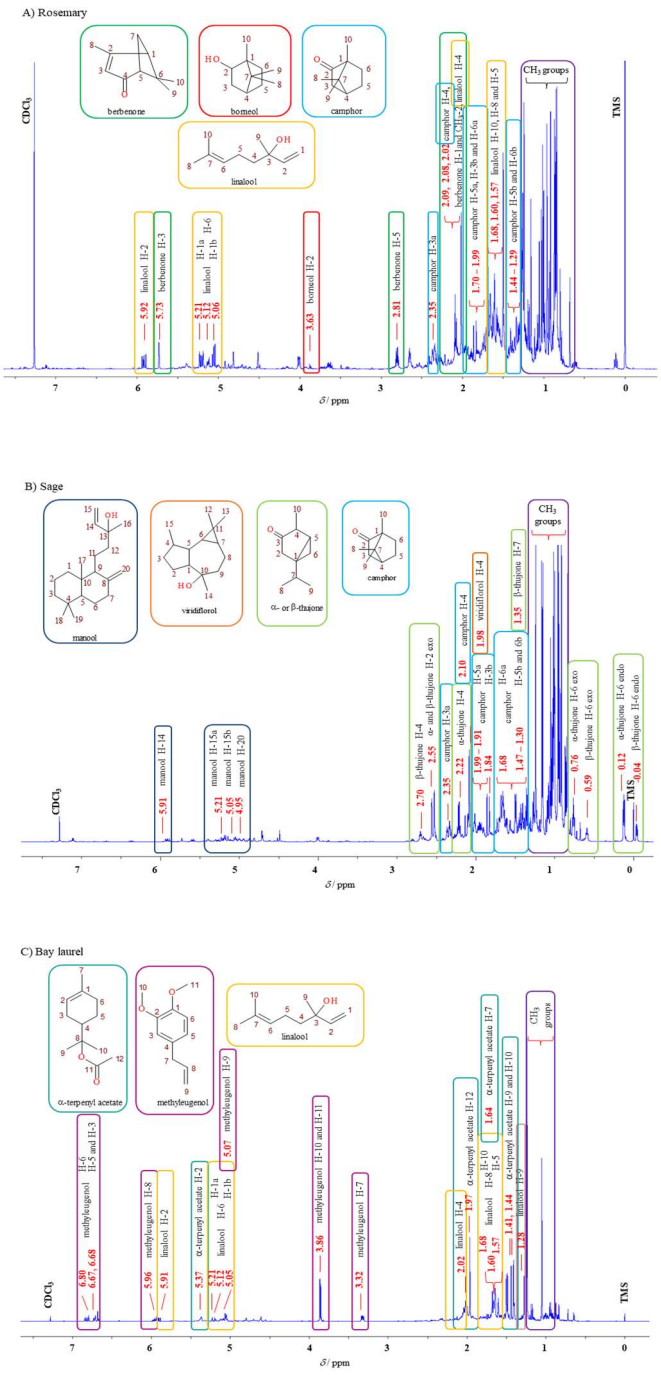

Figure S19. A) Rosemary, B) sage and C) bay laurel essential oils ^{1}H NMR spectra at 600 MHz in $CDCl_3$-*d*. Enumeration scheme used for the assignment of the NMR spectra is shown for every compound.

Supplementary file 2 of the manuscript:

Effect of Enzymatic, Ultrasound and Reflux Extraction Pretreatments on the Yield and Chemical Composition of Essential Oils

Anđela Miljanović[1], Ana Bielen[1,*], Dorotea Grbin[1], Zvonimir Marijanović[2], Martina Andlar[1], Tonči Rezić[1], Sunčica Roca[3], Igor Jerković[2], Dražen Vikić-Topić[3,4] and Maja Dent[1,*]

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Academic Editor: Petras Rimantas Venskutonis
Received: 8 September 2020; Accepted: 19 October 2020; Published: 20 October 2020

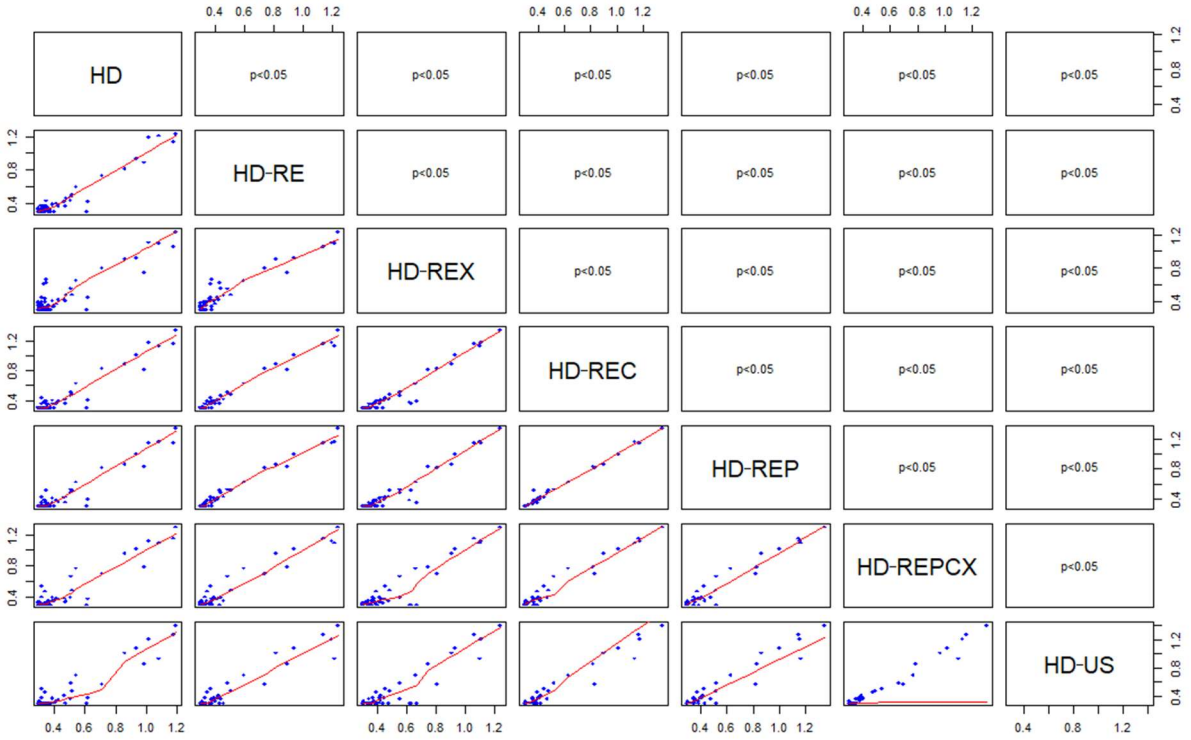

Figure S20. Scatter plot showing correlations between different pretreatments (on the diagonal) regarding chemical composition of sage essential oils. Significant p-values based on Spearman's rank test are shown above the diagonal, while bivariate scatter plots are shown below the diagonal.

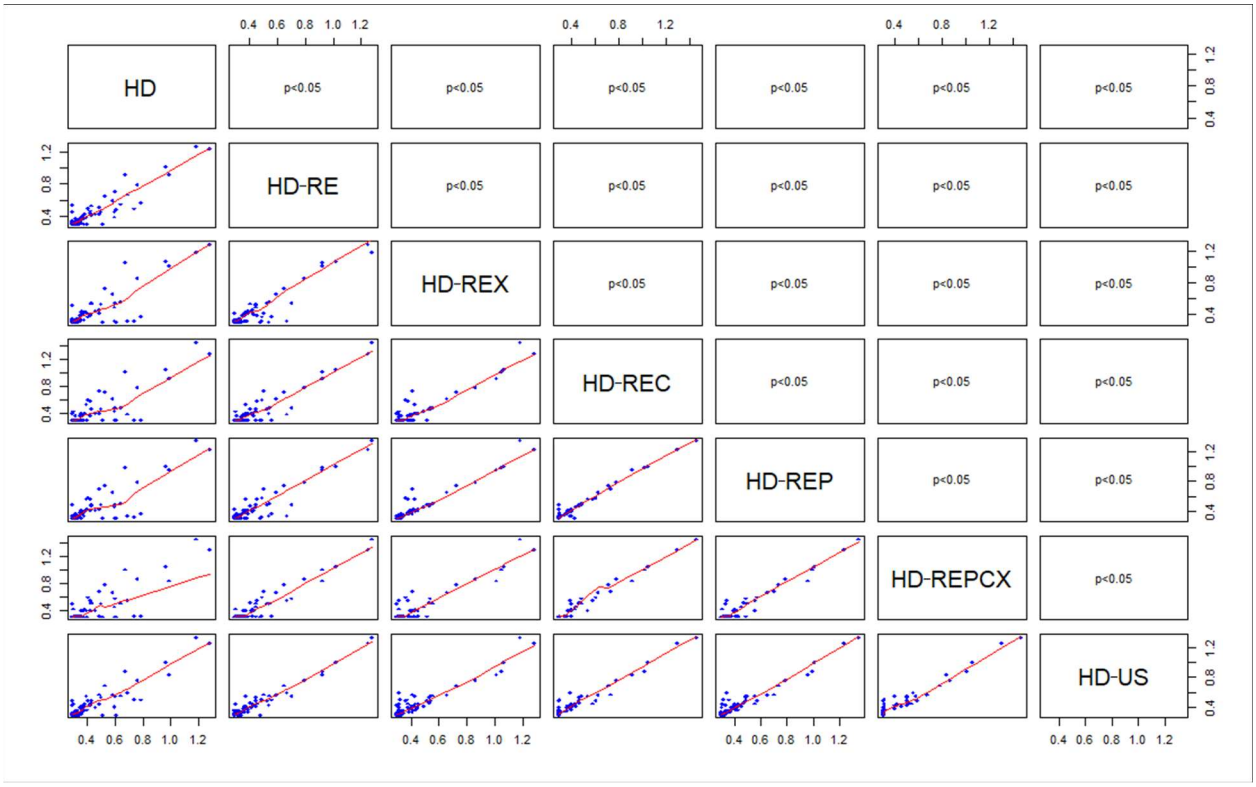

Figure S21. Scatter plot showing correlations between different pretreatments (on the diagonal) regarding chemical composition of bay laurel essential oils. Significant p-values based on Spearman's rank test are shown above the diagonal, while bivariate scatter plots are shown below the diagonal.

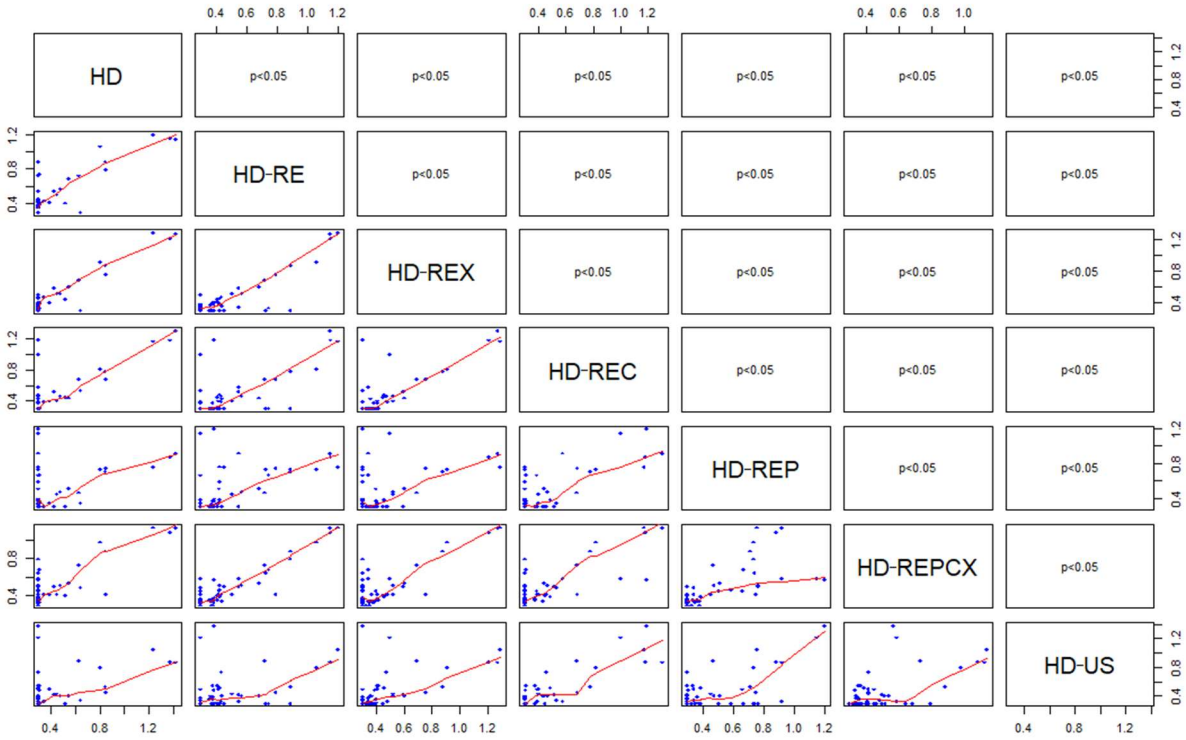

Figure S22. Scatter plot showing correlations between different pretreatments (on the diagonal) regarding chemical composition of rosemary essential oils. Significant p-values based on Spearman's rank test are shown above the diagonal, while bivariate scatter plots are shown below the diagonal.

Supplementary file 3 of the manuscript:

Effect of Enzymatic, Ultrasound, and Reflux Extraction Pretreatments on the Yield and Chemical Composition of Essential Oils

Anđela Miljanović [1], Ana Bielen [1,*], Dorotea Grbin [1], Zvonimir Marijanović [2], Martina Andlar [1], Tonči Rezić [1], Sunčica Roca [3], Igor Jerković [2], Dražen Vikić-Topić [3,4] and Maja Dent [1,*]

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Academic Editor: Petras Rimantas Venskutonis
Received: 8 September 2020; Accepted: 19 October 2020; Published: 20 October 2020

Table S1. Chemical composition of sage essential oils isolated by hydrodistillation with and without different pretreatments.

	group	compound	RI[1]	% total peak area						
				HD[2]	HD-RE[3]	HD-REX[4]	HD-REC[5]	HD-REP[6]	HD-REP CX[7]	HD-US[8]
monoterpenes	monoterpene hydrocarbons	*cis*-salvene	< 900	0.01	0.01	0.08	0.02	0.03	0.15	0.01
		tricyclene	929	0.03	0.04	0.01	0.01	0.01	0.10	0.01
		α-thujene	932	0.01	0.01	0.01	0.01	0.01	0.01	0.01
		α-pinene[S]	941	1.25	1.03	1.56	1.30	1.35	2.64	1.83
		camphene[S]	956	1.49	1.94	2.54	2.26	2.26	3.85	2.91
		verbenene	962	0.04	-[9]	-	-	-	-	-
		sabinene[S]	979	0.01	0.01	0.01	0.01	0.01	0.01	0.01
		β-pinene[S]	982	0.45	0.47	0.59	0.53	0.59	0.46	0.40
		β-myrcene[S]	992	0.08	0.12	0.34	0.28	0.25	0.53	0.37
		δ-car-3-ene[S]	1014	0.05	-	-	-	-	-	-
		α-terpinene[S]	1021	0.01	0.01	0.02	0.01	0.01	0.09	0.01
		p-cymene[S]	1030	0.19	0.22	0.47	0.46	0.39	0.99	0.90
		limonene[S]	1034	0.10	0.32	0.85	0.77	0.68	1.46	1.16
		γ-terpinene[S]	1063	0.03	0.01	0.12	0.02	0.01	0.08	0.01
		α-terpinolene	1091	0.06	0.01	0.01	0.01	0.02	0.05	0.01
	oxygenated monoterpenes	1,8-cineole[S]	1038	5.22	4.56	6.09	5.94	5.31	7.23	8.22
		linalool[S]	1104	2.17	0.61	0.79	0.59	0.51	0.37	0.39

		α-thujone*[S]	1110	13.52	15.33	15.16	20.15	19.91	18.18	23.48
		β-thujone*[S]	1121	6.57	6.63	6.45	8.33	8.03	8.51	10.22
		chrysanthenone	1129	0.21	-	-	-	-	0.04	-
		thujyl alcohol	1141	0.45	0.38	0.19	0.28	0.25	0.23	0.27
		camphor*[S]	1149	12.99	11.69	9.50	12.58	11.98	12.43	17.03
		pinocarvone	1167	0.07	-	-	-	-	-	-
		borneol[S]	1172	7.73	5.77	3.52	4.59	4.76	4.12	5.18
		trans-pinocarvone	1179	0.33	-	-	-	-	-	-
		4-terpineol[S]	1182	0.92	0.65	0.65	0.61	0.63	0.44	0.53
		p-cymen-8-ol[S]	1191	0.28	0.18	0.01	0.01	0.01	0.12	0.01
		α-terpineol[S]	1195	0.99	0.31	0.58	0.32	0.28	0.18	0.01
		myrtenol[S]	1200	0.37	0.13	-	-	-	0.05	-
		homomyrtenol	1208	0.39	-	-	-	-	-	-
		trans-carveol	1224	0.17	0.11	0.01	0.01	0.01	0.07	-
		geraniol[S]	1261	0.11	0.01	-	-	-	-	-
		bornyl acetate[S]	1287	1.31	1.20	1.04	1.04	1.30	1.09	1.00
		trans-sabinyl acetate	1294	0.61	0.51	0.37	0.34	0.44	0.39	0.16
		trans-carvyl acetate	1341	0.04	-	-	-	-	-	-
		α-terpenyl acetate	1355	0.25	0.68	2.25	0.34	1.32	-	-
	total monoterpenes			**58.51**	**52.95**	**53.22**	**60.82**	**60.36**	**63.78**	**74.23**
sesquiterpenes	sesquiterpene hydrocarbons	α-ylangene	1373	0.06	-	-	0.01		0.04	-
		α-copaene[S]	1378	0.18	-	0.12	0.02	0.01	0.11	-
		β-elemene[S]	1393	-	-	0.26	0.01	0.01	-	-
		trans-caryophyllene[S]	1421	1.21	0.73	1.59	0.93	1.03	0.51	0.30
		α-cadinene	1540	0.04	0.01	0.01	0.01	0.01	0.07	0.01
		α-guaiene	1441	0.01	0.01	0.22	0.03	0.03	0.01	0.01
		α-humulene[S]	1456	3.15	3.44	4.40	4.78	4.60	3.00	1.76
		alloaromadendrene[S]	1462	0.06	0.01	0.01	0.03	0.01	0.08	0.01
		α-amorphene	1478	0.32	0.19	0.28	0.27	0.20	0.25	0.01
		β-selinene	1488	0.05	-	0.16	0.01	-	-	-

		ledene[S]	1496	-	-	0.43	0.05	-	0.16	-
		α-muurolene	1501	0.13	-	0.18	0.01	-	0.07	-
		β-bisabolene	1510	0.07	-	-	-	-	-	-
		γ-cadinene	1515	0.26	0.07	0.19	0.10	0.09	0.10	0.01
		δ-cadinene[S]	1525	0.67	0.35	0.62	0.45	0.35	0.37	0.21
		trans-α-bisabolene	1545	-	0.01	-	-	-	-	-
		α-calacorene	1546	0.08	0.01	0.17	0.01	0.01	0.01	-
	oxyganeted sesquiterpenes	berbenone	1212	2.10	0.01	-	-	-	-	-
		spathulenol	1581	-	0.10	0.50	-	0.01	-	-
		caryophyllene oxide[S]	1584	1.00	0.86	1.01	0.62	0.71	0.25	-
		veridiflorol*	1594	10.13	14.39	10.60	11.70	12.63	10.64	6.52
		α-caryophylladienol	1640	0.31	-	0.29	0.05	0.01	-	-
		α-cadinol	1646	-	0.16	-	0.01	-	0.09	-
		β-eudesmol[S]	1654	0.10	0.18	0.46	0.02	-	0.06	-
		t-muurolol	1658	0.19	0.27	0.74	0.01		0.09	-
		(*E*,*E*)-farnesyl acetone	1919	0.15	-	-	-	-	-	-
		manool*	2055	8.30	13.74	10.80	12.99	12.24	11.47	14.33
	total sesquiterpenes			**28.57**	**34.54**	**33.04**	**32.12**	**31.95**	**27.38**	**23.17**
others	phenylpropane derivatives	thymol[S]	1296	0.04	0.36	0.01	0.01	0.01	0.06	0.01
		carvacrol[S]	1307	0.50	0.01	0.17	0.02	0.01	0.26	0.01
		eugenol[S]	1363	0.23	0.36	2.68	0.47	0.23	-	-
		methyleugenol[S]	1409	0.15	0.34	2.14	0.37	0.45	-	-
		elemicin	1561	-	-	0.27	-	-	-	-
	other compounds	methyl jasmonate[S]	1651	0.11	-	-	-	-	-	-
		pentadecanal[S]	1715	0.03	-	-	-	-	-	-
		hexadecan-1-ol[S]	1885	0.01	-	0.01	-	-	-	-
	total others			**1.07**	**1.07**	**5.28**	**0.87**	**0.7**	**0.32**	**0.02**
	total:			**88.15**	**88.56**	**91.54**	**93.81**	**93.01**	**91.48**	**97.42**

[1]Retention index on a MSD model 5975 C and HP-5MS; [2]HD - hydrodistillation without pretreatment (negative control); [3]HD-RE - hydrodistillation with reflux extraction pretreatment; hydrodistillation with reflux extraction

pretreatment assisted with enzymes: [4]HD-REX xylanase; [5]HD-REC cellulase; [6]HD-REP pectinase; [7]HD-REPCX pectinase + cellulase + xylanase; [8]HD-US hydrodistillation with ultrasonic pretreatment; [9](-) not detected; * - compounds confirmed by NMR, [S] - compounds identified with the standard.

Table S2. Chemical composition of bay laurel essential oils isolated by hydrodistillation with and without different pretreatments.

	Group	**Compound**	**RI[1]**	**% Total Peak Area**						
				HD[2]	HD-RE[3]	HD-REX[4]	HD-REC[5]	HD-REP[6]	HD-REPCX[7]	HD-US[8]
monoterpes	monoterpene hydrocarbons	α-thujene	932	0.04	0.04	0.02	0.01	0.14	0.01	0.09
		α-pinene[S]	941	0.56	1.12	0.32	2.05	1.77	1.88	1.10
		camphene[S]	956	0.28	0.21	0.08	0.23	0.25	0.02	0.23
		sabinene[S]	979	1.08	1.23	0.60	3.39	3.03	2.74	1.74
		β-pinene[S]	982	0.66	0.89	0.41	1.82	1.63	1.47	0.84
		β-myrcene[S]	992	0.11	0.04	0.03	0.01	0.19	0.02	0.12
		δ-car-3-ene[S]	1014	-[9]	0.05	-	-	0.04	0.01	0.07
		α-terpinene[S]	1021	0.05	0.01	0.07	0.01	0.19	0.02	0.15
		p-cymene[S]	1030	0.14	0.09	0.08	0.01	0.21	0.04	0.32
		limonene[S]	1034	0.01	0.01	0.05	0.69	0.02	0.62	0.76
		γ-terpinene[S]	1063	0.08	0.07	0.13	0.32	0.32	0.02	0.28
		α-terpinolene	1091	0.03	-	0.05	-	0.09	-	0.08
	oxygenated monoterpenes	1,8-cineole	1038	13.26	16.86	13.23	26.40	20.15	27.10	19.56
		cis-sabinene hydrate	1073	0.10	0.33	0.04	-	-	-	0.22
		linalool*[S]	1104	7.80	6.39	8.30	6.27	7.14	4.91	4.92
		α-thujone[S]	1110	3.51	1.09	0.09	0.01	0.09	0.02	1.18
		β-thujone[S]	1121	1.97	0.45	0.06	-	0.01	-	0.53
		cis-p-menth-2-en-1-ol	1127	-	-	0.09	-	0.05	-	0.06
		chrysanthenone	1129		0.13	-	-	-	-	0.06
		thujyl alcohol	1141	0.09	-	-	-	-	-	-
		terpenene-1-ol	1146	0.11	-	0.15	-	-	-	0.07
		camphor[S]	1149	2.90	2.69	0.05	0.48	0.20	1.57	1.93
		pinocarvone	1167	-	0.09	-	-	-	-	0.12
		borneol[S]	1172	2.04	3.05	1.45	1.09	1.06	1.74	1.55

		isopinocamphone	1178	-	0.20	-	-	-	-	-
		4-terpineol[S]	1182	1.83	1.93	2.55	2.23	2.46	2.65	1.71
		p-cymen-8-ol[S]	1191	-	0.08	-	-	-	-	0.05
		α-terpineol[S]	1195	3.76	4.25	5.26	4.06	4.17	5.27	3.84
		myrtenol[S]	1200	-	0.14	0.06	-	-	-	-
		homomyrtenol	1208	-	0.21	-	-	-	-	-
		nerol[S]	1233	0.35	0.48	0.56	0.01	0.35	0.01	0.21
		linalyl acetate[S]	1259	0.29	0.07	0.12	0.01	0.03	0.01	0.02
		geraniol[S]	1261	-	0.10	0.09	-	-	-	-
		bornyl acetate[S]	1287	1.97	0.83	1.11	0.74	0.83	0.83	0.64
		trans-sabinyl acetate	1294	0.15	-	-	-	-	-	
		δ-terpinyl acetate	1319	0.90	0.63	0.81	0.51	0.61	0.46	0.54
		2-acetoxy-1,8-cineole	1344	0.16	0.19	0.24	0.01	0.18	0.02	0.12
		α-terpenyl acetate*	1355	16.94	15.71	17.18	17.69	15.16	18.16	15.84
		neryl acetate	1367	0.23	0.13	0.23	0.01	0.18	0.01	0.12
		trans-cinnamyl acetate	1449	0.20	-	0.15	-	0.12	-	-
		spathulenol	1581	2.40	1.52	1.61	1.08	1.17	1.18	1.62
		manool	2055	2.10	1.03	-	-	-	-	-
	total monoterpenes			**66.1**	**62.34**	**55.27**	**69.14**	**61.84**	**70.79**	**60.69**
sesquiterpenes	sesquiterpenes hydrocarbons	α-ylangene	1373	0.15	0.14	0.23	0.02	0.23	0.01	0.26
		α-copaene[S]	1378	0.06	0.10	0.04	0.01	0.01	0.01	0.09
		β-cubebene	1391	0.06	-	0.05	-	-	-	0.04
		β-elemene[S]	1393	0.50	0.40	0.69	1.47	1.63	0.50	1.53
		trans-caryophyllene[S]	1421	1.40	2.47	3.37	3.19	3.49	3.99	2.92
		α-guaiene	1441	0.30	0.31	0.50	0.46	0.55	0.49	0.46
		guaia-3,7-diene	1446	0.07	0.12	0.17	0.01	0.17	0.01	0.18
		α-humulene[S]	1456	0.67	0.74	0.54	0.38	0.56	0.46	0.66
		alloaromadendrene	1462	0.13	0.14	0.15	0.01	0.13	0.02	0.18
		α-amorphene	1478	0.10	0.14	0.06	0.01	0.01	0.01	0.12

		germacrene D[S]	1482	0.30	0.37	0.65	0.54	0.59	0.02	0.63
		β-selinene	1488	0.38	0.33	0.59	0.44	0.48	0.51	0.59
		bicyclogermacrene	1496	1.18	0.83	1.39	0.96	1.07	1.19	1.40
		germacrene A	1505	0.71	0.58	1.43	0.93	0.94	1.25	0.87
		γ-cadinene[S]	1515	0.25	0.33	0.41	0.32	0.39	0.02	0.52
		δ-cadinene	1525	0.44	0.66	0.70	0.54	0.53	0.58	0.81
		trans-α-bisabolene	1545	0.32	0.25	0.42	0.01	0.31	0.02	0.20
	oxygenated sesquiterpenes	berbenone	1212	-	1.49	-	0.52	0.15	1.17	0.70
		elemol[S]	1553	0.13	0.09	0.12	0.02	0.12	0.01	0.15
		nerolidol[S]	1568	0.19	0.06	0.11	0.01	0.07	0.02	0.01
		caryophyllene oxide[S]	1584	0.70	1.39	1.50	0.92	1.07	1.19	1.07
		globulol[S]	1586	0.18	-	-	-	-	-	-
		veridiflorol	1594	4.14	1.64	0.37	0.01	0.30	0.02	1.16
		isospathulenol	1642	0.41	-	0.13	-	0.19	-	0.24
		α-cadinol	1646	0.35	0.37	0.49	0.01	0.40	0.01	0.39
		β-eudesmol[S]	1654	1.11	0.67	0.85	0.52	0.67	0.02	0.78
		α-eudesmol	1657	1.25	-	-	-	-	-	-
		t-muurolol	1658	-	0.83	1.30	-	1.06	0.01	1.06
	total sesquiterpenes			**15.63**	**14.45**	**16.26**	**11.31**	**15.12**	**11.54**	**17.02**
others	phenylpropane derivatives	p-allylanisole[S]	1199	0.08	-	-	-	-	-	0.08
		3-phenylpropenal	1275	0.06	-	0.14	-	0.06	-	-
		thymol[S]	1296	0.05	-	-	-	-	-	
		carvacrol[S]	1307	0.16	-	-	-	-	-	
		eugenol[S]	1363	2.76	6.33	9.23	8.42	7.82	8.20	5.64
		methyleugenol*[S]	1409	7.15	8.52	9.71	9.21	8.20	9.33	7.95
		cis-methylisoeugenol	1500	0.54	-	0.62	-	0.70	-	0.32
		elemicin	1561	0.32	0.48	0.75	0.51	0.58	0.01	0.37
	other compounds	(Z)-hex-3-en-1-ol[S]	< 900	-	-	0.06	-	-	-	-
		nonan-2-one[S]	1094	-	-	0.04	-	-	-	-
		decanal[S]	1207	-	-	0.03	-	-	-	-

		undecan-2-one[S]	1295	-	0.17	0.22	-	0.16	-	0.12
		pentadecan-2-one[S]	1699	-	-	0.08	-	-	-	0.09
		hexadecanal[S]	1818	-	-	0.11	-	-	-	-
	total others			**11.12**	**15.5**	**20.99**	**18.14**	**17.52**	**17.54**	**14.57**
	total:			**92.85**	**92.29**	**92.52**	**98.59**	**94.48**	**99.87**	**92.28**

[1]Retention index on a MSD model 5975 C and HP-5MS; [2]HD - hydrodistillation without pretreatment (negative control); [3]HD-RE - hydrodistillation with reflux extraction pretreatment; hydrodistillation with reflux extraction pretreatment assisted with enzymes: [4]HD-REX xylanase; [5]HD-REC cellulase; [6]HD-REP pectinase; [7]HD-REPCX pectinase + cellulase + xylanase; [8]HD-US hydrodistillation with ultrasonic pretreatment; [9](-) not detected; * - compounds confirmed by NMR, [S] - compounds identified with the standard.

Table S3. Chemical composition of rosemary essential oils isolated by hydrodistillation with and without different pretreatments.

	Group	**Compound**	**RI[1]**	**% Total Peak Area**						
				HD[2]	HD-RE[3]	HD-REX[4]	HD-REC[5]	HD-REP[6]	HD-REPCX[7]	HD-US[8]
monoterpenes	monoterpene hydrocarbons	α-pinene[S]	941	2.25	3.28	2.93	2.77	0.93	3.36	5.88
		camphene[S]	956	0.47	0.62	0.54	0.50	0.19	0.59	1.19
		verbenene[S]	962	-[9]	-	0.17	-	-	0.19	0.35
		sabinene[S]	979	-	-	-	-	-	0.08	-
		β-pinene[S]	982	-	0.27	-	-	-	0.28	0.29
		β-myrcene[S]	992	-	-	0.10	-	-	-	-
		p-cymene[S]	1030	0.01	0.42	0.42	0.01	0.02	0.15	1.03
		δ-car-3-ene[S]	1014	-	0.33	0.33	-	-	0.21	0.51
		limonene[S]	1034	0.01	0.58	0.57	0.02	0.18	0.25	1.49
	oxygenated monoterpenes	1.8-cineole[S]	1038	4.34	9.49	6.19	4.55	3.44	7.44	4.24
		linalool*[S]	1104	5.00	5.75	5.57	4.02	3.13	5.57	1.38
		filifolone	1105	-	0.38	0.47	-	-	0.29	0.29
		α-thujone[S]	1110	-	0.72	0.93	0.87	-	0.61	3.47
		β-thujone[S]	1121	-	0.38	0.53	0.38	-	0.35	1.49
		chrysanthenone	1129	0.20	0.72	0.99	0.45	-	0.58	0.14

		camphor*[S]	1149	15.38	13.82	17.58	12.77	3.73	11.56	9.01
		pinocarvone	1167	-	0.73	0.28	0.68	-	0.20	0.58
		borneol*[S]	1172	24.38	12.03	16.77	18.11	6.22	11.26	5.39
		isopinocamphone	1178	0.69	1.52	1.89	1.28	-	1.15	0.64
		4-terpineol[S]	1182	1.00	1.70	1.31	0.90	0.97	1.24	0.52
		p-cymen-8-ol[S]	1191	-	-	0.38	0.37	-	0.24	-
		α-terpineol[S]	1195	5.04	4.17	3.73	2.81	3.60	3.34	0.83
		myrtenol[S]	1200	1.28	0.56	0.78	0.78	-	0.53	-
		homomyrtenol	1208	2.39	-	-	1.39	0.19	1.05	0.23
		nerol[S]	1233	-	-	-	-	-	0.27	-
		geraniol[S]	1261	-	-	-	-	-	0.21	-
		bornyl acetate[S]	1287	-	0.41	-	-	0.33	0.23	0.47
		α-terpenyl acetate[S]	1355	-	3.31	-	-	3.46	2.38	-
		spathulenol	1581	-	0.64	-	-	3.81	1.28	-
	total monoterpenes			**62.44**	**61.83**	**62.46**	**52.66**	**30.2**	**52.17**	**39.42**
sesquiterpenes	sesquiterpene hydrocarbons	β-elemene[S]	1393	-	-	-	-	0.39	0.22	-
		trans-caryophyllene[S]	1421	1.53	2.84	2.00	0.76	1.28	1.47	0.56
		α-muurolene	1501	-	-	0.22	-	-	0.14	-
		α-humulene	1456	-	0.66	0.83	1.05	0.44	0.45	1.24
		α-guaiene	1441	-	-	-	-	-	0.11	-
		α-copaene	1378	-	-	0.36	-	-	0.13	-
		alloaromadendrene	1462	-	-	-	-	0.40	-	-
		β-selinene	1488	-	-	-	-	-	0.13	-
		α-copaene	1378	-	-	0.36	-	-	0.13	-

		α-amorphene	1478	-	-	0.40	-	-	0.13	-
		γ-cadinene	1515	0.01	0.43	0.49	0.02	0.32	0.28	0.02
		α-calacorene	1546	-	-	-	-	-	0.19	-
		germacrene A	1505	-	0.40	-	-	-	-	-
		δ-cadinene[S]	1525	0.81	1.22	1.31	0.50	0.83	0.60	0.69
	oxygenated sesquiterpenes	berbenone *	1212	21.76	12.17	14.40	13.24	5.55	9.93	5.56
		spathulenol	1581	-	0.64	-	-	3.81	1.28	-
		caryophyllene oxide	1584	-	0.67	-	-	1.88	0.92	-
		veridiflorol	1594	-	-	1.14	8.01	11.95	1.85	14.32
		α-caryophylladienol	1640	-	-	-	0.43	2.60	0.83	1.11
		α-cadinol	1646	-	0.63	0.47	-	1.21	0.82	-
		β-eudesmol	1654	-	0.84	-	-	3.80	1.15	-
		t-muurolol	1658	-	1.54	0.19	1.76	6.31	1.84	0.13
		manool	2055	-	0.45	-	13.47	13.65	1.71	22.05
	total sesquiterpenes			**24.11**	**23.53**	**21.81**	**39.24**	**56.43**	**25.1**	**45.68**
others	phenylpropane derivatives	thymol[S]	1296	-	-	0.22	-	-	0.20	-
		carvacrol[S]	1307	-	-	-	-	-	0.32	-
		eugenol[S]	1363	-	5.70	-	-	3.44	4.24	-
		methyleugenol[S]	1409	0.01	3.56	0.17	0.02	2.65	2.82	-
		elemicin	1561	-	0.46	-	-	0.46	0.39	-
	other compounds	oct-1-en-3-ol	983	-	-	0.24	-	-	-	-
		methyl jasmonates	1651	-	-	0.30	-	-	0.62	-
		hexadecan-1-ol[S]	1885	-	-	-	-	0.23	0.13	-
	total others			**0.01**	**9.72**	**0.93**	**0.02**	**6.78**	**8.72**	**0**

total:	**86.56**	**95.07**	**85.2**	**91.92**	**93.41**	**85.99**	**85.1**

[1]Retention index on a MSD model 5975 C and HP-5MS; [2]HD - hydrodistillation without pretreatment (negative control); [3]HD-RE - hydrodistillation with reflux extraction pretreatment; hydrodistillation with reflux extraction pretreatment assisted with enzymes: [4]HD-REX xylanase; [5]HD-REC cellulase; [6]HD-REP pectinase; [7]HD-REPCX pectinase + cellulase + xylanase; [8]HD-US hydrodistillation with ultrasonic pretreatment; [9](-) not detected; * - compounds confirmed by NMR, [S] - compounds identified with the standard.

Appendix 1 of the manuscript:

Effect of Enzymatic, Ultrasound, and Reflux Extraction Pretreatments on the Yield and Chemical Composition of Essential Oils

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Academic Editor: Petras Rimantas Venskutonis
Received: 8 September 2020; Accepted: 19 October 2020; Published: 20 October 2020

Experimental details and analysis of nuclear magnetic resonance (NMR) analysis spectra

The NMR spectra were recorded on a Bruker Avance 600 spectrometer using a 5-mm broad band probehead equipped with z-gradient coils, operating at 600.130 MHz for ^{1}H and 150.903 MHz for ^{13}C. All spectra were measured from $CDCl_3$-d with tetramethylsilane (TMS) as the internal standard at 25 °C. Individual resonances were assigned on the basis of their chemical shifts, multiplicity, signal intensities, and by using correlation signals in the spectra of 2D NMR techniques: correlation spectroscopy (^{1}H-^{1}H COSY), total correlation spectroscopy (^{1}H-^{1}H TOCSY), heteronuclear multiple quantum coherence (^{1}H-^{13}C HMQC) and heteronuclear multiple bond correlation (^{1}H-^{13}C HMBC).

One-dimensional ^{1}H and ^{13}C NMR spectra were recorded at Bruker AV600 by using 32K and 64K data points and spectral widths of 12019 Hz and 39370 Hz for proton and carbon (APT) experiments, respectively. Digital resolution was 0.37 Hz and 0.60 Hz per point, respectively. The number of scans was 128–256 for ^{1}H and ca. 34000 for 13C APT spectra. Two-dimensional homonuclear ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY spectra were recorded by using 2048 points in f2 and 512 increments in f1 dimension. Increments were obtained by 8 and 24 scans each, with 9615 Hz spectral width, and by using a relaxation delay of 1.0 and 1.5 s, respectively. The zero filling of f1 data was performed to 1024 points. The digital resolution was 4.69 and 18.75 Hz per point in f2 and f1 domains, respectively. TOCSY spectra were obtained with the mixing time of 60 ms. The inverse ^{1}H-13C correlation experiments, HMQC and HMBC were recorded with 2048 points in f2 dimension and 256 increments in f1 dimension, and were subsequently zero-filled to 1024 points. For each increment, 128 (HMQC) and 256 (HMBC) scans were collected, using relaxation delay of 1.0 s. The spectral widths were 9615 Hz (f2) and 36240 Hz (f1), with the corresponding resolution of 4.69 and 141.48 Hz per point in f2 and f1 dimensions, respectively. In HMBC spectra, the additional delay of 65 ms was used for detecting the long-range C–H couplings.

The atom signals of the most terpenes observed by GC-MS analysis in more than 5% of total peak area, were also obtained in recorded NMR spectra. Their assignation was made by signal chemical shifts and multiplicity in the ^{1}H and ^{13}C NMR spectra, and by the cross peaks in ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HMBC. Due to the large spin splitting within the molecules, some atom signals came in the proton spectra as very low intensity multiplet. The signal detection in spectra was also difficult by their large overlap in the higher magnetic field due to the similarity of the chemical structures of the observed molecules. As the result of that 1,8-cineole and α-terpineol could not be detected with certainty. The most useful for assignation were the long-range cross peaks in the HMBC spectra. Fig. 3 shows the spectra of rosemary (Fig. 3A), sage (Fig. 3B) and bay laurel (Fig. 3C) extracts with labelled atom signals of compounds found in the mixture. In the same figure, the structures of the compounds with the numbering used in NMR spectroscopy are presented. Atom signals of the linalool were fully assigned in the proton spectra because of the cross peaks found in HMBC: H-1, H-2, H-9 to C-3; H-5, H-8, H-10

to C-6; H-8, H-10 to C-7. In COSY spectrum its H-1 to H-2 cross peak was observed. Recorded TOCSY revealed H-4 – H-5 – H-6 spin system of the linalool. The methyleugenol atom signals were revealed also by HMBC cross peaks: H-5 to C-7; H-6 to C-2, C-4; H-7 to C-3, C-4, C-8, C-9; H-10 to C-2; H-11 to C-1. The COSY experiment revealed three vicinal couplings in molecule: H-5 to H-6; H-7 to H-8; H-8 to H-9. Atom signals of camphor were observed thanks to the deshielded C-2 atom (219.5 ppm) in the ^{13}C APT spectra, and HMBC cross peaks observed between: H-3b to C-2, C-5, C-1; H-8, H-9 to C-7; H-10 to C-2, C-6, C-7. The α-terpenyl acetate signals were obtained because of the acetyl group signals: methyl H-12 at 1.97 ppm and carbonyl C-11 at 170.8 ppm. In recorded HMBC spectra cross peaks between: H-2 to C-4, C-7; H-9, H-10 to C-4, C-8, C-10, were also found. Chemical shifts and multiplicities of other α-terpenyl acetate signals (H-3a, H-3b, H-4, H-5a, H-5b, H-6, H-7) in our spectra agree with literature data [1]. Signals of the α- and β-thujone were in recorded spectra confirmed by cross peaks in COSY and/or TOCSY spectra. For α- thujone: H-5 to H-6 endo and H-6 exo; H-6 endo to H-6 exo; H-6 endo and H-6 exo to H-7; H-4 to H-10, couplings were found. For β-thujone: H-6 endo to H-6 exo and H-6 endo and H-6 exo to H-7 were observed. Thujones signals were also confirmed by literature data [2]. All atom signals of berbenon, borneole, manool and veridiflorol were not observed in recorded NMR spectra, but each of the above molecules have some signals that confirmed their presence in the spectra of the investigating mixtures. Berbenone was assigned because of its characteristic C-2 (170.2 ppm), H-3/C-3 (5.73 ppm, 121.6 ppm), C-4 (204.2 ppm) and H-1/C-1 (2.08 ppm, 49.4 ppm) atom signals in ^{1}H and ^{13}C NMR spectra. Cross peaks found in the HMBC spectra were between: H-1 to C-4; H-5 to C-2, C-4; H-8 to C-2. At borneol molecule, H-2 atom signal at 3.63 ppm was easily detected because it does not overlap with other signals. In TOCSY spectra, H-2 to H-3 cross peak was observed, as well as methyl H-10 to C-2 in HMBC spectra. At manool molecule deshielded ^{1}H atom signals of H-8, H-14, H-15a and H-15b were found. In COSY spectra H-14 to H-15 cross peak was observed. In HMBC spectra H-14 and H-15 to C-13 cross peaks were found. Other signals were found by comparison with literature data [3]. Veridiflorol was also only partially assigned, since all ^{1}H atom signals fall in the range of 0.8 to 1.9 ppm where they overlap with the other molecule signals of the mixture (e.g. α-thujone and camphor). According to the signal intensity and coupling constant (6.5 Hz) the one at 0.93 ppm was attributed to veridiflorol methyl H-15 atom, and the cross peaks of H-5 to H-6 and H-4 to H-15 atoms in the COSY spectrum was detected [4]. The observed NMR spectra were compared with data published in the Biological Magnetic Resonance Data Bank database where possible [5].

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Supplementary file 1 of the manuscript

Essential Oils of Sage, Rosemary and Bay Laurel Inhibit the Life Stages of Oomycete Pathogens Important in Aquaculture

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Table S1. Volatile composition of essential oils determined by GC-MS.

Compound	RI[1]	% total peak area		
		Rosemary	Bay laurel	Sage
(*Z*)-hex-3-en-1-ol	< 900	0.03	-	-
cis-salvene	< 900	-	-	0.14
tricyclene	929	0.16	-	0.20
α-thujene	932	0.03	0.23	0.08
α-pinene	941	10.79	2.48	2.96
camphene	956	2.41	0.33	5.15
sabinene	979	0.04	4.90	0.33
oct-1-en-3-ol	981	0.27	-	0.01
β-pinene	982	0.58	2.28	1.50
octan-3-one	990	0.15	-	-
β-myrcene	992	1.10	0.39	0.36
α-phellandrene	1010	0.25	0.06	0.04
δ-car-3-ene	1014	1.72	0.10	-
α-terpinene	1021	0.26	0.23	0.12
p-cymene	1030	1.82	0.36	0.49
limonene	1034	3.36	0.87	1.26
1.8-cineole	1038	7.29	26.79	12.53
γ-terpinene	1063	0.30	0.62	0.24
α-terpinolene	1091	0.65	0.10	0.18
linalool	1104	4.37	6.95	1.13
filifolone	1108	0.48	-	-
α-thujone	1110	0.80	0.37	20.34
β-thujone	1121	0.11	0.08	1.87
chrysanthenone	1131	1.78	-	-
trans-pinocarveol	1147	0.19	-	-
camphor	1149	11.71	0.39	23.86
pinocarvone	1169	0.33	-	-
borneol	1172	8.94	0.91	4.14
isopinocamphone	1181	1.67	-	-
4-terpineol	1182	1.02	2.25	0.61
p-cymen-8-ol	1191	0.27	-	-
α-terpineol	1195	2.05	4.12	0.34
myrtenol	1201	0.48	-	-
nopol	1209	1.12	-	-
berbenone	1212	6.11	-	0.23
trans-carveol	1224	0.09	-	-
nerol	1233	-	0.28	-
β-citronelol	1234	0.16	-	-
geraniol	1261	0.15	-	-
3-phenylpropenal	1275	-	0.06	-
bornyl acetate	1287	0.80	0.66	1.16
trans-sabinyl acetate	1294	-	-	0.07
undecan-2-one	1295	-	0.10	-
thymol	1296	0.11	-	-
carvacrol	1307	0.25	-	-
α-terpenyl acetate	1355	0.17	13.18	0.48
eugenol	1363	0.23	4.37	0.07
α-ylangene	1373	0.18	0.14	-
α-copaene	1378	0.52	0.05	-
β-cubebene	1391	-	0.03	-

β-elemene	1393	-	0.83	-
methyleugenol	1409	0.34	8.79	0.37
α-gurjunene	1413	-	0.10	-
trans-caryophyllene	1421	2.84	1.81	1.74
α-guaiene	1441	-	0.28	-
guaia-3.7-diene	1446	-	0.12	-
α-humulene	1456	1.24	0.40	3.17
alloaromadendrene	1462	0.04	0.17	0.18
α-amorphene	1478	-	0.07	-
γ-muurolene	1481	0.52	-	-
germacrene D	1482	-	0.28	-
β-selinene	1488	-	0.40	-
ar-curcumene	1489	0.07	-	-
β-eudesmene	1490	0.10	-	-
bicyclogermacrene	1496	-	0.91	-
zingiberene	1498	0.30	-	-
cis-methylisoeugenol	1500	-	0.25	-
α-muurolene	1504	0.25	-	-
α-bulnesene	1509	-	0.55	-
β-bisabolene	1513	0.19	-	-
γ-cadinene	1515	0.53	0.36	-
δ-cadinene	1525	1.17	0.34	0.07
trans-α-bisabolene	1545	-	0.14	-
α-calacorene	1546	0.13	-	-
elemicin	1561	-	0.33	-
spathulenol	1581	0.04	1.07	-
caryophyllene oxide	1584	1.18	0.84	-
veridiflorol	1594	2.57	-	10.31
α-cadinol	1646	0.27	-	-
β-eudesmol	1654	-	0.50	-
α-bisabolol	1689	0.27	-	-
hexadecan-1-ol	1886	0.06	-	-
farnesyl acetone	1924	0.19	-	-
manool	2055	2.25	-	1.42
Total %		89.6	92.2	97.2

[1]Retention index on HP-5MS

Supplementary file 1 of the manuscript

Bioactive compounds in fluid propolis preparations inhibit different life stages of pathogenic oomycetes *Aphanomyces astaci* and *Saprolegnia parasitica*

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Table S1. Polyphenol composition of propolis samples determined by UPLC-MS2.

group	compound	P1	P2
		µg/mL	
flavonols	rutin	2.19 ± 0.01	1.84 ± 0.03
	K-3-R	-	2.96 ± 0.14
	Q-3-G	-	0.45 ± 0.04
	K-3-G	-	11.44 ± 0.07
	Q-dimetileter	0.19 ± 0.01	0.34 ± 0.02
	ishoramnetin	0.24 ± 0.03	0.40 ± 0.01
	rhamnetin	1.22 ± 0.03	0.77 ± 0.02
	galangin	6.96 ± 0.13	7.00 ± 0.14
	total flavonols	**10.80**	**25.20**
flavones	apigenin	182.59 ± 1.61	119.53 ± 2.77
	chrysin	734.27 ± 1.97	838.38 ± 3.97
	luteolin	30.10 ± 0.31	19.11 ± 0.89
	total flavones	**946.97**	**977.01**
flavanones	sakuranentin	0.48 ± 0.03	-
	naringenin	1.77 ± 0.10	1.09 ± 0.01
	pinocembrin	34.74 ± 0.02	15.78 ± 0.46
	pinobanksin	0.26 ± 0.01	0.11 ± 0.01
	total flavanones	**37.25**	**16.98**
phenolic acids and derivatives	ferulic acid	23.08 ± 0.33	39.89 ± 1.31
	isoferulic acid	24.82 ± 1.20	49.90 ± 1.94
	caffeic acid	5.41 ± 0.03	4.75 ± 0.04
	p-coumaric acid	27.31 ± 1.50	43.20 ± 0.87
	cinnamic acid	30.44 ± 1.63	58.20 ± 0.41
	CAPE-caffeic acid phenylethyl ester	2.30 ± 0.01	2.95 ± 0.08
	p-HBA acid	2.32 ± 0.23	2.49 ± 0.14
	total phenolic acids and derivatives	**115.69**	**201.39**
others	vanilin	39.15 ± 0.52	22.44 ± 0.83

(-) – not detected

Table S2. Volatile composition of propolis samples determined by GC-MS.

Group	Compound	RI	Samples (% total peak area)	
			P1	P2
monoterpene hydrocarbons	α-pinene	943	-	1.11
	camphene	958	-	1.41
	β-pinene	984	-	0.55
	β-myrcene	995	-	0.40
	p-cymene	1031	-	0.80
	limonene	1034	-	1.66
oxygenated monoterpenes	1,8-cineole	1037	-	4.55
	α-thujone	1101	-	6.79
	β-thujone	1113	-	5.08
	camphor	1152	-	2.78
	menthone	1162	-	4.07
	neoisomenthol	1165	-	0.78
	menthol	1183	-	3.88
	pulegone	1237	-	0.07
	carvone	1250	-	0.64
	piperitenone	1252	-	0.19
	bornyl acetate	1288	-	0.47

	methyl acetate	1289	-	4.21
sesquiterpene hydrocarbons	α-ylangene	1375	-	0.48
	α-copaene	1378	1.74	-
	β-bourbonene	1387	-	0.72
	α-cedrene	1408	-	0.22
	trans-β-caryophyllene	1422	-	1.60
	α-guaiene	1434	-	2.20
	α-humulene	1456	-	2.70
	aromadendrene	1463	-	1.18
	α-amorphene	1479	-	1.42
	ar-curcumene	1485	1.41	1.38
	β-selinene	1489	-	1.37
	α-muurolene	1502	1.80	1.88
	γ-cadinene	1517	2.75	2.32
	cis-calamenene	1525	6.84	-
	δ-cadinene	1526	-	4.94
	α-cadinene	1540	-	0.38
	α-calacorene	1546	-	1.27
oxygenated sesquiterpenes	caryophyllene oxide	1585	-	0.14
	γ-eudesmol	1636	0.26	-
	β-eudesmol	1654	5.05	-
	α-eudesmol	1657	5.28	-
others	ethyl acetate	< 900	-	3.18
	3-methylbut-2-en-1-ol	< 900	-	0.23
	benzaldehyde	970	2.38	1.05
	benzyl alcohol	1046	4.99	2.15
	2-phenylethanol	1121	4.60	0.02
	benzoic acid	1162	12.97	-
	ethyl benzoate	1169	4.77	4.02
	ethyl octanoate	1198	-	0.67
	ethyl nonanoate	1292	-	0.87
	2-methoxy-4-vinylphenol	1312	-	0.32
	ethyl benzenepropanoic acid	1346	3.23	2.34
	ethyl decanoate	1391	-	1.73
	vanillin	1407	2.78	-
	(*E*)-ethyl cinnamate	1464	1.58	0.78
	ethyl dodecanoate	1595	18.91	7.99
	benzyl benzoate	1767	3.92	0.31
	ethyl tetradecanoate	1794	3.73	0.72

RI - Retention index on a MSD model 5975 C and HP-5MS
(-) – not detected

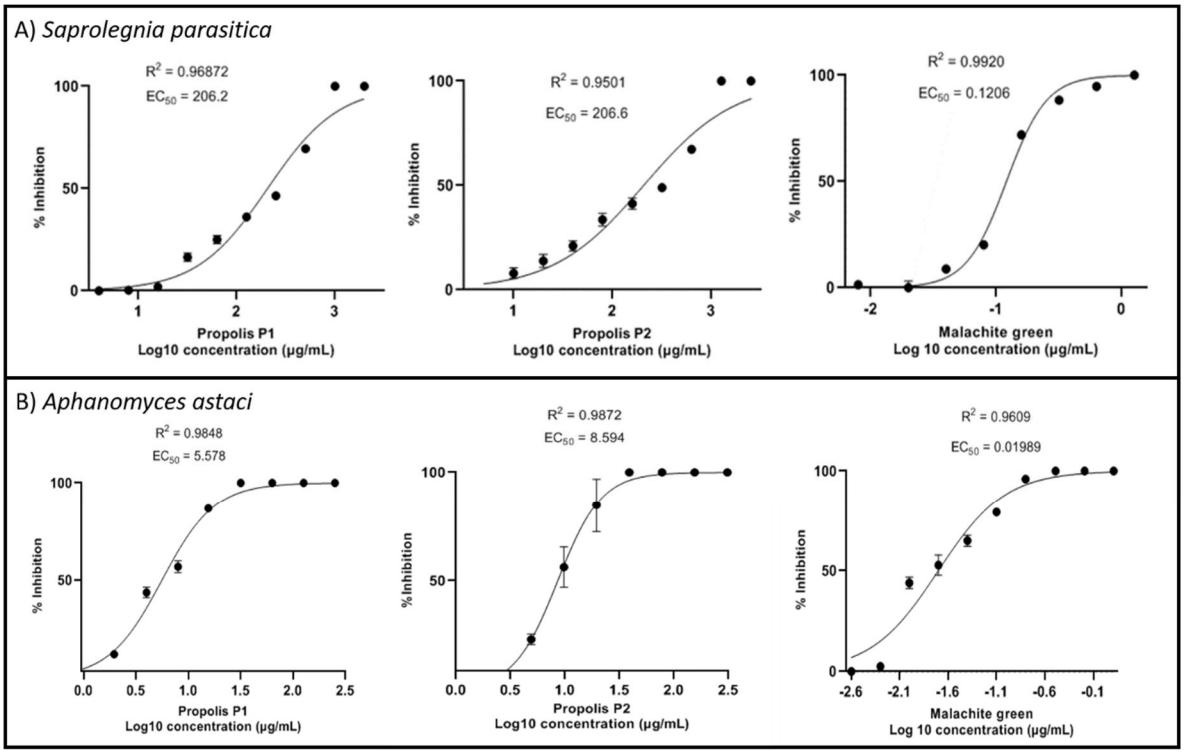

Figure S1. Mycelial growth inhibition curves.

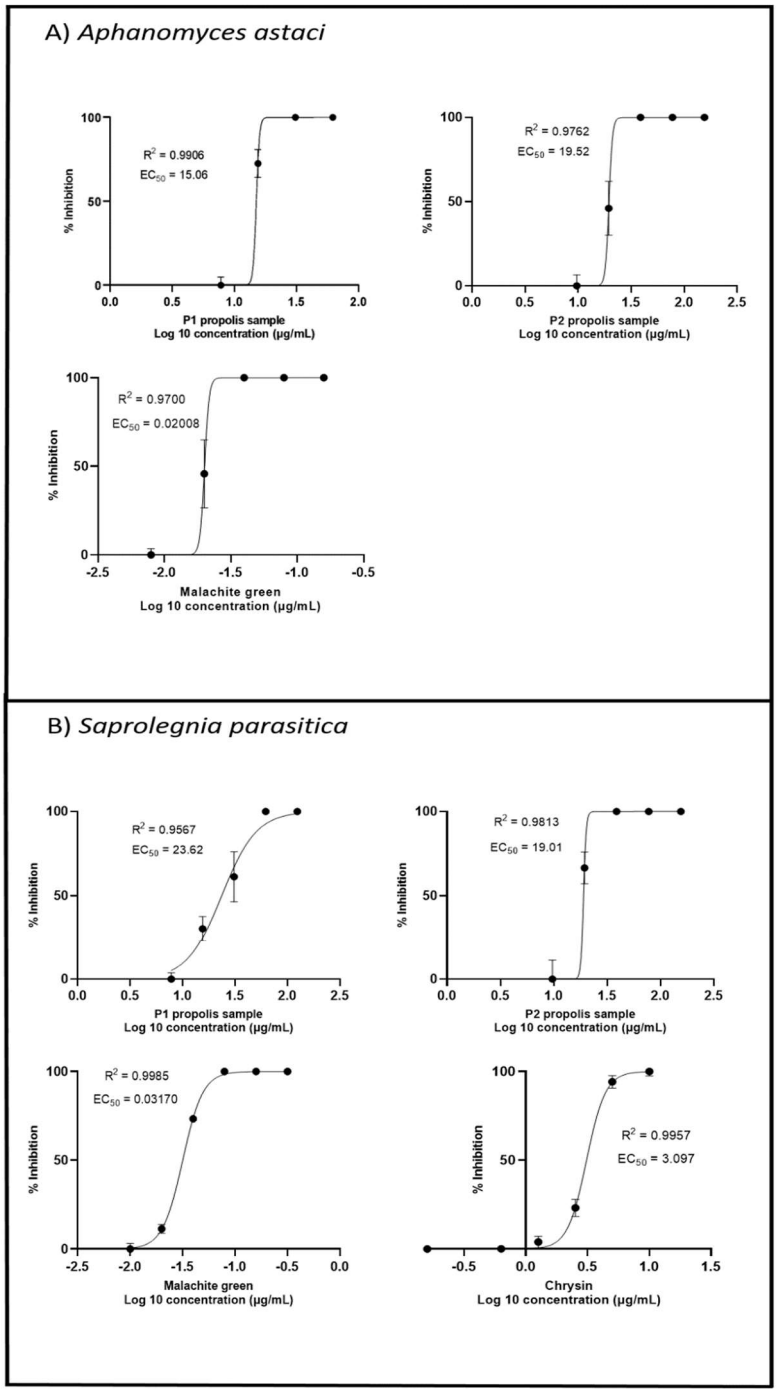

Figure S2. Zoospore germination inhibition curves.

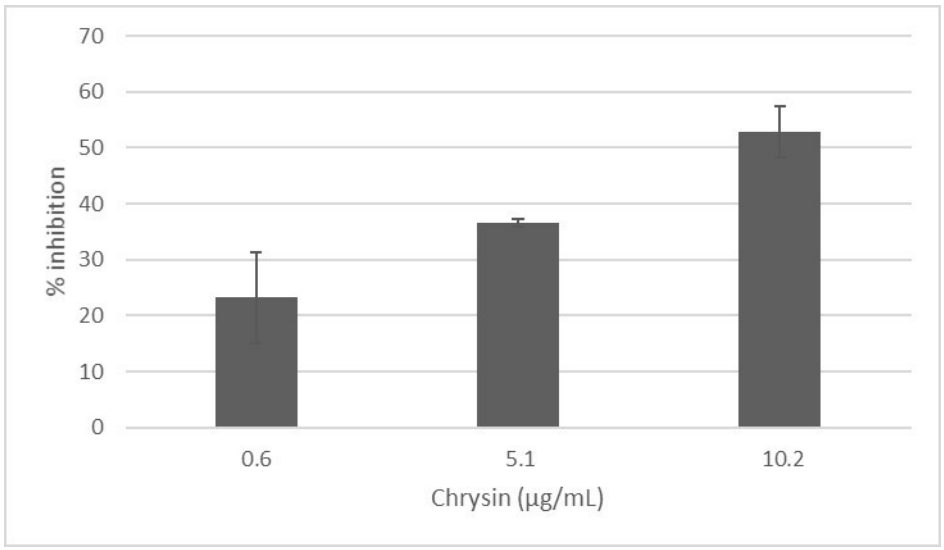

Figure S3. Inhibition of *A. astaci* zoospore germination with chrysin.

A) B)

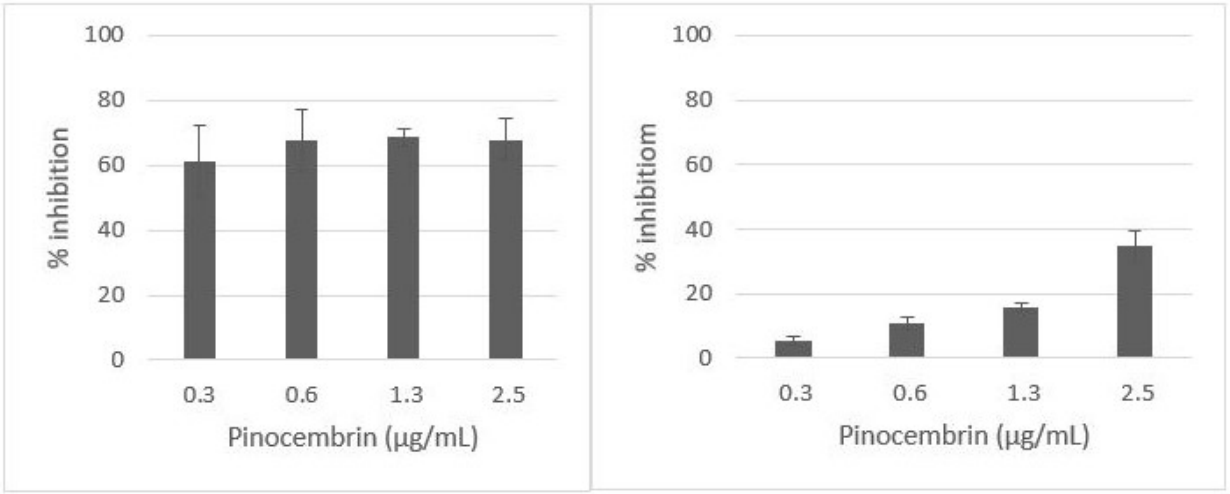

Figure S4. Inhibition of *A. astaci* (A) and *S. parasitica* (B) zoospore germination with pinocembrin.

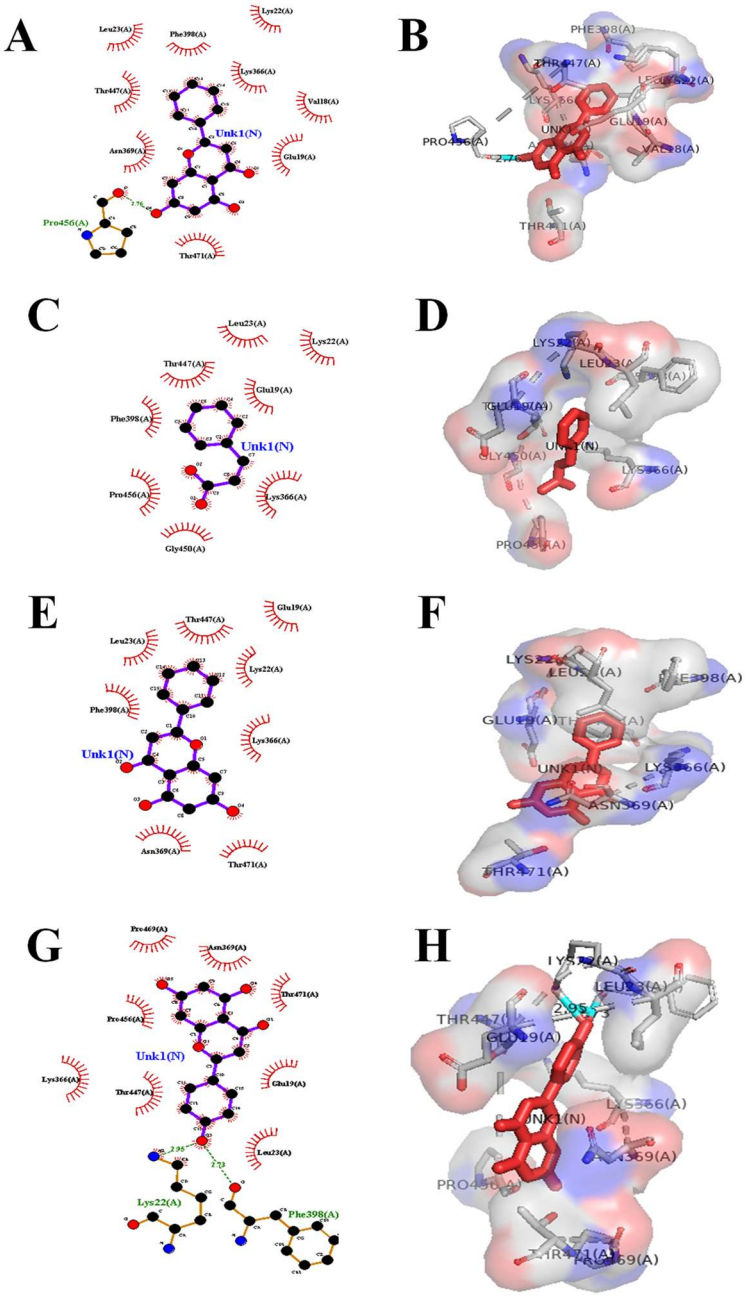

Figure S5. 2D and 3D structure showing endochitinase protein of *Aphanomyces astaci* mycelium interacting with chrysin (A-B), cinnamic acid (C-D), pinocembrin (E-F), and apigenin (G-H).

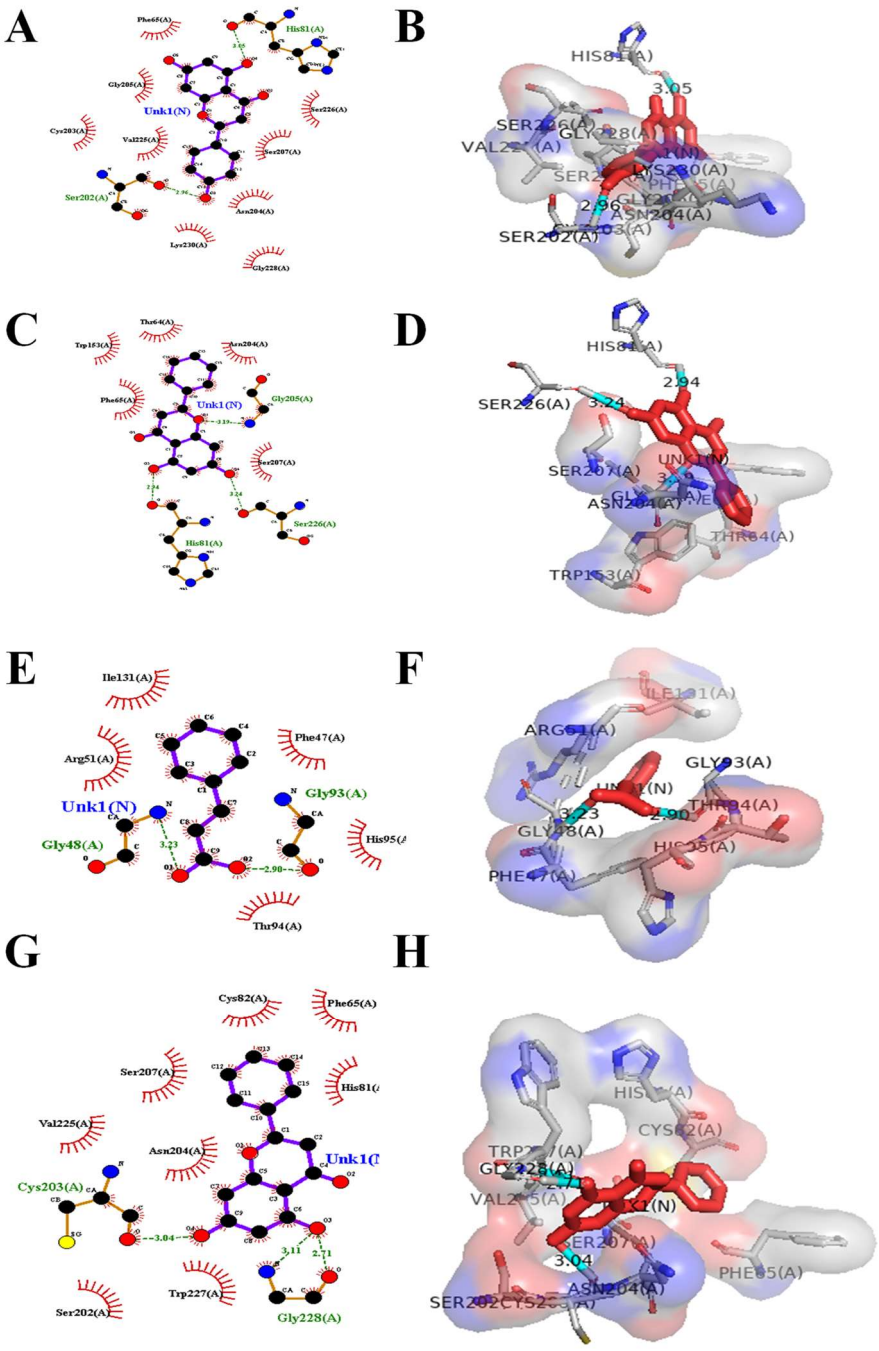

Figure S6. 2D and 3D structure showing trypsin proteinase of *Aphanomyces astaci* mycelium interacting with chrysin (A-B), cinnamic acid (C-D), pinocembrin (E-F), and apigenin (G-H).

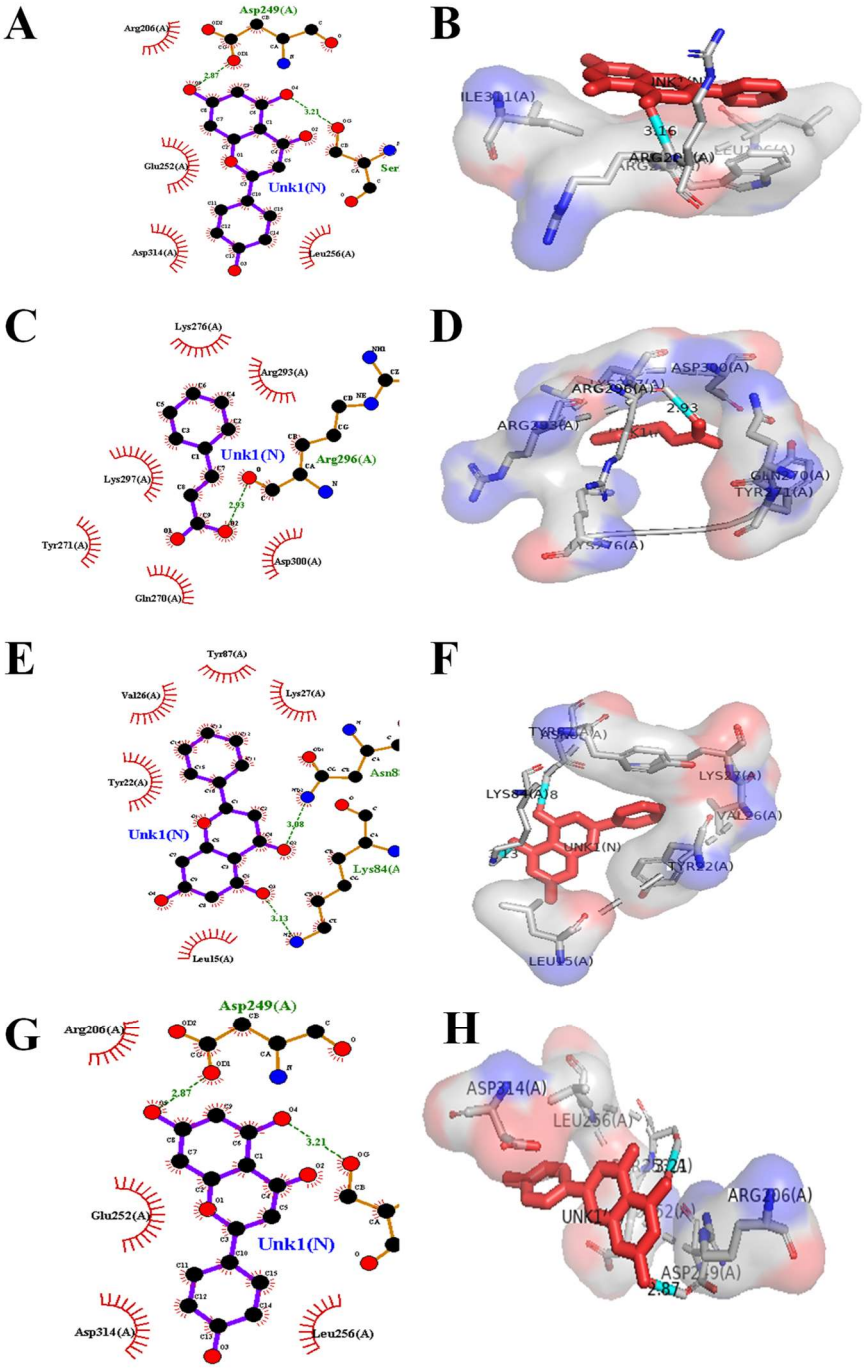

Figure S7. 2D and 3D structure showing laminlike protein of *Saprolegnia parasitica* cysts interacting with chrysin (A-B), cinnamic acid (C-D), pinocembrin (E-F), and apigenin (G-H).

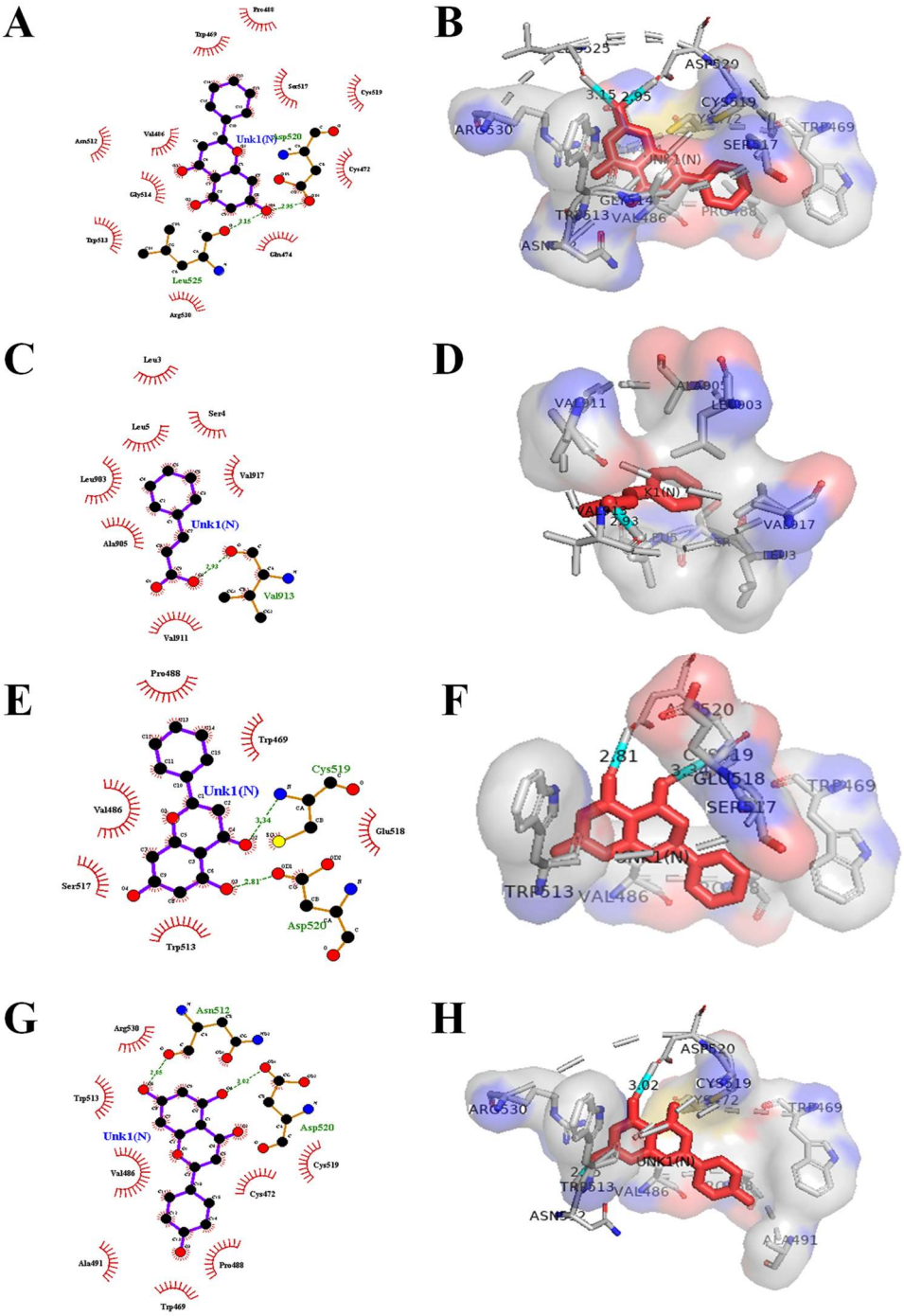

Figure S8. 2D and 3D structure showing thrombospondin protein of *Saprolegnia parasitica* cysts interacting with chrysin (A-B), cinnamic acid (C-D), pinocembrin (E-F), and apigenin (G-H).

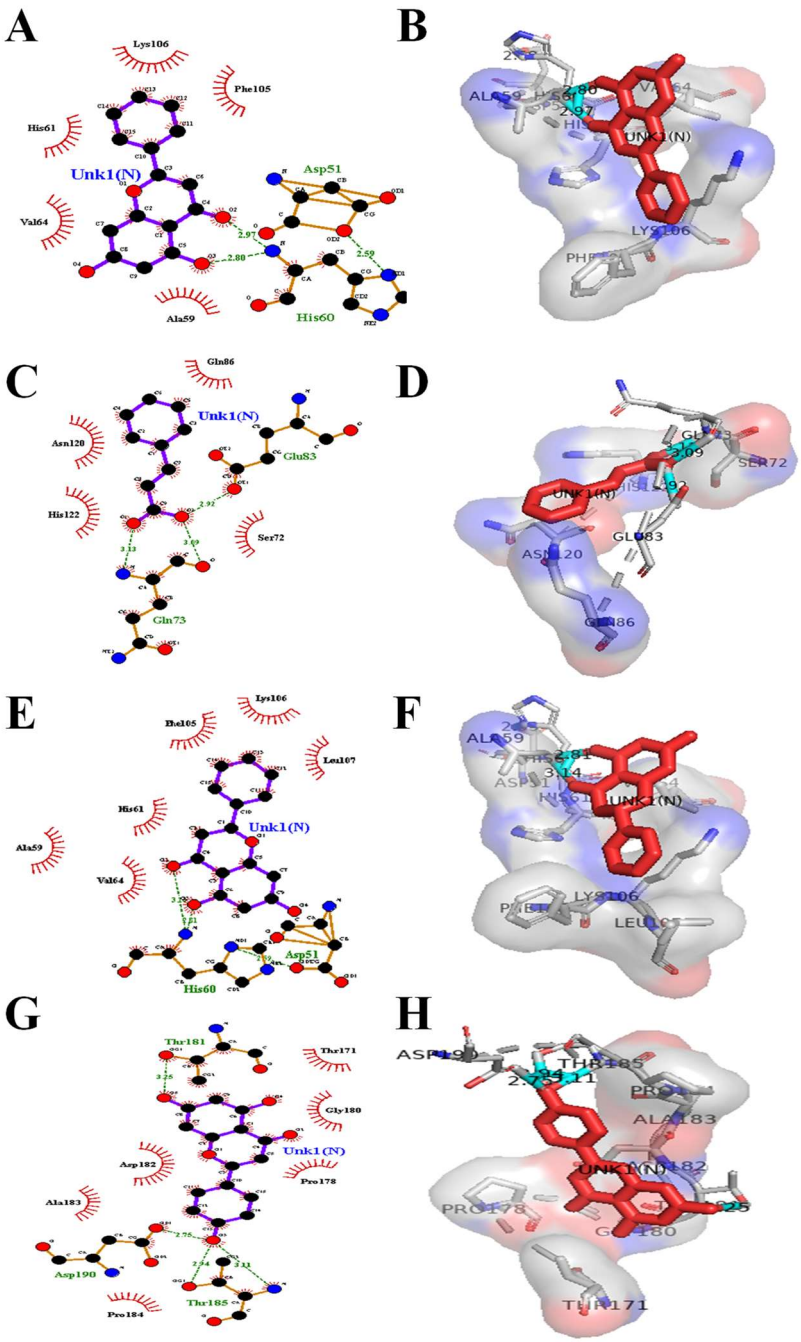

Figure S9. 2D and 3D structure showing host targeting protein 1 of *Saprolegnia parasitica* cysts interacting with chrysin (A-B), cinnamic acid (C-D), pinocembrin (E-F), and apigenin (G-H).

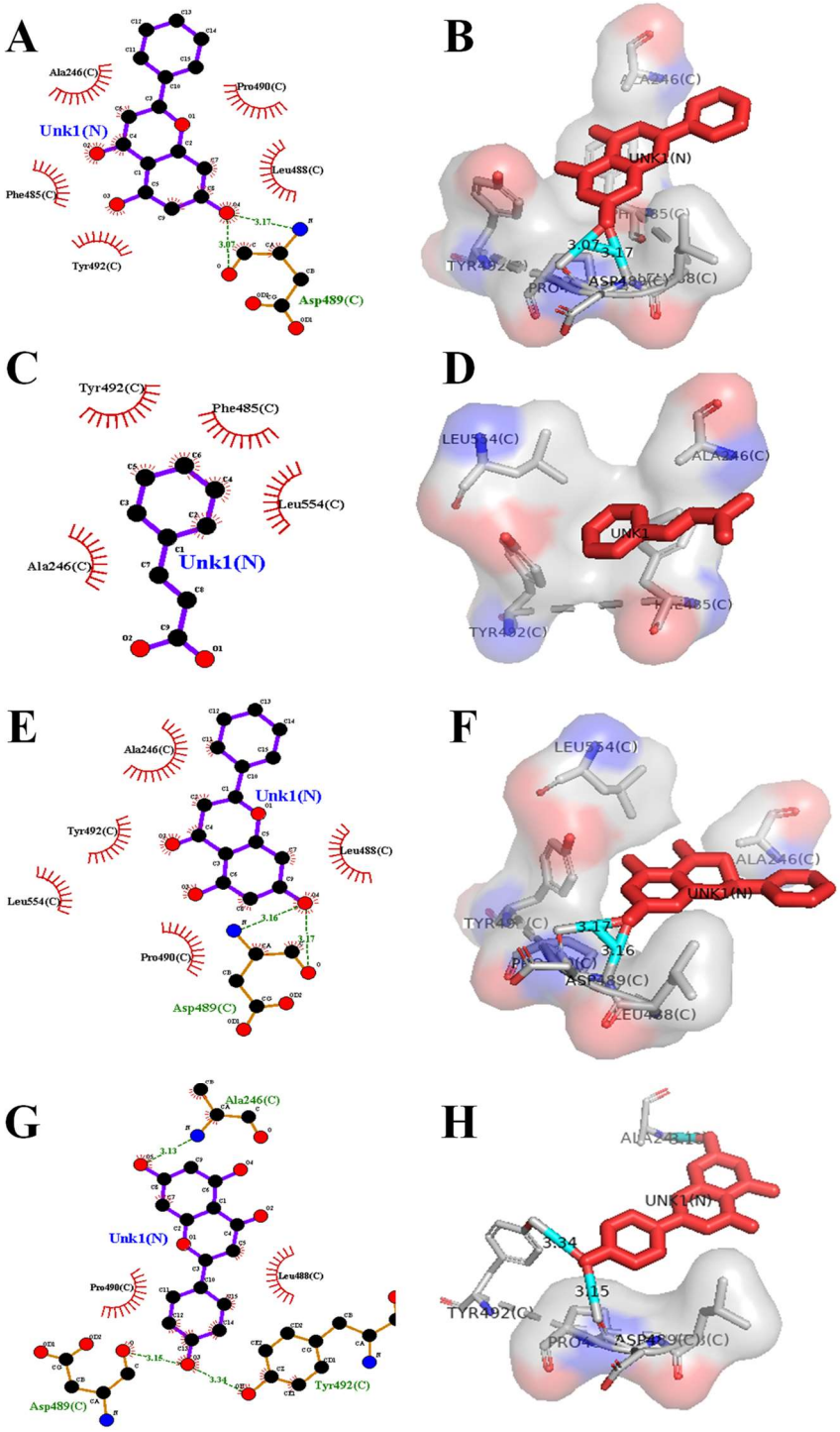

Figure S10. 2D and 3D structure showing V type proton ATPase of *Saprolegnia parasitica* mycelium interacting with chrysin (A-B), cinnamic acid (C-D), pinocembrin (E-F), and apigenin (G-H).

7. CURRICULUM VITAE

Anđela Miljanović, mag. ing. techn. aliment., graduated from primary and secondary school in Tomislavgrad, Bosnia and Herzegovina. In 2014, she graduated from the Faculty of Food Technology and Biotechnology at the University of Zagreb. In 2016, she finished the programme for acquiring teaching competences at the Croatian Catholic Polytechnic, University of Zagreb. During her studies, she was awarded the Rector's Award for a student paper "Volatile compounds of Istrian prosciutto aroma". Since 2018, she has been working as a research assistant in the Laboratory for Biology and Microbial Genetics at the Faculty of Food Technology and Biotechnology as a PhD student within the team of the Croatian Science Foundation project "Interactions of the freshwater pathogenic oomycetes and their environment" (InteractOomyc) (PI: Assoc. prof. Ana Bielen, PhD).

Anđela is first author or co-author of six scientific papers indexed in the SCI-expanded database. She participated in many international and national congresses where she presented her research results. She trained scientifically and professionally in five courses and workshops in Croatia and abroad. She is a member of the Croatian Microbiological Society. She has participated in the supervision of final (3) and diploma (3) theses. She is also involved in popularising science and participated in the 2021 Science Festival in Zagreb.

List of published papers:

1. **Miljanović, A.,** Bhat, R. A. H., Tandel, R. S., Pavić, D., Grbin, D., Dent, M., Marijanović, Z., Jerković, I., Pedisić, S., Maguire, I., Bielen, A. (2022) Bioactive compounds in fluid propolis preparations inhibit different life stages of pathogenic oomycetes *Aphanomyces astaci* and *Saprolegnia parasitica*, *Aquaculture* **552**, 737982. https://doi.org/10.1016/j.aquaculture.2022.737982.
2. Pavić, D., Grbin, D., Gregov, M., Ćurko, J., Vladušić, T., Šver, L., **Miljanović, A.,** Bielen, A. (2022) Variations in the sporulation efficiency of pathogenic freshwater oomycetes in relation to the physico-chemical properties of natural waters. *Microorganisms* **10**, 520. https://doi.org/10.3390/microorganisms10030520
3. **Miljanović, A.,** Grbin, D., Pavić, D., Dent, M., Jerković, I., Marijanović, Z., Bielen, A. (2021) Essential Oils of Sage, Rosemary, and Bay Laurel Inhibit the Life Stages of Oomycete Pathogens Important in Aquaculture, *Plants* **10**(8), 1676. https://doi.org/10.3390/plants10081676.
4. Pavić, D., **Miljanović, A.,** Grbin, D., Šver, L., Vladušić, T., Galuppi, R., Tedesco, P., Bielen, A. (2021) Identification and molecular characterization of oomycete isolates from trout farms in Croatia, and their upstream and downstream water environments, *Aquaculture* **540**, 736652. https://doi.org/10.1016/j.aquaculture.2021.736652
5. **Miljanović, A.,** Bielen, A., Grbin, D., Marijanović, Z., Andlar, M., Rezić, T., Roca, S., Jerković, I., Vikić-Topić, D., Dent, M. (2020) Effect of Enzymatic, Ultrasound, and Reflux Extraction Pretreatments on the Yield and Chemical Composition of Essential Oils, *Molecules* **25**, 4818. https://doi.org/10.3390/molecules25204818
6. Pedisić, S., Zorić, Z., **Miljanović, A.,** Šimić, D., Repajić, M., Dragović-Uzelac, V. (2018) Retention of Bioactive Compounds During Domestic Processing of „Croatian Domestic" Garlic (*Allium sativum* L.), *Food Techn. Biotechnol.* **56**(4), 590-596. https://doi.org/10.17113/ftb.56.04.18.5709